Cellular and Molecular Basis of Autoimmunity in Vitiligo Pathogenesis

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Dedicated to my parents and family…..

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List of Abbreviations

3'UTR	3'-untranslated region
5,6-DHI	Dihydroxyindole
5,6-DHICA	Dihydroxyindole carboxylic acid
5'UTR	5'untranslated region
6-ANAD	6-amino nicotinamide
6BH4	6(R)-L-erythro-5,6,7,8- tetrahydrobiopterin
8-OHdG	8-hydroxydeoxyguanosine
ACE	Angiotensin- converting enzyme
AChE	Acetylcholine esterase
ADCC	Antiboy dependent cellular cytotoxicity
AIRE	Autoimmune regulator
AIS1	Autoimmunity susceptibility locus 1
AP-1	Activator Protein-1
ARMS-PCR	Amplification refractory mutation system-polymerase chain reaction
ATF-6	Activating transcription factor-6
AV	Active vitiligo
bFGF	Basic fibroblast growth factor
BH4	Tetrahydrobiopterin
bp	Base pair (s)
BSF-2	B cell stimulatory factor-2
C	Control
CAF1	Chromatin assembly factor 1

CAT	Catalase
CCL-20	C-C Motif Chemokine 2
CD	Cluster of Differentiation
CHOP	CCAAT-Enhancer-Binding Protein
CI	Confidence interval
CLA	Cutaneous Lymphocyte Antigen
CNTF	Ciliary Neurotropic Factor
COMT	Catechol O-Methyl Transferase
COX2	Cyclooxygenase-2
CRT	Calreticulin
CSA	Complex segregation analysis
CTLA4	Cytotoxic T Lymphocyte-Associated Antigen-4
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
CYP	Cytochrome P450
DCT	Dopachrome Tautomerase
DHPR	Dihydro pteridine reductase
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DOPA	Dihydroxyphenylalanine
DTNB	5-5' dithiobis (2-nitrobenzoic) acid
ECSOD	Extracellular SOD
EDTA	Ethylene diamine tetra acetic acid

EGF	Epidermal growth factor
EGTA	Ethylene glycol-O, - O-bis (2 aminoethyl) N,N,N',N' tetra acetic acid
eIF	Eukaryotic initiation factor
EIF2AK1	Eukaryotic translation initiation factor 2-alpha kinase 1
ELISA	Enzyme linked inmmuno sorbent assay
ER	Endoplasmic reticulum
FADD	FAS-Associated Death Domain
FOXO	Forkhead class O
FT	Fourier Transform
G6PD	Glucose 6 phosphate dehydrogenase
G6PO4	Glucose 6 phosphate
GM-CSF	Granulocyte macreophagecolony stimulating factore
Gp100/ PMEL17	Glycoprotein 100/ pre melanosome protein
GPX	Glutathione peroxidise
GRP78	78kDa glucose regulated protein
GV	Generalized vitiligo
GWAS	Genome-wide association studies
H_2O_2	Hydrogen peroxide
Hb	Hemoglobin
HBSS	Hank's Balanced Salt Solution
HEPES	N-2-hydroxyethly piperazine-N-2-ethane sulphonic acid
HGH	Human growth factor
HLA	human leucocyte antigen

HMB-45	Homatropine Methylbromide 45
HMGA2	High mobility group A2 protein
HSP	Heat shock protein
HWE	Hardy-Weinberg equilibrium
ICAM	Intercellular adhesion molecule-1
IFNG	Interferon-γ
IgG	Immunoglobulin G
IKK	Inhibitor of nuclear factor kappa
IL	Interleukin
IL-1R	Interleukin 1 receptor
IL-1RN	IL-1 Receptor Antagonist
IRE-1α	Inositol requiring protein 1 α
JAK-STAT	Janus kinase-signal transducer and activator of transcription
Kb	kilobase (s)
KDa	Kilo Dalton
L	Lesional
LC	Langerhans cell
LD	Linkage disequilibrium
LIF	Leukemia Inhibitory Factor
LMP	Low molecular weight polypeptide
LPO	Lipid peroxidation
LV	Localized vitiligo
MC1R	Melanocortin 1 receptor
MCHR1	Melanin Concentrating Hormone Receptor 1

MCP-1	Monocyte Chemoattractant Protein-1
MDA	Malondialdehyde
MHC	Major histocompatibility complex
miRNA	MicroRNA
MITF	Microphthalmia transcription factor
MITF-APE1	Micropthalmia Associated Transcription Factor- Human Apurinic/Apyrimidinic Endonuclease-1
mM/M	millimolar/Molar
MNC	Mono-Nuclear Cells
MnSOD	Manganese superoxide dismutase
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium salt
MYG-1	Melanocyte proliferating gene 1
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NCC	Neural crest cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NHM	Normal human melanocytes
NK	Natural Killer Cell
NLRP1	NACHT leucine-rich repeat protein 1
nm	Nanometer
NSV	Non-segmental vitiligo
OD	Optical density
OR	Odds ratio

PAGE	Polyacrylamide Gel Electrophoresis
PAH	Phenylalanine Hydroxylase
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PDC	Plasmacytoid dendritic cells
PDGF	Platelet-derived growth factor
PEGylated	Polyethylene glycol
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PI3K	Phosphoinositide-3-kinase
PI3K/Akt	Phosphatidylinositol 3-kinase/ a serine/threonine kinase
PL	Peri-Lesional
PRDXs	Peroxiredoxins
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
PUVA	Psoralen and Ultraviolet A
RFLP	Restriction fragment length polymorphism
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
rRNAs	ribosomal RNAs
SCF	Stem cell factor

SDS	Sodium dodecyl sulphate
sIL-2R	Soluble interleukin 2 receptor
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
STATs	Signal Transducers and Activators of Transcription
SV	Stable Vitiligo
TAE	Tris acetate EDTA
TAP	Transporter associated with antigen processing
TCA	Trichloroacetic acid
TEMED	N,N,N',N' tetramethylethylenediamine
TGFβ	Transforming growth factor β
Th1	Type 1 T helper cell
TMB	Tetra methyl bezidine
TNFA	Tumor necrosis factor-α
TNFB	Tumor necrosis factor-β
TNFR-2	Tumor Necrosis Factor Receptor 2
TRAF-2	TNF receptor-associated factor 2
Tregs	Regulatory T cells
Tris	Tris (hydroxymethyl) aminomethane
TYR	Tyrosinase
TYRP1	Tyrosinase related protein 1
U	Unit
UPR	Unfolded protein response

UVB	Ultraviolet B
VIT-1	Vitiligo-associated protein 1
VLA-2	Vascular cell adhesion molecule-1 (VCAM-1) binding integrin
VNTR	Variable number of tandem repeats
XBP-1	X-box binding protein 1
α-MSH	Melanocyte Stimulating Hormone
µg/mg	Micro gram/ milligram
µL/mL	Micro litre/ millilitre

Note: The full forms of several rarely used abbreviations have been described within the text.

OBJECTIVES:

(1) Isolation and culture establishment of primary normal human melanocytes from epidermal human skin.

(2) To study the transcript levels of the immune regulator genes: *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* in vitiliginous and control skin.

(3) To study the dose dependent effect of the interleukins: TNF-α, IL-1α, IL-6 and IL-10 on *in vitro* cultured melanocyte cell death, expression of melanin synthesis genes and immunoregulatory genes.

(4) Effect of receptor activation of TNFR1, IL1R1, IL6R and IL10R upon treatment of TNF-α, IL-1α, IL-6 and IL-10 respectively on *in vitro* cultured melanocytes.

(5) To study the role of PARP upon H_2O_2 stimulation of *in vitro* cultured melanocytes.

(6) Genotyping of candidate genes: *IL1RN* intron *2* VNTR, *IL6* and *IL10* in vitiligo patients and controls from Gujarat population with possible genotype-phenotype correlation.

"Some diseases take life but this just ruins it"

Stephen Rothman

1.1 Vitiligo

Vitiligo is a multifactorial polygenic skin disorder caused due to selective destruction of melanocytes associated with a complex pathogenesis, linked with both genetic and non-genetic factors. The precise *modus operandi* for vitiligo pathogenesis has remained elusive although being under investigation for several years. Castro *et al.,* (2005) have reported that vitiligo contributes around 35% among various dermatological disorders. Worldwide prevalence of vitiligo is ~0.5–1% (Ezzedine *et al.,* 2012); ~0.5-2.5 % in India (Handa and Kaur, 1999) and the states of Gujarat & Rajasthan have the highest prevalence i.e. around 8.8% (Valia and Dutta, 1996).

Usually, the incidence of vitiligo starts before the age of 20 years and affects both genders without any biasness (Taïeb and Picardo, 2009; Ezzedine *et al.,* 2012). As such vitiligo is hardly a disease of medical significance but there is more of a social stigma attached to it. It is just a cosmetic disfigurement without any health complications; still, it has a profound effect on quality of life, especially in younger patients (Silverberg and Silverberg, 2014). Most patients exhibit early age of onset (<20 years), and often face stigmatization during their personal, professional, and psychological development and, girls, in particular, are subjected to ostracization from the marital point of view (Mehta *et al.,* 1973; Parsad *et al.,* 2003; Silverberg and Silverberg, 2014; Al-Shobaili, 2014). A significant number of patients feel stressed by their condition and experience anxiety and depression leading to low self-esteem and fear of social isolation (Porter *et al.,* 1978; Nogueira *et al.,* 2009). Vitiligo can happen without predilection for gender or race and may occur anywhere on the body, but it's more likely to develop in some of these areas:

- Skin that's exposed to the sun, such as the face or hands,
- Skin that has folds, such as the elbows, knees, or groin,
- The skin around the eyes, nostrils, belly button, and genital areas.

In vitiligo patients, skin melanocytes are partially or completely lost, and several theories have been put forward to explain the etiology of vitiligo such as oxidative stress,

autoimmune, neural and genetic hypotheses (Agrawal *et al.,* 2001; Shajil *et al.,* 2006; Kemp *et al.,* 2001; Ongenae *et al.,* 2003).

Vitiligo is majorly considered as an autoimmune disorder due to its frequent association with other autoimmune disorders as well as the presence of anti-melanocyte antibodies in the sera of vitiligo patients (Laddha *et al.,* 2014). Several factors inclusive of intrinsic and/or extrinsic factors are responsible for an enigmatic loss of melanocytes in vitiligo. A single dominant pathway appears unlikely to account for all cases of melanocyte loss in vitiligo and apparently, a complex interaction of genetic, environmental; biochemical and immunological events are likely to generate a permissive milieu. It is most likely that loss of melanocytes in vitiligo occurs through a combination of pathogenic mechanisms that act in concert. A generally accepted hypothesis is that the genetic factors render the melanocyte susceptible to developing apoptosis and immune presentation after oxidative stress that, in turn, predisposes individuals to develop vitiligo (Koshoffer and Boissy, 2014; Laddha *et al.,* 2013).

Histological investigations have demonstrated the presence of inflammatory infiltrate of mononuclear cells in the upper dermis and at the dermal-epidermal junction of the peri-lesional skin of vitiligo patients (Picardo *et al.,* 2010). The initiation mechanism of this micro-inflammatory reaction is still not clear, however, local triggers are reported to signal the innate immune system of skin that initiate adaptive immune responses targeting melanocytes (Kroll *et al.,* 2005; van den Boorn *et al.,* 2011). Alternatively, melanocytes exposed to oxidative stress in vitiligo patients could produce self-antigens and/or cytokines to activate an autoimmune response (Koshoffer and Boissy, 2014). Oxidative stress may play a role in vitiligo onset, while autoimmunity contributes towards the disease progression (Laddha *et al.,* 2013; Laddha *et al.,* 2014).

1.1.1 Historical aspects of Vitiligo

Vitiligo is a disease that was observed very early in the history of mankind and most ancient civilizations and religions have documented a lack of the pigmentation in the skin. Vitiligo was described as Swetakustha in Atharva Veda (1500-1000 BC) and was confused with leprosy from biblical times (Nair, 1978), hence the reason for the social and psychological stigma attached to white spots on the skin. The earliest mention of

patchy skin disease that can be interpreted as vitiligo dates back to approximately 1500 B.C. (Panja, 1977).

In the Atharva Veda, particular reference was made to a disease called Kilas. The term "Kilas" comes from Sanskrit word kil, which means "white," in the sense of "casting away.'' In a 1905 translation of the Atharva Veda, Kilas was identified as vitiligo. In the same book, a plant with black seeds is mentioned as being used by Indians in an attempt to restore normal color to discolor skin: "O plant, thou produced even color! Render this (spot) its uniform color." Ancient medical literature indicates that the plant generally used was Bavachee or Psoraleacorylifolia. Later it was discovered to contain psoralene, a photodynamically active furocoumariane. In the sacred Buddhist book, 'Vinaypitah' (624-544 B.C.) also the word 'Kilas' is mentioned in reference to those affected by leucoderma.

Around 4000 years of known history since man became aware of distressing white spots on the skin, its exact etiology is still under investigation (Panja, 1977). Many centuries went by and vitiligo continued to be one of the most important depigmentation ailments worldwide provoking discrimination or segregation in certain cultures, and its pathogenesis still remains an enigma.

1.2 Classification of Vitiligo

Vitiligo is most often classified clinically according to the extent and distribution of depigmentation (Figure 1). It has been proposed that the segmental and focal presentations of the disease constitute a separate subgroup to the non-segmental forms of vitiligo (Taieb and Picardo, 2010). Because, compared to focal and segmental vitiligo non-segmental forms show a later age of onset, a stronger association with autoimmunity and unstable results following autologous grafting (Table 1). Generalised vitiligo is often referred to as Non-segmental vitiligo (Faria *et al.*, 2014).

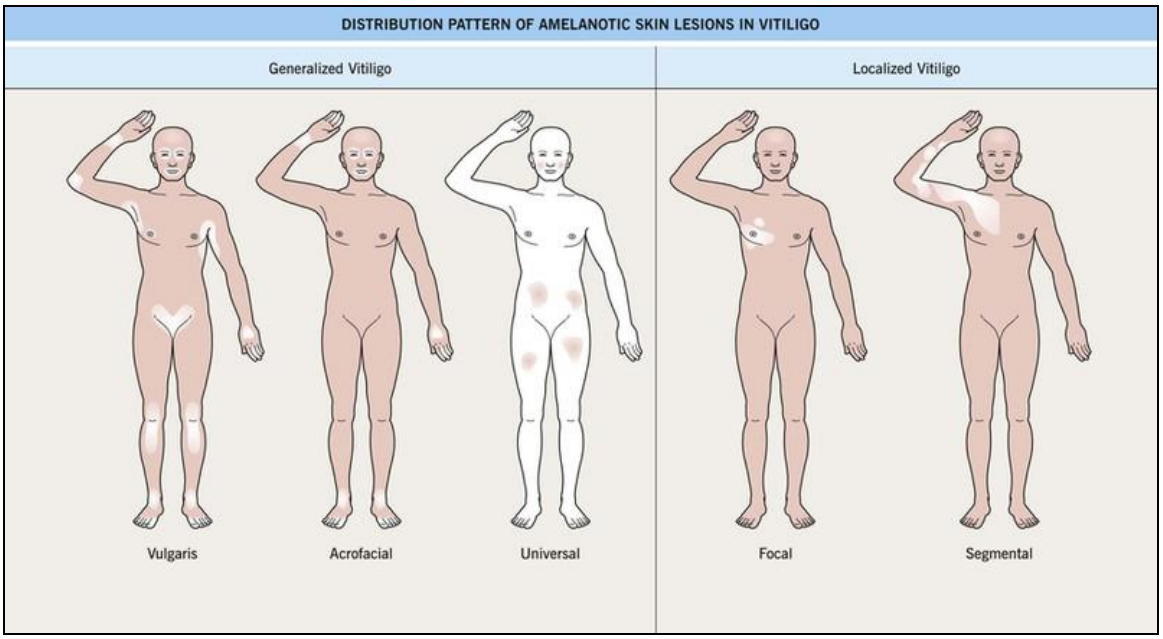


Figure 1: Clinical types of vitiligo

According to the review conducted by the Vitiligo Global Issues Consensus Conference (Ezzedine 2012; Faria *et al*., 2014) vitiligo can be classified into the following clinical forms:

I. Non-segmental vitiligo (NSV): It comprises of acrofacial, mucosal, generalized or common, universal, and mixed forms besides rare forms.

a. Acrofacial: It can affect the face, head, and hands and feet, and preferably involves the perioral region and the extremities of digits.

b. Mucosal: It affects the oral and genital mucosae. Furthermore, areas of mucosa may also be affected in patients with acrofacial, common, or universal forms; when it involves only one mucosal site, it is classified as indeterminate.

c. Generalized or common: It can affect any part of the tegument, mainly hands, fingers, face and trauma-exposed areas. Macules/patches are often symmetrical.

d. Universal: It affects the largest extent of tegument (80-90% of body surface), and it is the most common form in adulthood. The generalized form usually precedes universal.

e. Mixed: It is due to the concomitant involvement of segmental and non-segmental vitiligo. Most often, the segmental form precedes NSV.

f. Rare forms: These types were also considered unclassifiable: vitiligo punctate, minor and follicular.

II. Segmental Vitiligo: It can affect one, two or multiple segments. The unisegmental form is the most common one and consists of one or more white macules on one side of the body and there is also the involvement of body hair (leukotrichia) besides rapid onset of the condition.

Unclassifiable forms or undetermined vitiligo

a) Focal: Isolated white macule without segmental distribution. This form can evolve to segmental or NSV forms.

b) Mucosal: It is seen when only one mucosa is affected.

Table 1: Clinical distinction between the segmental and non-segmental forms of vitiligo and their various characteristics and prevalence (Taieb and Picardo, 2009)

	Non-segmental	Segmental
Prevalence	72-95%	5-28%
Distribution	Symmetrical	Unilateral
Onset	Any age	Early
Course	Variable rate of growth with new lesions throughout life	Rapid initial growth with non-progression within 2 years
Etiology (most plausible)	Autoimmune	Neurochemical

1.3 Skin

Skin is the largest organ of the human body and is the main component of an innate immune system. The skin layers are mainly represented by the epidermis and dermis (Figure 2). The epidermis is multilayered structure and composed of different cell

populations; keratinocytes are the major populating cells and comprise ~95% of epidermal cell population while melanocytes are ~3-5% of epidermal cell pool.

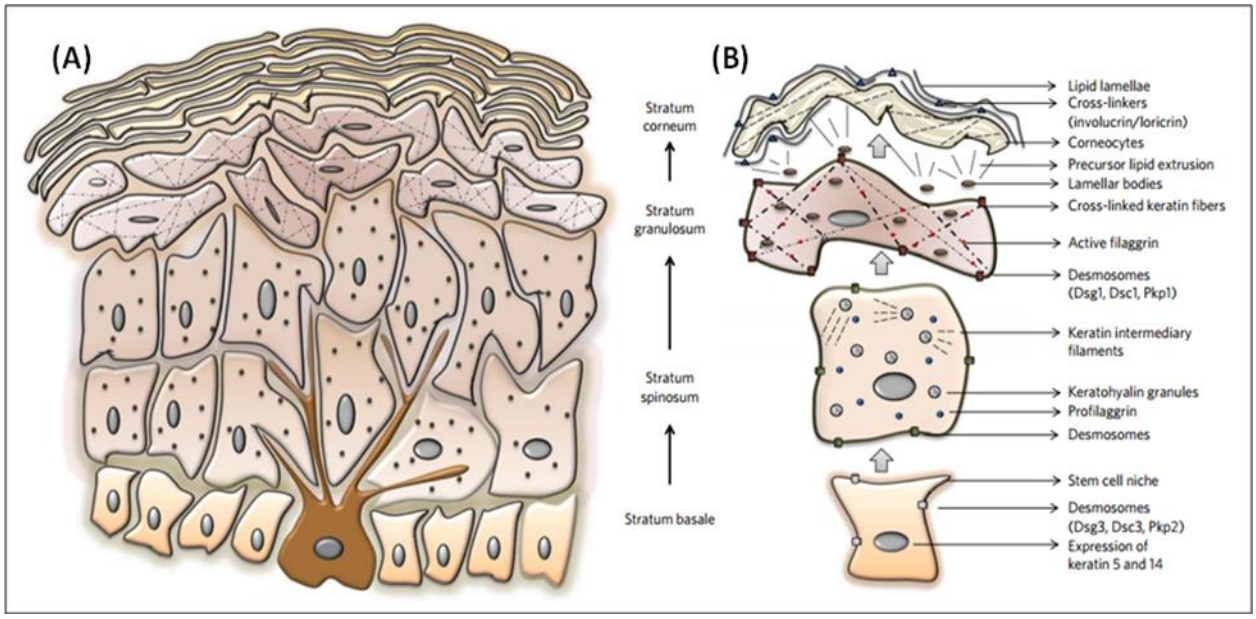


Figure 2: Structure of the skin: A) the different layers and components of human skin. B) Layers of the epidermis (Nararajan *et al*., 2014).

1.3.1 Epidermis

The epidermis is composed of the outermost layers of the skin. It forms a protective barrier over the body's surface responsible for homeostasis and prevents pathogens from entering (McGrath *et al*., 2004). It is composed of proliferating basal and differentiated suprabasal keratinocytes. It also helps the skin to regulate body temperature. Keratinocytes are the major cells, constituting 95% of the epidermis (McGrath *et al*., 2004) while Merkel cells, melanocytes, and Langerhans cells are also placed interstitially. The epidermis is devoid of blood vessels and can be further subdivided into the following strata or layers (beginning with the outermost layer)

- Stratum corneum
- Stratum lucidum
- Stratum granulosum
- Stratum spinosum
- Stratum germinativum

Basement membrane

The epidermis and dermis are separated by a thin sheet of fibres called the basement membrane, and is made through the action of both tissues. The basement membrane controls the traffic of the cells and molecules of the dermis and epidermis but also serves through the binding of a variety of cytokines and growth factors, as a reservoir for their controlled release during physiological remodeling or repair processes (Iozzo, 2005).

Dermis

It is the layer of skin beneath the epidermis that consists of connective tissue and cushions the body from stress and strain. It provides tensile strength and elasticity to the skin through an extracellular matrix composed of collagen fibrils, microfibrils, and elastic fibres, embedded in proteoglycans (Breitkreutz *et al*., 2009). It harbours many mechanoreceptors. It also contains the hair follicles, sweat glands, sebaceous glands, apocrine glands, lymphatic vessels and blood vessels.

Hypodermis

The hypodermis lies below the dermis. Its purpose is to attach the skin to underlying bone and muscle as well as supplying it with blood vessels and nerves. It consists of loose connective tissue and elastin. The main cell types are fibroblasts, macrophages, and adipocytes.

1.3.2 Skin Pigmentation- Melanocytes, Melanin synthesis pathway

Melanocytes are neural crest derived specialized cells that majorly reside in the basal layer of the epidermis and produce melanin. Melanin is a protective, skin darkening, polymeric pigment, capable of ultra violet (UV) absorption and protects cells against different kinds of genotoxic stresses (Lin and Fisher, 2007; Hill *et al*., 1997). Melanocytes are of ectodermal origin and arise during gastrulation of the embryo at the dorsal edge of the neural plate (Thomas and Erickson, 2008). Once emerged, they migrate intensively to specific sites where they differentiate into a wide range of lineages including peripheral neurons, endocrine cells, bone, cartilage, connective tissue, melanocytes and many more (Anderson, 2000; Lin and Fisher, 2007; Adijanto *et al.*, 2012). Apart from skin, melanocytes are also present in a range of other sites where their role as pigment-producing cells is less understood; such as the choroid layer of the eye,

the Harderian gland, the anal canal, and in the stria vascularis of the inner ear where they play a vital role in the endolymph-controlled generation of action potential and suggesting their crucial role in hearing (Steel and Barkway 1989; Tachibana, 1999).

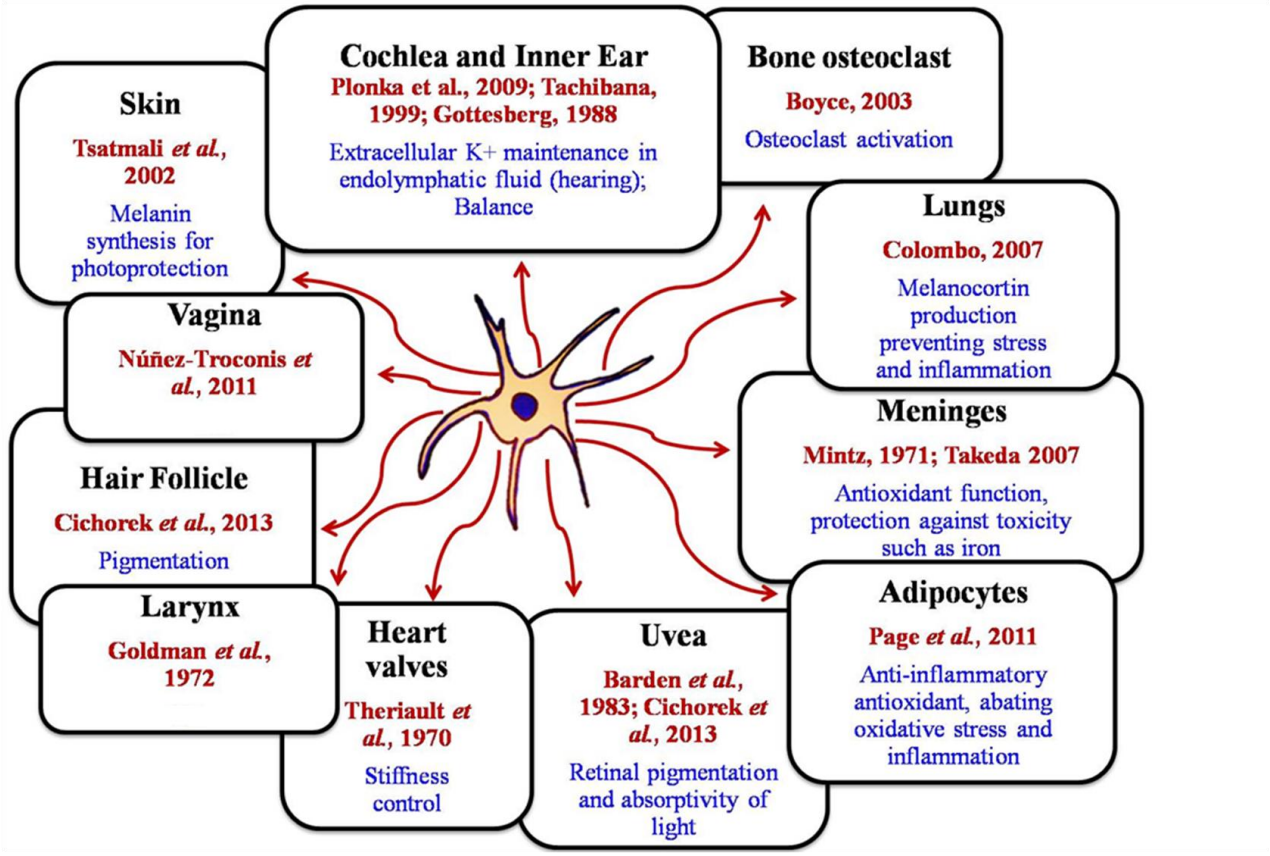


Figure 3: Different locations of melanocytes in the human body and their functions.

The exact functions of melanocytes are not well explored but their presence is important for the integrity and proper functioning of various tissues as illustrated in Figure 3. The term melanocyte was coined by Meyerson in the year 1889 whereas the true discovery of pigmentary cells 'melanocytes' began around 1940-1960 (Westerhof, 2006). The mechanisms by which multi-potent neural crest cells are assigned to evolve into different lineages remain unclear. But precursors for melanocytes, referred to as melanoblasts, originating from bi-potent glial melanoblast progenitors have been seen to occur through the action of Microphthalmia Associated Transcription Factor (MITF)-M and SRY-related HMG-box (Sox)-10 signaling (Dupin *et al.*, 2000; Mollaaghababa and Pavan, 2003). Reservoirs for melanocyte precursor stem cells 'melanoblasts' are primarily

located at hair follicles and are also present in the dermal stem cells which are capable of melanocyte differentiation (Prasad *et al*., 2012). These melanoblasts are therefore responsible for persistent pigmentation of the skin, hair, and eye.

1.4 Melanin synthesis pathway

Pheomelanin and eumelanin are the two pigments formed during melanogenesis. Pheomelanin and eumelanin differ not only in color but also in the size, shape, and packaging of their granules (Slominski *et al.*, 2004). Both melanins are derived from a common tyrosinase-dependent pathway with the same precursor, tyrosine. The obligatory step is hydroxylation of tyrosine to dopaquinone, from which L-DOPA can also be derived (Figure 4, Land *et al.*, 2000). From dopaquinone, the eumelanin and pheomelanin pathways diverge. Two enzymes crucial to eumelanogenesis are the tyrosinase-related proteins TRP1 (also known as GP75 or b-locus) and TRP2 (also known as dopachrome tautomerase, DCT) (Figure 4). Pheomelanin is derived from conjugation by thiol-containing cysteine or glutathione. As a result, pheomelanin is more photo labile and can produce, among its by-products, hydrogen peroxide, superoxide and hydroxyl radicals, and all known as triggers of oxidative stress, which can cause further DNA damage. Individual melanocytes typically synthesize both eumelanin and pheomelanin, with the ratio of the two being determined by a balance of variables, including pigment enzyme expression and the availability of tyrosine and sulphydryl containing reducing agents in the cell.

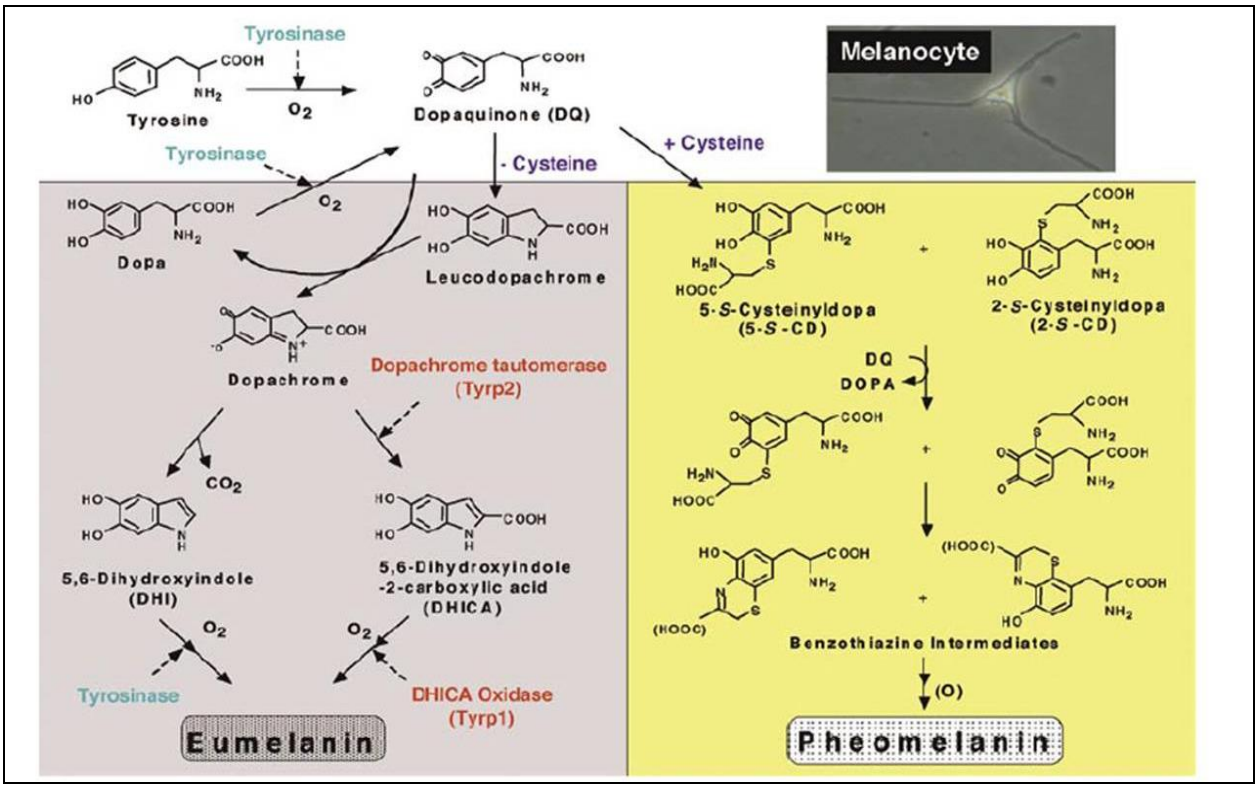


Figure 4: Production of Eumelanin and Pheomelanin in Melanocytes (Slominski *et al*., 2004).

As shown in Figure 4, once melanin is produced, the melanosomes are transferred to the neighboring keratinocytes. The size of these organelles and their numbers are important in determining pigmentation. The melanosomes in the black skin are larger than their counterparts in white skin and are packaged as single units rather than in groups. This has the effect of retarding their degradation in the keratinocytes and contributes to a higher level of skin pigmentation. It appears that association of melanosomes with microtubules and actin filaments via motor proteins, such as kinesin, dynein, and myosin V, is important for melanosome movement along the dendrites and for subsequent transfer to keratinocytes. Melanocyte dendricity and contact with keratinocytes is likely to be essential for the transfer of melanin containing melanosomes. A recent study showed that Activation of the protease activated receptor 2 (PAR-2), which is expressed only on keratinocytes, increases melanin transfer to keratinocytes (Seiberg *et al.*, 2000). The synthesis of melanin takes place in the melanosome. The proteins that are required for melanin biogenesis are found in the melanosome and therefore termed melanosomal

proteins. In addition to this MITF-M exists which is a master transcriptional regulator of melanogenesis and melanocyte survival. It regulates various melanosomal proteins such as TYR, TYRP1, DCT, MART etc. which plays an important role in melanin synthesis pathway (Levy *et al*., 2006). They are classified into two major groups (Rad *et al*., 2004; Hoashi *et al*., 2005).

1. Tyrosinase (TYR), tyrosinase-related protein 1 and 2 (TRP- 1 and TRP- 2), are the enzymes that catalyze the biochemical steps of melanin biosynthesis.

2. MART-1, Pmel17, Rab7, Rab27 etc. are the proteins that have a role in retaining melanosomal structures and/or transporting melanogenic proteins or melanin pigments. Once the melanocyte is stimulated, there is an upregulation, synthesis, and activation of these enzymes as the melanosome is going through its maturation process to enable melanin synthesis (Berson *et al*., 2001).

1.4.1 Functions of the Melanosomal Proteins

Tyrosinase

Tyrosinase is a glycoprotein located in the melanosomal membrane. It has an inner melanosomal domain that contains the catalytic region (~ 90% of the protein), followed by a short transmembrane domain and a cytoplasmic domain composed of approximately 30 amino acids (Sánchez-Ferrer *et al*., 1995). Histidine residues present in the inner (catalytic) portion of tyrosinase bind copper ions that are required for tyrosinase activity. The tyrosinase cytoplasmic domain, specifically the motif EXXQPLL (glutamic acid-X-X-glutamine-proline-leucine-leucine, where 'X' stands for any amino acid), directs tyrosinase trafficking into the melanosomes (Marmol and Beermann, 1996). In addition, PKC-β phosphorylates two serine residues on this domain, a modification required for tyrosinase activation (Hearing and Jimenez, 1987). Tyrosinase can also be indirectly activated by tyrosine hydroxylase isoenzyme I (THI) that was shown to be present in melanosomes and to catalyze L-dopa synthesis, thus activating tyrosinase (Hearing, 1993). In addition, the enzyme phenylalanine hydroxylase can contribute to tyrosinase activation because it catalyzes the conversion of L-phenylalanine to L-tyrosine, providing a substrate for tyrosinase (Schallreuter, 1994).

Tyrosinase mutations including missense, nonsense, frame shift and deletion mutations that lead to inactivation of the enzyme are the cause of oculocutaneous albinism, a group

of hereditary disorders characterized by melanin deficiency or absence. Tyrosinase mutations may affect glycosylation of the protein, interfering with enzyme maturation; the copper (Cu) binding sites disrupting enzymatic activity; or the cytoplasmic domain, preventing enzyme activation (Gillbro and Olsson, 2011).

Tyrosinase-Related Protein 1 (TRP-1*)*

Tyrosinase-related protein 1 is expressed specifically in melanocytes and functions in melanin synthesis within melanosomes (Kobayashi *et al*., 1998). In the lumen of the endoplasmic reticulum (ER), TRP-1 helps to fold tyrosinase properly via chaperon-type interactions and functions to stabilize tyrosinase Kobayashi *et al*., 1994 by forming an enzymatic complex (these enzymes contain a cysteine-rich epidermal growth factor motif thought to be involved in protein–protein interactions). A mutation in TRP-1 can prevent tyrosinase export from the ER and/or affect the catalytic functions of TYR and its stability (Kobayashi *et al*., 1998,).

Tyrosinase-Related Protein 2 (TRP-2)

Tyrosinase-related protein 2 is an important regulatory enzyme that plays a pivotal role in the biosynthesis of melanin and in the rapid metabolism of its toxic intermediates. The enzymatic activity of TRP-2 determines whether the eumelanin or pheomelanin pathway is preferred for pigment biosynthesis (Sturm *et al*., 1995). Active and accelerated levels of TRP-2 increase the efficiency of eumelanin production through the isomerization of dopachrome to DHICA rather than the spontaneous conversion of dopachrome to DHI, which is a toxic intermediate thought to be the most effective in suppressing the growth of the cell. Pheomelanin pigments are visually dominant when TRP-2 levels are lower because of diminished flow of dopaquinone through dopachrome to eumelanin (Gillbro and Olsson, 2011).

Pmel17/gp100

The major scaffold protein Pmel17 is delivered to the premature melanosome, going on to proteolytic cleavage into several fragments; this then forms the fibrillar matrix of the organelle that enables the synthesis and deposition of melanin (Du *et al*., 2003).

MART1/MelanA, Pmel17

MART1 forms a complex with Pmel17 and affects its expression, stability, trafficking, and the processing that is required for melanosome structure and maturation. MART1 is

indispensable for Pmel17 function and thus plays an important role in regulating mammalian pigmentation (Busam and Jungbluth, 1999).

Rab7 and Rab27

Rab27a is a member of the small GTPase Rab27 subfamily, which controls transport and exocytosis of lysosome-related organelles (LRO) in specific cell types (Izumi *et al*., 2003). Mutations in the RAB27A gene are associated with the ashen phenotype and Griscelli syndrome type II in mice and humans respectively (Anikster *et al*., 2002; Bahadoran *et al*., 2003).

Another member of the family of Rab GTPases associated with LRO is Rab7, which is involved in microtubule-based transport of late endosomes/lysosomes (Bucci *et al*., 2000; *et al*., 2001), major histocompatibility complex class II compartments (Jordens *et al*., 2001), Jordens cytolytic granules and phagosomes (Guignot *et al*., 2004).The minus-end microtubule-based motor complex dynein– dynactin is recruited to the Rab7-containing compartments through its effector Rab7-interacting lysosomal protein (RILP) (Jordens *et al*., 2001). Consequently, minus-end transport increases and compartments accumulate around the microtubule-organizing center (MTOC). Proteomics and immunofluorescence analyses showed that Rab7 is also associated with the melanosomal membrane. Moreover, Hirosaki *et al*. (2002) demonstrated that Rab7 is involved in the transport of the melanosome-specific proteins tyrosinase and tyrosinase-related protein 1 (Tyrp1), both involved in melanin synthesis, from the trans-Golgi network to melanosome.

Microphthalmia Associated Transcription Factor-M (MITF-M)

The Microphthalmia Associated Transcription Factor (MITF) is a member of the basic helix-loop-helix-leucine zipper (bHLH-LZ) transcription factor family having nine isoforms, fundamentally governing and regulating a broad range of genes important for melanin synthesis, cell cycle regulation, migration, and survival. The MITF-M is melanocyte specific while nine other isoforms are found in varying amounts in different cell types (Levy *et al.*, 2006; Wang *et al.*, 2011; Widlund and Fisher, 2003; Goding, 2000, Tassabehji *et al.*, 1994). The MITF gene was first discovered while working with spotted mutant mice (Hertwig, 1942) and was then histologically characterized to be occurring due to selective ablation of functional melanocytes, instead of defective pigmentation within viable melanocytes (Hodgkinson *et al.*, 1993; Hallsson *et al.*, 2004).

A dysfunctional MITF, exhibit defects in pigment cells of the skin, eye, hair follicles and inner ear, abnormalities in MITF also affects several other cell types such as bone resorbing osteoclasts, retinal pigment epithelial cells, and mast cells (Steingrímsson *et al.*, 2004).

1.4.2 Melanogenesis

Melanosomes are specialized subcellular organelles in which melanin is synthesized and deposited (Orlow, 1995). There are four stages in the maturation of melanosome (Figure 5):

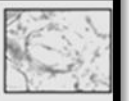
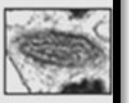
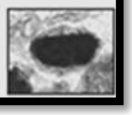
Stages	Description	Electron micrograph
I	Spherical; no melanin deposition	
II	Oval; obvious matrix in the form of parallel longitudinal filaments; minimal deposition of melanin; high tyrosinase activity	
III	Oval; moderate deposition of melanin; high tyrosinase activity	
IV	Oval; heavy deposition of melanin; electron-opeque, minimal tyrosinase activity	

Figure 5: Description and electron micrographs of the four major stages of eumelanin melanosome maturation within melanocytes and keratinocytes.

As shown in Figure 5 the following changes occur in the eumelanin melanosomes.

Stage I, the "premelanosome" a spherical organelle with ill defined matrix filaments is seen;

Stage II, the typical elliptical shape of the melanosome is filled with a well defined filamentous or laminar matrix;

Stage III, deposition of electron opaque melanin occurs in this matrix;

Stage IV, complete opacification of melanosomal contents takes place by the melanin deposited therein (Orlow, 1995). The transition to stage II melanosomes involves

elongation of the vesicle and the appearance of distinct fibrillar structures. The production of internal matrix fibers and the maturation from stages I to II melanosomes depend on the presence of a structural protein termed Pmel 17 or gp100. A melanosomal protein called MART 1 forms a complex with Pmel 17 and thus plays an important role in melanogenesis by regulating the expression, stability, trafficking, and processing of Pmel 17, which in turn regulates the maturation of melanosomes (Hoashi *et al.*, 2005).

1.5 Etiopathogenesis Of Vitiligo

The etiopathogenesis of vitiligo has not been fully understood and several theories have been proposed (Passerson and Ortonne, 2005; Dell'Anna and Picardo, 2006; Westerhof and d'Ischia, 2007; Schallreuter *et al.,* 2008; Boissy and Spritz, 2009). So far there is no universally accepted hypothesis. Vitiligo corresponds to a 'syndrome' rather than a disease, with different but not mutually exclusive pathways resulting in melanocyte failure or its death. Genetic factors may determine which particular pathway is predominant in a patient. Le Poole *et al*., (1993) proposed the 'convergence' theory which was later refined by Schallreuter *et al*., (2008) that increased concentration of endogenous or exogenous phenol/catechol around the melanocytes competes with tyrosine for tyrosinase binding sites producing abnormal substrates, curtailing melanin production. These aberrant substrates generated during melanogenesis create reactive quinones and disturb redox balance, which impairs cell functions and bind covalently to the catalytic center of tyrosinase, impairing or inactivating the enzyme and further reducing melanogenesis (Passerson and Ortonne, 2005). Analogous detrimental effects occur with other reactive itermediates and enzymes due to excessive ROS. Another proposal emphasizes the immunogenicity of melanosomal proteins and melanocytes, with aberrant T-cell attack resulting in melanocyte destruction by apoptosis (Kobayashi *et al.,* 1998). Within these polar views of vitiligo pathogenesis, there is speculation that ROS and the immune system may interact synergistically so that both mechanisms might be relevant. Further hypotheses focus on cytokines, calcium imbalance, hormones, neural dysregulation, infections and melanocytorrhagy in the causation of vitiligo (Gauthier *et al*., 2003; Alikhan *et al*., 2011; Le Poole *et al*., 1993; Namazi, 2007).

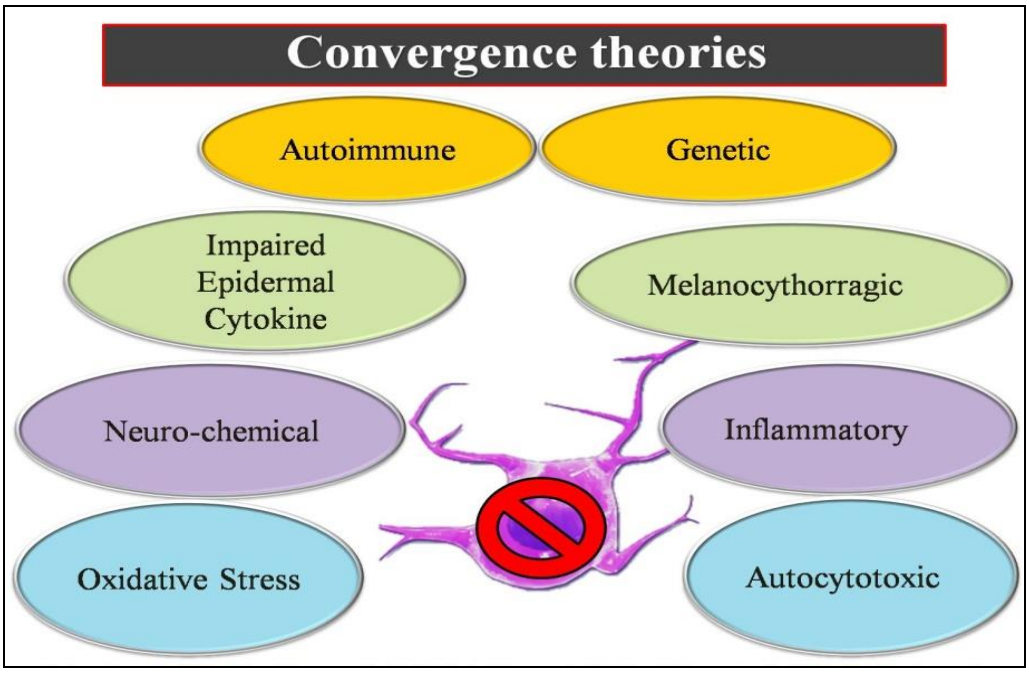


Figure 6: Different hypothesis proposed for vitiligo etiopathogenesis (Gauthier *et al*., 2003; Alikhan *et al*., 2011; Le Poole *et al*., 1993; Namazi, 2007).

1.5.1 Auto-cytotoxic Theory

Melanocytes due to their high metabolic load of melanin synthesis are under oxidative stress (Denat *et al.*, 2014). Toxic metabolites such as vitiligo inducing phenols or quinones or from intrinsic melanin synthesis pathways may accumulate and lead to the damage of melanocytes in genetically susceptible individuals (Hann and Chun, 2000). As tyrosine, itself a phenol, enters into the melanin synthesis pathways, electrically unstable by-products are generated with the potential to damage other cellular substrates (Schallreuter *et al.*, 1994). A defect in the melatonin receptor is also capable of resulting in toxic by-products leading to cellular damage (Hann and Chun, 2000).

1.5.2 Oxidative Stress Theory

Lesional and non-lesional skin from vitiligo patient's exhibit abnormally low levels of catalase, (Schallreuter *et al.*, 1991) correlating with high H_2O_2 levels throughout the epidermis (Schallreuter *et al.*, 1999). H_2O_2 accumulation also leads to inactivation of catalase, reducing its functionality. The resulting deranged melanin synthesis pathways involve reduced glutathione peroxidase activity (Schallreuter *et al.*, 1996), reduction in the functioning of glucose-6-phosphate dehydrogenase along with a concomitant

decrease in tyrosinase activity. Increased levels of accumulating epidermal H_2O_2 also lead to a concomitant increase in the superoxide dismutase levels (SOD), especially of SOD2 and SOD3 along with higher lipid peroxidation levels (Schallreuter *et al.*, 2004; Agrawal *et al.*, 2004; Shajil and Begum, 2006; Glassman, 2011). However, a recent report by Zailaie (2017) has shown a non-significant difference in the H_2O_2 levels between vitiligo patients and controls. Moreover, oxidative stress and antioxidant imbalance are well documented in vitiligo patients as shown in Tables 2 and 3.

Table 2: Reports on H_2O_2 in vitiligo (Glassman, 2011)

Source	Level	Reference(s)
Catalase	Decreased	Mansuri *et al*., 2017; Schallreuter *et al.*, 1991; Maresca *et al.*, 1997; Dell'Anna *et al.*, 2001; Agrawal *et al*., 2004; Shajil and Begum, 2006; Dammak, *et al.*, 2009; Shajil *et al*., 2007
GPX/ GSH	Decreased	Mansuri *et al*., 2016; Beazley *et al.*, 1999; Dell'Anna *et al.*, 2001; Agrawal *et al*., 2004; Shajil and Begum, 2006; Shajil *et al*., 2007
H_2O_2	Increased	Schallreuter *et al.*, 1999
Peroxynitrite	Increased	Salem *et al.*, 2009
TNF-α	Increased	Moretti *et al.*, 2002; Laddha *et al*., 2012
Oxidized pterins	Increased	Rokos *et al.*, 2002
$6BH_4$ recycling	Decreased	Schallreuter *et al.*, 1994b; Schallreuter *et al.*, 2001; Rokos *et al.*, 2002
iNOS	Increased	Salem *et al.*, 2009

Source	Level	Reference(s)
Hcy	Increased	Shaker *et al.*, 2008
Monoamine oxidase A	Increased	Schallreuter *et al.*, 1996
SOD	Increased	Koca *et al.*, 2004; Picardo *et al.*, 1994; Agrawal *et al.*, 2004; Yildirim *et al.*, 2004; Hazneci *et al.*, 2005; Shajil and Begum, 2006; Khan *et al.*, 2009; Dammak, *et al.* ,2009; Laddha *et al.*, 2013
Thioredoxin reductase	Decreased	Gibbons *et al.*, 2006
Xanthine oxidase	Increased	Shalbaf *et al.*, 2008
Catecholamines	Increased	Westerhof *et al.*, 2007
GTP-cyclohydrolase I	Increased	Schallreuter *et al.*, 1994; Chavan *et al.*, 2009
Vitamin E	Decreased	Agrawal *et al.*, 2004; Jain *et al.*, 2008; Khan *et al.*, 2009
Monoamine oxidase A	Increased	Schallreuter *et al.*, 1996
NADPH oxidase	Increased	Schallreuter *et al.*, 1999
Photooxidation of pterins	Increased	Rokos *et al.*, 2002
TYRP1	Decreased	Jimbow *et al.*, 2001

Table 3: **Oxidative stress and Antioxidant status in vitiligo patients** (Agrawal *et al.*, 2004)

LPO	Increase
SOD	**Increase**
CAT	Decrease
GPx	Decrease
G6PDH	Decrease
GST	NS
GR	NS
GSH	Decrease

1.5.3 Melanocytorrhagy Theory

This theory proposes that imbalance in cytokines levels leads to increased ICAM-1 levels (intercellular adhesion molecule-1), which act on melanocytes causing their circularisation resulting in the weak anchorage of these cells in their niche. Any minor friction and/or other stress can, therefore, induce upward migration and loss through the induction of immune cells promoting the selective destruction of melanocytes (Gauthier *et al.*, 2003).

1.5.4 Autoimmune Hypothesis Of Vitiligo

Melanocytes also contribute to the skin immune system, secreting a wide range of signal molecules and responding to cytokines and growth factors. Melanocytes can phagocytize and eliminate exogenous antigens, which have penetrated the skin barrier (Le Poole *et al.*, 1993), and they can process and present antigens in the form of peptides with HLA (human leucocyte antigen) class II molecules to T-cells, triggering an adaptive immune response. Melanosomal proteins are involved in this antigen processing (Le Poole and Luiten, 2008). Activation of T-cells by melanocytes has shown by the secretion of co-stimulatory molecules like ICAM (intercellular adhesion molecule)-1 and LFA (leucocyte fusion-associated molecule)-3 (Le Poole *et al.*, 1993). Case reports on inflammatory vitiligo furnished the involvement of T cells in the pathogenesis of vitiligo

(Buckley, 1953; Michaelsson, 1968). Immune-mediated responses are consistently observed in progressive vitiligo at the periphery of depigmenting patches. Histopathological investigations of the peri-lesional skin suggest lymphocyte involvement in the depigmentation process. Immunohistochemical studies have also confirmed the presence of infiltrating T cells and their frequent opposition to peri-lesional melanocytes in skin biopsies from vitiligo patients (Le Poole *et al*, 1996; Yagi *et al*, 1997). Notably, similar *in situ* T cell infiltrates, primarily $CD8^+$ T cells, have also been detected in generalized vitiligo (Gross *et al*, 1987; Badri*et al*, 1993; Abdel-Naser*et al*, 1994; van den Wijngaard *et al*, 2000; Sanchez-Sosa *et al.,* 2013; Bertolotti *et al.*, 2014). T cells are more prevalent in vitiligo peri-lesional skin than in surrounding non-lesional skin. The lymphocyte infiltrates consists essentially of $CD8^+$ T cells with occasional $CD4^+$ T cells (Le Poole *et al.,* 1996). The prevalence of cytotoxic T cells and their co–localization with surviving melanocytes suggest T cell mediated cytotoxicity towards the melanocytes (Wankowicz-Kalinska *et al.,* 2003). Various other studies including ours show decrease in systemic $CD4^+$ T-cells and an increase in $CD8^+$ T-cells with consequent decrease in $CD4^+/CD8^+$ ratio in vitiligo patients, suggesting role of $CD8^+$ cells in melanocyte death (Grimes *et al.,* 1986; Halder *et al.,* 1986; Nigam *et al.*, 2011; Dwivedi *et al.,* 2013). Several studies have shown a defective functionality and decreased the frequency of regulatory T cells (Tregs) in vitiligo patients suggesting the unchecked activation of $CD8^+$cells (Dwivedi *et al.*, 2013a; Lili *et al.,* 2012). Furthermore, Bertolotti *et al*. (2014) have also reported the presence of IFNα secreting plasmacytoid dendritic cells (pDC) in the infiltrate of progressive vitiligo. IFN*α* induces the expression of *MxA*, which encodes a guanosine triphosphate (GTP)-metabolizing protein. Association of MxA with the expression of chemokine (C-X-C motif) ligand 9 (*CXCL9*) correlates well with the recruitment of chemokine (C-X-C motif) receptor $3^+(CXCR3^+)$ immune cells. Further, they also showed increased expression of *MxA* in the peri-lesional skin in close opposition to surviving melanocytes within the T-cell infiltrate. In contrast, MxA was not evident in lesional skin, suggesting that IFN-*α* production is an early event in the progression of the disease. An autoimmune aspect of vitiligo pathogenesis is strongly supported by the presence of autoreactive T-cells (Ongenae *et al.,* 2003; Le Poole *et al.,* 1996). They target melanocyte-specific antigens, such as melan-A/MART1, Gp100/Pmel

17 (a melanosomal matrix glycoprotein), tyrosinase (Fishman *et al.,* 1993; Song *et al.,* 1994; Kemp *et al.,* 1997), TRP1 and TRP2 (Wankowicz-Kalinska *et al.,* 2003; Palermo *et al.,* 2001) that are localized primarily on melanosomes (Kemp *et al.,* 1998a; Kemp *et al.,* 1998b; Hearing *et al.,* 1999).

Auto-antibodies against melanocyte antigens have also been detected in the sera of vitiligo patients (Alkhateeb *et al.,* 2003, Pradhan *et al.,* 2013; Laddha *et al.,* 2014). The transcription factors SOX9 and SOX10 have also been identified as melanocyte auto antigens (Hedstrand *et al.,* 2001). Even auto antibodies against HLA Class I molecules have been detected in vitiligo (Ongenae *et al.,* 2003). A positive correlation has also been seen between the level of melanocyte antibodies and disease progression in vitiligo (Harning *et al.,* 1991; Naughton *et al.,* 1986). Overall, these studies point to a new innate immune pathway for vitiligo progression.

Genes within MHC class II region are associated with several autoimmune diseases (Pamer and Cresswell, 1998). This highly polymorphic region includes several genes involved in the processing and presentation of antigens to the immune system including low molecular weight polypeptide 2 and 7 (LMP2 and LMP7) and transporter associated with antigen processing 1and 2 (TAP1 and TAP2). Though *LMP/TAP* gene cluster is located on MHC class II region of chromosome 6, it is involved in antigen presenting function of MHC class I molecule. Different researchers have reported an association of *LMP7* and *TAP1* with susceptibility to vitiligo (Casp *et al.,* 2003). LMP2 and LMP7 are also involved in the degradation of ubiquitin tagged cytoplasmic proteins to peptides while, TAP1 and TAP2 are involved in transportation of peptides into the endoplasmic reticulum for exposure to nascent MHC class I molecules (Pamer and Cresswell, 1998). MHC-I molecules are critical in the regulation of cytotoxic effector functions of natural killer (NK) cells and T cells. MHC-I molecules present antigens to cytotoxic T cells and are part of the recognition signals that regulate activation of NK cells (Ulianich *et al.,* 2011). Usually, antigenic peptides are generated by proteasomal degradation of cytosolic proteins and consequently translocated to ER by TAP. In the ER, assembly of MHC class I α chain, β2-microglobulin and peptides are guided by chaperones (Pamer *et al.,* 1998). On the other hand, antigenic peptides are associated with the chaperones GRP94, GP96, PDI and calreticulin (Nieland *et al.,* 1996; Lammert *et al.,* 1997; Spee and Neefjes, 1997;

Nicchitta, 1998). Functional class I-peptide complexes are then transported to the cell surface. A necessary condition for the successful completion of this complex process is glycosylation and correct folding of MHC class 1 heavy chain in the ER. A failure in the above results in their slow or inefficient transport to the cell surface (Carreno *et al.,* 1995; Degen *et al.,* 1992; Noessner *et al.,* 1995; Rajagopalan and Brenner, 1994; Parham, 1996). ER stress may result in reduced expression of MHC class I on the cell surface, thereby preventing the recognition of cells by the adaptive and innate immune system (Gleimer *et al.,* 2003; Hickman-Miller *et al.,* 2004). It has been reported that defects in the expression of different varients of the MHC class I antigen processing machinery, such as the proteasomal subunits LMP2 and LMP7 and the peptide transporters TAP1 and TAP2, account for impaired MHC class I surface expression (Casp *et al.,* 2003). In addition, an inappropriate functioning or expression of LMP7 might inhibit antigen processing and presentation, leading to a loss of peripheral tolerance to self-antigens and occurrence of several autoimmune diseases (Casp *et al.,* 2003). In this context, Ulianich *et al*., (2011) have shown ER stress induced decrease/reduction in surface expression of MHC class I in thyroid cells. This effect was accompanied by activation of NK cells and their cytotoxicity to thyroid cells by increased IFN-γ production. Together, these data indicate ER stress induced a reduction in MHC class I expression and reduced NK-cells self-tolerance. It has been shown that IFN-γ induces LMP and TAP subunits (Casp *et al.,* 2003). Taken together these results suggest IFN-γ induces expression of MHC-I, MHC-II, and TAP on melanocytes. Recently it has been shown that IFN-γ induces senescence in melanocytes (Wang *et al.,* 2014). IFN-γ signaling impedes maturation of melanosomes by intensive down regulation of a few pigmentation genes that lead to IFN-γ–mediated hypo- pigmentation of melanocytes (Natrajan *et al.,* 2014). IFN-γ and TNF-α induce the expression of *ICAM1* on melanocytes (Yohn *et al.,* 1990). Our previous lab studies also showed increased levels of IFN-γ, TNF-α, TNF-β and *ICAM1* in vitiligo patients (Dwivedi *et al.,* 2013b; Laddha *et al.,* 2012; Laddha *et al.,* 2013). Further, levels of ICAM-1 were found to be upregulated in melanocytes of the peri-lesional skin of vitiligo patients and melanocyte- T cell binding was enhanced by elevated expression of ICAM-1 on melanocytes (Al Badri *et al.,* 1993). A melanocyte is in close association with ~32 keratinocytes in the epidermal melanin unit. Keratinocytes synthesize 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. In numerous cell types, TNF-α induces apoptosis by the activation of the receptor-mediated apoptotic pathway and also inhibits melanocyte stem cell differentiation (Alghamdi *et al.,* 2012). NACHT leucine-rich repeat protein 1 (NLRP1), known to be involved in inflammation and apoptosis (Martinon *et al.,* 2007), modulates the response of cells towards cytokines such as IL-1β, IFN- and TNF-α. Previously, the increased expression of *NLRP1* in vitiligo patients has been reported from Gujarat (Dwivedi *et al.,* 2013). Bassiouny *et al*., (2011) reported higher levels of IL17 in both the lesional skin and sera of vitiligo patients and its positive correlation with disease progression. Zhao *et al.,* (2006) found decreased levels of cytokine IL10 in vitiligo patients. All the above studies indicate the significant role of immune mechanisms in the progression of vitiligo which is further corroborated by the use of immunosuppressive treatments for vitiligo (Lepe *et al.,* 2003).

1.5.4.1 Impaired Cytokine Theory

Amidst various hypotheses proposed for vitiligo pathogenesis, an autoimmune theory is widely explored where cytokines are the key mediators for cellular communication and networking. Cytokines have crucial functions in the development, differentiation, and regulation of immune cells. As a result, dysregulation of cytokine production or their action is thought to have a central role in the development of autoimmunity (O'Shea *et al*., 2002). Melanocytes express and react to a panoply of cytokines and growth factors and hence they can be considered immuno-competent and immunomodulatory in nature. Moreover, keratinocytes themselves can produce and release pro-inflammatory cytokines such as Interleukin IL-6, IL-1α, and TNF-α which in turn promote the expression of adhesion molecules on the melanocyte membrane such as ICAM-1, promoting further lymphocyte recruitment (Dell'Anna and Picardo, 2006). Epidermal as well as systemic cytokine imbalance between Th1 and Th2 types & pro and anti inflammatory cytokines is well evident in vitiligo. Various cytokines and their possible role in melanocyte biology and vitiligo pathogenesis are described below:

1.5.4.1.1 Interferon gamma (IFN-γ)

IFN-γ is a type II interferon. It is an important activator of macrophages and inducer of MHC Class II molecules. Aberrant IFN-γ expression has been associated with a myriad of inflammatory and autoimmune diseases. Studies by Natarajan *et al.,* (2014) and Son *et al.,* (2014) have reported that IFN-γ inhibits melanogenesis *via* down regulating tyrosinase (*TYR*) and *MITF-M* expression as well as maturation of the melanosome in melanocytes (Yang *et al.,* 2015). A complex interplay of cytokines exists in the skin microenvironment and reports suggest that IFN-γ synergizes with TNF-α and TNF-β in inhibiting the proliferation of various cell types including melanocytes. IFN-γ may participate in the homing of $CD8^+$ T cells to the skin through local induction of chemokines and expression of adhesion molecules on endothelial cells (Bromley *et al.,* 2008). IFN-γ is Th1 cytokine capable of C-X-C Motif Chemokine (CXCL)-10 induction, which further promotes the migration of auto-reactive T cells into the epidermis implicating its importance in disease progression (Rashighi *et al.,* 2014). IFN-γ is able to induce apoptosis in melanocytes, and thus IFN-γ-mediated apoptosis may represent an alternative mechanism through which $CD8^+$ T cells kill their targets in vitiligo (Yang *et al*., 2015). It has been established that IFN-γ is required for depigmentation in mouse models of vitiligo (Gregg *et al.,* 2010; Harris *et al.,* 2012; Harris, 2015) IFN-γ and IFN-γ-induced chemokines, most specifically CXCL10 and its cognate receptor C-X-C Motif Chemokine Receptor (CXCR)-3 are found to be increased in lesional skin and serum samples of patients with vitiligo, and also seen to be upregulated on autoreactive T cells in the blood and skin of vitiligo patients (Rashighi *et al*., 2014; Ezzedine *et al*., 2015; Harris, 2015)**.** Corroborated by mechanistic experiments in mouse models demonstrating the functional requirement of IFN-γ/CXCL10 axis in both, disease progression and maintenance; it is plausible that IFN-γ can be therapeutically targeted to reverse depigmentation (Ezzedine *et al*., 2015; Harris, 2015).

1.5.4.1.2 Tumor Necrosis Factor (TNF)-α and TNF-β

TNF-α is a pro-inflammatory cytokine involved in Th1 mediated response and plays an important role in immune homeostasis. *TNFA* gene resides in close proximity of Human Leucocyte Antigen (HLA) which is one of the most polymorphic sites in human genome. Melanocytes, keratinocytes, and fibroblasts which are predominant in epidermal

microenvironment are capable of TNF-α synthesis which acts both in autocrine as well as paracrine manner and suppress growth and proliferation of melanocytes (Moretti *et al.*, 2002 a). Laddha *et al.*, 2012b, Namian *et al*., 2009; Salinas-Santander *et al*., 2012; Aydingoz *et al*., 2014).

Increased levels of TNF-α have been reported in the lesional skin samples of vitiligo patients (Moretti *et al.*, 2002 a; Grimes *et al.*, 2004; Birol *et al.*, 2006). Laddha *et al*., (2012 b) have shown increased transcript levels of *TNFA* in PBMCs. However, Yu *et al.*, (1997), Singh *et al.*, (2012) and Birol *et al.*, (2006) found no significant difference in TNF-α levels in serum/plasma samples of vitiligo patients. Over all studies suggest that levels of TNF-α definitely play an important role in vitiligo pathogenesis. We have also reported the association of genetic polymorphisms and increased transcript levels of *TNFB* in vitiligo patients and found the same to be correlated with disease activity (Laddha *et al*., 2013 c). TNF-α and TNF-β have found to reduce the expression of the pigment cell-associated antigens Homatropine Methylbromide (HMB)-45 and K.1.2, and enhance the expression of Vascular cell adhesion molecule-1 (VCAM-1) binding integrin (VLA)-2, ICAM-1 and HLA class I antigens and strongly induce HLA-DR (Krasagakis *et al*., 1995). Moreover, reports have shown that TNF-α inhibits melanocyte proliferation and tyrosinase activity in primary cultured melanocytes (Lee *et al*., 2013a; Swope *et al*., 1991). Up regulation of ICAM-1, which is inducible by TNF-α recruit melanocyte directed $CD8^+$ T cells provide an explanation for the selective destruction of melanocytes in vitiligo (Yohn *et al*., 1990).

TNF-β association with vitiligo is limited to a few studies including ours, where significant association of intron 1 (+252A/G) and exon 3 (C/A) polymorphisms of *TNFB* and vitiligo along with increased levels of *TNFB* and *ICAM1* in vitiligo patients is reported (Laddha *et al*., 2013c; Al-Harthi *et al*., 2013). The intron 1 (+252A/G) polymorphism affects a phorbol ester-response element and distinguishes the two alleles suggesting an influence of this SNP on TNFB plasma levels (Messer *et al*., 1991). *In vitro* SNP validation data have shown that *TNFB* exon 3 C/A (Thr26Asn) polymorphism is associated with an increase in the induction of several cell adhesion molecules including ICAM1 (Ozaki *et al*., 2002). Additionally, *in vitro* studies on Normal Human

Melanocyte (NHM) showed that TNF-β stimulation increased ICAM1 expression on melanocytes cell surface (Kirnbauer *et al*., 1992).

1.5.4.1.3 Interleukin (IL)-17

IL-17 is a cystine-linked homodimeric proinflammatory cytokine produced by Th17 cells, which forms a distinct subset of the $CD4^+$ T-cell lineage. IL-17 stimulates the production of IL-1β, TNF-α, and IL-6 (Kolls and Linden, 2004; Liang *et al*., 2006). Th17 cells have been identified in autoimmune skin inflammatory disorders such as psoriasis and atopic dermatitis (Asarch *et al*., 2008; Fitch *et al*., 2009). Bassiouny and Shaker demonstrated elevated IL-17 levels in lesional skin and serum of patients with vitiligo (Bassiouny and Shaker, 2011). A few studies have also reported high serum levels of IL-17 in patients with vitiligo (Khan *et al*., 2012; Zhou *et al*., 2015; Esmaeili *et al*., 2011; Habeb *et al*., 2013). Another study showed a positive correlation between serum IL-17 levels and the extent of the depigmentation patch area in vitiligo, thus suggesting that Th17 cells are involved in vitiligo (Basak *et al*., 2009). Kotobuki *et al*., (2012) have reported infiltration of Th17 cells in addition to $CD8^+$ cells in lesional skin of vitiligo patients. They have also demonstrated that IL-17 affected adversely on the function of melanocytes and dramatically induced IL-1β, IL-6 and TNF-α production in skin-resident cells such as keratinocytes and fibroblasts (Kotobuki *et al*., 2012).

1.5.4.1.4 Interleukin (IL)-1 gene cluster

The IL-1 family consists of the cytokines IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RN), mapped on chromosome 2q14 (Smith *et al*., 2000a; Patterson *et al*., 1993). The importance of IL-1 regulation is evident by the presence of natural antagonist, decoy receptor and other IL-1 family members for its fine regulation. Increased levels of IL-1α and IL-1β are reported in vitiligo (Birol *et al*., 2006; Tu *et al*., 2003; Dani *et al*., 2017). Moreover, our earlier studies have shown increased levels of *IL1B* in non-lesional skin of vitiligo patients indicating its important role in disease progression. However, there was no difference in *IL1A, IL1R1* and *IL1RN* transcript levels in skin samples of vitiligo patients compared to controls (Singh *et al*., 2015; Mansuri *et al*., 2016). Moreover, an association of promoter polymorphisms and transcript levels of *IL1B* with vitiligo was

observed in our previous studies (Laddha *et al*., 2014 a). Additionally, our *in vitro* studies on primary melanocytes have shown that IL1-α decreases melanocyte viability along with its receptor activation (IL1R1). Further, *IL1RN, IL1A, IL1B, IL6, TNFA, ICAM1* showed significantly increased expression while *MITF-M* showed significantly decreased expression upon IL-1α stimulation on NHM; whereas *TYR,* Tyrosinase related protein *(TYRP)1, IL8* and *IL1R1* showed no difference (Singh *et al*., 2016). Similarly, Kotobuki *et al.*, (2012) have shown MITF suppression upon exogenous stimulation of IL-1β on NHM. Currently, there is a paucity of data correlating *IL1RN* polymorphism and vitiligo, where available reports Lee *et al*., (1995b) and Pehlivan *et al*., (2009) showed lack of association which may be attributed to their low sample size warranting further investigation.

1.5.4.1.5 Interleukin (IL)-2

IL-2 exerts a wide spectrum of effects on the immune system, playing a crucial role in the regulation of both immune activation and homeostasis (Gaffen *et al*., 2004). IL-2 signals through the IL-2 receptor, a complex consisting of three chains – α, β and γ. It is a pleiotropic cytokine that drives T-cell growth, augments Natural killer (NK) cells' cytolytic activity, induces the differentiation of regulatory T (T reg) cells and mediates activation-induced cell death (Liao *et al*., 2011). An increased level of soluble interleukin 2 receptors (sIL-2R) is reported in skin as well as sera of vitiligo patients and in few cases was associated with onset of disease (Kasumagic-Halilovic *et al*., 2016; Shi *et al*., 2013 a; Yeo *et al*., 1999; Caixia *et al*., 1999; Honda *et al*., 1997). Moreover, increased serum IL-2 levels have been reported by Khan *et al*., (2012) and Kasumagic-Halilovic *et al*., (2016) in vitiligo patients as compared to controls.

1.5.4.1.6 Interleukin (IL)-6

IL-6 plays an important role during the transition from innate to acquired immunity. Increased levels of IL-6 in the skin (Moretti *et al.*, 2009) and serum (Farhan *et al*., 2014; Singh *et al.*, 2012; Yu *et al*., 1997; Tu *et al*., 2003) have been reported in vitiligo patients. IL-6 secreted by neighboring keratinocytes in epidermal melanin unit is reported to be a paracrine inhibitor of growth and proliferation of melanocytes (Swope *et al.*, 1991).

Moreover, IL-6 also induces and enhances ICAM-1 expression on melanocytes, which promotes melanocyte-leukocyte attachment (Yohn *et al.*, 1990). Additionally, Toosi *et al*., (2012) have reported that exposure of vitiligo inducing phenols (4-tertiary butyl phenol and monobenzyl ether of hydroquinone) to NHM increased expression of X-box binding protein (XBP) 1, XBP1 activation increases IL-6 and IL-8 production. There is a paucity of data on the study of *IL6* genetic variants and vitiligo susceptibility. A single report by Aydingoz *et al*., (2014) have observed the lack of association of *IL6* polymorphism (-174G/C) and vitiligo. Various pro-inflammatory cytokines which are reported to be increased in vitiligo have been found to induce *IL6* expression upon exogenous treatment for e.g. TNF-α induced IL-6 synthesis in glioma cells and human cardiac fibroblasts (Turner *et al*., 2007); IL6 and IL6 receptor modulation by IFN-γ and TNF-α in human monocytic cell line (Sanceau *et al*., 1991).

Kamaraju *et al*., (2002) have demonstrated the inhibition of melanogenesis in melanoma cells by IL-6 through the suppression of MITF-M and tyrosinase expression. MITF is a master transcriptional regulator of melanogenesis and melanocyte survival (Vachtenheim and Borovansky, 2010). Over expression of IL-6 by monocytes and macrophages has systemically profound effects, seen to induce MITF-M suppression (Choi *et al*., 2005).

1.5.4.1.7 Interleukin (IL)-10

IL-10 acts as an anti-inflammatory cytokine wherein Zhao *et al*., (2010) and Ala *et al*., (2015) showed significant decrease in the expression of IL-10 in vitiligo. In addition, Taher *et al*., (2009) have found increased levels of IL-10 after application of topical tacrolimus treatment to patients with vitiligo. On the contrary Grimes *et al*., (2004) and Aydingoz *et al*., (2015) have reported increased levels of *IL10* in skin and serum samples of patients with vitiligo respectively; and after topical application of tacrolimus, Grimes *et al*., (2004) did not find any significant difference in the *IL10* gene expression levels. Genetic variants study of *IL10* -1082, -592 and -819 promoter polymorphisms revealed association of *IL10* single nucleotide polymorphism (SNP) with vitiligo susceptibility (Aydingoz *et al*., 2015, Abanmi *et al*., 2008).

1.5.4.1.8 Interleukin (IL)-4

IL-4 is a hallmark cytokine for Th1 and Th2 polarization inducing differentiation of naive helper T cells (Th0 cells) to Th2 cells. Stimulation of IL-4 leads to further IL-4 production by Th2 cells exhibiting positive feedback loop (Sakol *et al*., 2008). IL-4 levels were decreased in peri-lesional skin, when compared to non-lesional and lesional skin (Wang *et al*., 2011) and significantly lower *IL4* transcript levels were reported by Nouri-Koupaee *et al*., (2015) in vitiligo patients. Suppressing the expression of early cytokines e.g. IL-4, IFN-γ, IL-2 and IL-10, has been found to be effective in vitiligo treatment (Grassberger *et al*., 1999; Nihei *et al*., 1998). On the contrary, Imran *et al*., (2012) have reported increased transcript as well as protein levels of IL-4 in vitiligo patients along with possible genotype phenotype correlation of intron 3 variable number of tandem repeats (VNTR) and -590 C/T (rs2243250) promoter polymorphism. However, Pehlivan *et al*., (2009) did not report any significant association of *IL4* -590 promoter polymorphism with vitiligo.

1.5.4.1.9 Interleukin (IL)-8

Interleukin-8 [C-X-C Motif Ligand (CXCL8)] is a chemo attractant cytokine recruiting neutrophils in inflammatory regions further which might play an important role in the onset of vitiligo. IL-8 is produced by monocytes, mast cells, fibroblasts, endothelial cells, dendritic cells as well as keratinocytes (Luger *et al*., 1990). TNF-α has been shown to induce *IL8* mRNA expression in melanoma cells (Mohler *et al*., 1996) and up regulates IL-8 receptor expression in NHM (Norgauer *et al*., 2002). IL-1β and TNF-α stimulation on NHM showed increased expression of IL-8 both at transcript as well as protein levels. Dani *et al*., (2017) and Miniati *et al*., (2014) have shown increased *IL8* transcript levels in lesional skin of vitiligo patients. Furthermore, anti melanocyte antibodies induce HLA-DR and ICAM-1 expression on melanocytes along with release of IL-8 (Li *et al*., 2000). Moreover, Toosi *et al*., (2012) have shown that vitiligo inducing phenols lead to increased expression of IL-8 in NHM. An immunomodulator 'Imiquimod' promotes secretion of IL-6, IL-8 and IL-10, which are pro-inflammatory and pro-apoptotic cytokines that may cause vitiligo (Dahl *et al*., 2002). Significant increase in spontaneous

production of IL-6 and IL-8 in mononuclear cells (MNC) isolated from vitiligo patients was observed (Yu *et al*., 1997). These changes may enhance the antigen-presenting activity of the cells and potentiate the antigen-specific immune effector cell attack resulting in melanocytotoxicity.

1.5.4.1.10 Interleukin (IL)-21

IL-21 is produced by Th17 cells and other activated $CD4^+$ T cells with pleiotropic functions (Caruso *et al*., 2009). Shi and Erf (2012) have shown increased production of IL-21 in addition to IL-10, IFN-γ with progressive melanocyte loss in the Smyth line (SL) of chicken vitiligo model. Moreover, increased levels of IL-21, IL-17A and Th-17 cells are reported in the sera of non-segmental vitiligo patients (Zhou *et al.,* 2015). Dysregulated cytokine milieu in the skin microenvironment provides a suppressive environment for Treg cell differentiation, migration and function (Ben Ahmed *et al*., 2012; Elela *et al*., 2013). Serum levels of IL-22 were found to be non-significant in vitiligo patients when compared to controls (Zhou *et al.,* 2015).

1.5.4.1.11 Interleukin (IL)-23

IL-23 is a cytokine secreted by activated dendritic and phagocytic cells, dermal Langerhans cells in addition to keratinocytes and play an important role in autoimmune disorders (Piskin *et al*., 2006). IL-23 induces the differentiation of Th17 cells in a pro-inflammatory context, especially in the presence of transforming growth factor (TGF)-β and IL-6 (Maddur *et al*., 2012). IL-23R is expressed by inflammatory macrophages, which are activated to produce IL-1, TNF-α, and IL-23 itself (Duvallet *et al*., 2011). However, there are inconsistent reports associating IL-23 and vitiligo. Vaccaro *et al*., (2015) and Wang *et al*., (2010) have shown increased serum levels of IL-23 whereas Osman *et al*., (2015) and Zhou *et al*., (2015) have shown non-significant difference of IL-23 levels. There was a significant positive correlation of IL-23 serum levels with disease duration and extent of vitiligo and disease activity (Vaccaro *et al*., 2015).

1.5.4.1.12 Interleukin (IL)-33

Vaccaro *et al*., (2016) and Li *et al*., (2015) have shown increased levels of IL-33 in serum and skin respectively in vitiligo patients. IL-33 levels are found to be co-related with disease activity (Vaccaro *et al*., 2016). IL-33 is newly discovered IL-1 family member which binds to interleukin 1 receptor-like 1 protein (ST2) present on keratinocytes in addition to other epithelial cells. Apoptotic keratinocytes secrete IL-33 in response to combined TNF-α and IFN-γ stimulation. IL-33 has been reported to inhibit stem cell factor (SCF) and basic Fibroblast Growth Factor (bFGF) and augment TNF-α and IL-6 expression in keratinocytes (Li *et al*., 2015).

1.5.4.1.13 Granulocyte-macrophage colony stimulating factor (GM-CSF)

GM-CSF is a part of the family of hematopoietic cytokines associated with modulation of the immune system. Moretti *et al.,* (2002) have shown decreased expression of GM-CSF in skin of vitiligo patients which are consistent with various other studies (Yu *et al*., 1997; Abdellatif *et al*., 2015). On the contrary, Tu *et al*., (2003) have found increased serum levels of GM-CSF. GM-CSF secreted by keratinocytes plays an essential role in the maintenance of melanocyte proliferation and UVA-induced pigmentation in the epidermis (Moretti *et al.,* 2002; Imokawa *et al*., 1996). Other melanogenic cytokines, including stem cell factor and endothelin-1 (EDN-1), are also lowered in depigmented lesions (Moretti *et al*., 2002).

1.5.4.1.14 Regulatory T cell (Treg) and transforming growth factor (TGF)-β

TGF-β is an important immunoregulatory cytokine mainly produced by immune cells inclusive of T-regulatory $CD4^+$ cell subset (Treg). TGF-β plays an important role in conversion of inducible Treg cells by Forkhead Box P3 (FOXP3) induction on $CD4^+CD25^-$ cells (Fu *et al*., 2004). Various reports including ours suggest decreased levels of skin and blood Treg cells in vitiligo with concomitant decrease in FOXP3 expression which is a marker for Treg cells identification (Dwivedi *et al*., 2015). However, various reports suggest both increased (Zhou *et al*., 2015; Tembhre *et al*., 2013) as well as decreased levels of TGF-β in vitiligo patients (Basak *et al*., 2009; Khan

et al., 2012; Tu *et al*., 2011; Osman *et al*., 2015). Moreover, Gambichler *et al*., (2007) have reported decreased levels of TGF-β upon topical therapies and phototherapy which are frequently used for vitiligo treatment. Fully restored depigmentation in h3TA2-IFN-γ deficient mice with the Treg cell depletion correlates with increased IL-17 expression in autoreactive T cells (Chatterjee *et al*., 2014).

1.5.4.1.15 PRO2268

This gene of unidentified function lies adjacent to a region containing the *IFNG-IL26-IL22* gene cluster (12q14 chromosomal region) and its gene product plays crucial role in vitiligo pathogenesis. Douroudis *et al*., (2011a and 2011b) reported increased levels of PRO2268 in vitiligo patients and psoriasis vulgaris skin respectively. Moreover, *PRO2268* (rs10784680) has shown to confer genetic susceptibility towards vitiligo (Douroudis *et al*., 2011a).

The reports of various polymorphisms in vitiligo patients and controls, along with genotype-phenotype correlation, sample size have been discussed in Table 4.

Table 4: Reports of various polymorphisms in vitiligo patients and controls

Cytokine	**Sample Type/ Sample Size: Patients (Controls) & Ethnicity**	**Genotype-Phenotype correlation**	**Salient features of polymorphism**
Interleukin 1 *(IL1)*			
**IL1B* promoter -511 C/T (rs16944) (Laddha *et al.*, 2014)	PBC/ 448 (785) Indian (Gujarat)	Increased *IL1B* in vitiligo patients, AV and females.	TT genotype showed significantly increased *IL1B*
ILRN	PBC/	-	These tandem VNTR might act

intron 2 VNTR (rs2234663) (Pehlivan *et al.*, 2009)	48 (50) Turkish		as putative binding sites for transcription factors. (Tarlow *et al.*, 1993)
ILRN (rs2234663) (Lee *et al.*, 1995)	PBC/ 31 (79) Korean	-	
Interleukin 4 *(IL4)*			
**IL4* intron 3 VNTR (rs2243250) promoter -590 (rs2243250) (Imran *et al.*, 2012)	PBC/Serum 1101 (1141) Indian (Gujarat & North India)	Increased what in Gujarat (low prevalence in North India)	The three tandem repeat alleles enhance *IL*4 transcription (Nakashima *et al.*, 2002). -590 (rs2243250) C to T transition is associated with enhanced promoter strength
IL4 promoter -590 (rs2243250) **IL4R* exon (rs1801275) (Q155R) (Al-Shobaili *et al.*, 2012)	Whole blood/ 96 (87) Arab	N/A	Q551R : The R576 allele was associated with higher levels of expression of CD23 by interleukin-4 than the wild-type allele (Hershey *et al.*, 1997)
IL4 promoter -590 C/T (rs2243250) (Pehlivan *et al.*, 2009)	PBC/ 48 (50) Turkish	Increased susceptibility to vitiligo	
Interleukin 6 (*IL6*)			
promoter -174 C/G (rs1800795) (Aydıngoz *et al.*, 2014)	PBC/ 105 (211) Turkish	N/A	Regulatory effect on its transcript as well as protein levels with respect to G allele (Fishman *et al.*, 1998)
Interleukin 10 (*IL10*)			
*promoter -1082 (rs1800896) (Aydıngoz *et al.*, 2014)	Serum/ 105 (211) Turkish	N/A	-1082 GG, -819 CC & -592 CC promoter alleles are associated with higher *IL10*

*promoter -1082 C/G (rs1800896) * -819 C/T (rs1800871) * -592C/A (rs1800872) (Abanmi *et al.*, 2008)	Whole blood/ 83 (101) Arab	N/A	production. (Asadullah *et al.*, 2003)
Transforming growth factor, beta receptor II (*TGFBR2*)			
*intron variant C/T (rs2005061) C/G (rs3773645) G/A (rs3773649) (Yun *et al.*, 2010)	Whole blood /233 (415) Korean	N/A	-
Interferon-γ (*IFNG*)			
intron 1 +874 A/T (rs2430561) * CA microsatellite (rs3138557) (Dwivedi *et al.*, 2013)	PBC/ 517 (881) Indian (Gujarat)	N/A increased IFN-γ & ICAM-1, higher in females	Allele 2, with 12 CA repeats is associated with *in vitro* constitutive high IFN-γ production (Dwivedi *et al.*, 2013). Presence of *IFNG* (+874A/T) polymorphism creates a putative NF-kB binding site showing preferential binding to the T allele corresponding to increased IFN-γ production (Pravica *et al.*, 2000).
intron 1 +874 A/T (rs2430561) (Namian *et al.*, 2009)	PBC/ 176 (545) Iranian	N/A	
NACHT, LRR and PYD domains-containing protein 1 (*NLRP1*)			
*promoter A/G (rs2670660) T/C (rs6502867) A/T (rs12150220) (Dwivedi *et al.*, 2013)	PBC/ 537 (645) Indian (Gujarat)	N/A	*NLRP1* (rs2670660) and (rs6502867) SNPs are associated with increased *NLRP1* mRNA expression.(Dwivedi *et al.*, 2013)
*promoter A/G (rs1008588) A/G (rs2670660)	PBC/ 26 (61)	N/A	

T/A (rs11078587) T/C (rs8182352) A/T (rs12150220) A/G (rs16954840) T/C (rs8074853) T/C (rs6502867) (Alkhateeb *et al.*, 2010)	Jordanian Arab		
intron variant *G/A (rs6502867) T/C (rs2670660) *A/G (rs8182352) A/G (rs8074853) T/C (rs16954840) T/A (rs11078587) C/T (rs1008588) (Jin *et al.*, 2007)	PBC/ 66 (93) Caucasian/ Romanian		
Melanocyte proliferating gene 1 (*MYG1*)			
*promoter -119 (rs1465073) (Dwivedi *et al.*, 2013)	PBC/ 846 (726) Indian (Gujarat)	N/A	-119C/G promoter poly-morphisms in the mitochondrial signal of *MYG1* have a functional impact on the regulation of the *MYG1* gene. (Philips *et al.*, 2010)
*promoter -119 (rs1465073) (Philips *et al.*, 2010)	Skin biopsies/ 124 (325) & Europeans	Expressed more in AV	
Tumor Necrosis Factor-β *(TNFB)*			
* intron 1 +252 G/A (rs909253) (Al-Harthi *et al.*, 2013)	Whole blood/ 123(200) Saudi	Marginally increased expression	rs909253 and rs1041981 are found to influence *TNFB* expression *in vitro* (Messer *et al.*, 1991; Whichelow *et al.*, 1996)
* intron 1 +252	Whole blood	Increased	

A/G(rs909253); IVS1 +90 A/G exon 3 C/A (rs1041981) (Laddha *et al.*, 2013)	524 (592) Indian (Gujarat)	*TNF*-β and *ICAM*-1	
Tumor Necrosis Factor-α *(TNFA)*			
* promoter -308 G/A (rs1800629) (Al-Harthi *et al.*, 2013)	Whole blood/ 123 (200) Saudi	N/A	The promoter polymorphisms at positions: -238, -308, -857, and -1031 may lead to a higher rate of *TNFA* gene transcription whereas -863 leads to decrease in the *TNFA*.
*promoter -308 G/A (rs1800629) (Salinas-Santander *et al.*, 2012)	Whole blood/ 198 (395) North eastern Mexican population	N/A	
*promoter -308 G/A (rs1800629) (Laddha *et al.*, 2012)	Whole blood/ 977 (990) Indian (Gujarat)	Increased *TNF*-α transcript protein	
* -238 G/A (rs361525), -857 C/T (rs1799724), *-1031 (rs1799964) (Laddha *et al.*, 2012)	Whole blood 524 (592) Indian (Gujarat)	-238: Increased susceptibility -857: Increased susceptibility -1031: Decreased susceptibility to vitiligo	

*promoter -308 G/A (rs1800629) (Namian *et al.*, 2009)	PBC/ 176 (545) Iranian	N/A	
promoter -308 G/A (rs1800629) (Yazici *et al.*, 2006)	Whole blood/ 61 (123) Turkish	N/A	
Cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*)			
exon 1 +49 (rs231775) * 3'UTR CT60 (rs3087243) (Dwivedi *et al.*, 2011)	PBC/ 437(746) Indian (Gujarat)	N/A GG genotype shows higher phenotypic occurrence	Increased *sCTLA-4 / flCTLA-4* mRNA ratio with +49AG and CT60GG genotypes in vitiligo patients suggest that G allele confers lower *sCTLA-4* transcript levels. (Dwivedi *et al.*, 2011 ; Atabani *et al*. 2005)
*exon 1 +49 (rs231775) Pehlivan *et al.*, 2009	PBC/ 48 (50) Turkish	Susceptibility increased in patients with GG genotype, while reduced in AA phenotype	
3'UTR CT60 (rs3087243) exon 1 +49 (rs231775) (rs1863800) (rs11571302) (rs11571297) (rs10932037) LaBerge *et al.*, 2008	PBC/ saliva/ 712(none) Caucasian	N/A	
3'UTR (rs3087243) (rs11571302) (rs11571297)	Whole blood/ 100 (140)	N/A	

exon 1 +49 (rs231775) (Blomhoff *et al.*, 2005)	UK		
exon3, 106 bp allele (Kemp *et al.*, 1999)	Whole blood/ 74 (173) British	Susceptibility observed only for patients who have other autoimmune disease condition	
C-C Chemokine Receptor 5 (*CCR5*)			
32 bp deletion (Pehlivan *et al.*, 2009)	PBC/ 48 (50) Turkish	N/A	32 base pair deletion (CCR5-delta32) that introduces a premature stop-codon into the CCR5 locus (Bream *et al.*, 1999)
Protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*)			
*1858T C/T (rs2476601) (La Berge *et al.*, 2008)	PBC/ saliva/ 126 (none) Caucasian	N/A	The C1858T substitution causes a change R to W at codon 620 preventing the binding of P1 proline-rich motif to the SH3 domain of Csk. *In vitro* experiments have shown that the T-allele binds less efficiently to Csk than the C-allele, suggesting that T-cells expressing the T-allele may be hyper-responsive, and consequently, individuals carrying this allele may be prone to autoimmunity (Bottini
1858T C/T (rs2476601) (Laddha *et al.*, 2008)	Whole blood/ 126 (140) Indian (Gujarat)	N/A	
*missense R620W, codon620 1858T C/T (rs2476601) (Canton *et al.*, 2005)	Whole blood/ 165(304) Caucasian	Expression observed typically in GV	

			et al., 2004; Begovich *et al.*, 2004)
Autoimmune Regulator (*AIRE*)			
*C–103T,*C4144G, *T5238C, *G6528A, *T7215C, T11787C, (Tazi-Ahnini *et al.*, 2008)	Whole blood 86 (63) Caucasian	T11787C, susceptibility increased only for those who have other autoimmune disease along with vitiligo	*AIRE* C–103T, G6528A, T7215C and T11787C haplotype CGCC gives the most stable mRNA secondary structure, suggesting that *AIRE* CGCC mRNA could have a longer half-life and produce more AIRE protein, leading to dysregulation of downstream genes. (Tazi-Ahnini *et al.*, 2008)
Glutathione Peroxidase (*GPX*)			
* exon 1 R5P G/C (rs8179169) *exon 1 L6P T/C (rs4991448) exon2 A194T C/T (rs6446261) (Mansuri *et al.*, 2016)	PBC/ 521 (614) Indian (Gujarat)	decreased GPx1 activity decreased GPx1 activity N/A	R/P genotype forR5P SNP; L6P SNP, P/P and L/P genotypes, showed significantly decreased GPX1 activity and decreased stability of L6P and A194T variants. (Mansuri *et al.*, 2016)
codon 200 P to L (rs1050450) (Casp *et al.*, 2002)	PBC/ 126 (143) Indian (Gujarat)	N/A	
Superoxide Dismutase (*SOD*)			
**SOD1* (Laddha *et al.*, 2013) C/T I40T, C/T V82V (rs11556619) A/G N87S(rs11556620), C/T N140N (rs1804449)	PBC/ 950 (1650) Indian (Gujarat)	Increased susceptibility to vitiligo observed	The SOD2 T/C (V16A) polymorphism in the mitochondrial targeting sequence may influence the efficiency of SOD2 transport followed by the alteration of

**SOD2* (Laddha *et al.*, 2013) C/T T58I (rs35289490),C/T L84F(rs11575993), C/T V16A (rs4880)		Altered oxidative metabolism along with increased susceptibility to AV	SOD2 activity in Val allele carriers. (Sutton *et al.*, 2003)
**SOD3* (Laddha *et al.*, 2013) G/A R213G(rs8192291)		increased susceptibility to vitiligo	
Melanocortin-1 Receptor (*MC1R*)			
* G274A (V92M), A488G (R163Q) (Na *et al.*, 2003)	PBC/ 114 (111) Korean	Presence of both SNPs together causes higher vitiligo susceptibility	V92M is commonly associated with poor tanning. (Valverde *et al.*, 1995)
Transporter associated with Antigen Processing 1 (*TAP1*)			
TAP1 exon10 A/G (Casp *et al.*, 2003)	Whole blood/ 230 (188) Caucasian	-	*TAP1* exon 10 A>G leads to variation in TAP1 protein from Aspartic acid to Glycine at position 637 (Quadri and Singal,1998)
Proteasome subunit beta type-8 (PSMB8)/ Low Molecular Mass Polypeptide 7 (*LMP7*)			
LMP7 intron 6 G/T (Casp *et al.*, 2003).	Whole blood/ 230 (188) Caucasian	Heterozygous, MHC class 1 Ag	-
**LM7/PSMB8* exon 2 A/C (Dani *et al.*, 2017).	1320 (752) North Indian		*PSMB8* exon 2, in codon 49, substitution of C to A leads to amino acid change from

			glutamine to Lysine
Estrogen Receptor 1 (*ESR1*)			
*intron 1 C/T (rs2234693), *exon 4 C/G (rs1801132), *exon 8 A/G (rs2228480) (Jin *et al.*, 2004)	Serum/ 120 (254) Korean	Expressivity higher in Female GV patients.	-
Uncharacterized protein PRO2268 (*PRO2268*)			
*(rs10784680) (Douroudis *et al.*, 2011)	PBC/ 100 (194) Caucasian	Increased susceptibility to vitiligo	-
Endothelin 1 (*EDN1*)			
G5665T, T-1370G C +70G, G-231A Bingül *et al.*, 2016.	PBC/ 100 (185) Turkish	Decreased susceptibility to vitiligo	GG genotype of T-1370G polymorphism is associated with the low production of *EDN1*. (Barden *et al.*, 2001)
*intron 4 G/A (rs2071942) * exon 5 G/T (rs5370) (Kim *et al.*, 2007)	Cultured keratinocytes from skin 312 (313) Korean	-	
Toll Like Receptor (*TLR*)			
**TLR2* R753Q	Whole blood/ 100 (100) Turkish	N/A	TLR2 R753G could lead to diminished activation of intracellular signaling pathways (Xiong *et al.*, 2012)
**TLR4* D299G (Karaca *et al.*, 2012)	Whole blood/ 100 (100) Turkish	N/A	D299G is in the leucine-rich repeat (LRR) domain of Exon 3 which is associated with the recognition of pathogen-associated molecular patterns (PAMPs) (Smirnova *et al.*, 2000).

X-box binding protein 1 (*XBP1*)			
*-116 G/C (rs2269577) Ren *et al.*, 2009 *-116 G/C (rs2269577) (Birlea *et al.*, 2011)	PBC/ 319(294) Chinese 2629 (1392) Caucasian	Increased expression of *XBP1* in the lesional skin of patients carrying the risk associated C allele.	*XBP1*-116 C/G SNP changing the consensus motif ACGT into AGGT at nucleotide−116 of the *XBP1*, identified by Kakiuchi *et al.*, and it associated with significantly decreased XBP1 transcriptional activity (Kakiuchi *et al.*, 2003)

1.6 Cytokine interplay affecting melanin synthesis via Microphthalmia Associated Transcription Factor (MITF)-M

The MITF is a master transcriptional regulator of melanogenesis and melanocyte survival (Levy *et al*., 2006). It has been shown that exogenous stimulation of vitiligo inducing phenols to melanocytes causes increased expression of IL-17 and TNF-α with a concomitant increase in the expression of cytokines CXCL1 and 3, IL-6, IL-8, and C-C Motif Chemokine (CCL)-20 (Toosi *et al*., 2012). Kotobuki *et al*., (2012) have reported that a combination of cytokines i.e. TNF-α, IL-1β, IL-6 and IL-17 could inhibit melanin production. Cytokines and oxidative stress are also reported to regulate the melanin synthesis pathway and one of the target molecules for this could be MITF. It is a positive regulator of TYR, TYRP1, TYRP2 and Dopachrome Tautomerase (DCT) and is crucial for lineage commitment of melanocytes during differentiation from neuronal crest derived cells (Widlund and Fisher; 2003). MITF-M is a melanocyte specific isoform and plays an important role in melanocyte biology. However, there is paucity of studies correlating vitiligo and MITF.

Vitiligo patients show reduced expression of MITF-M in perilesional (Kitamura *et al*., 2004) and lesional as well as non lesional skin compared to controls (Kingo *et al*., 2008). Various pro inflammatory cytokines and H_2O_2 have been reported to suppress melanin

synthesis. TNF-α, which is increased in vitiligo down regulates MITF expression in NHM (Wang *et al.*, 2013; Skov *et al.*, 1998). IL-17 can dramatically amplify the inhibitory effect of TNF-α on melanogenesis (Wang *et al.*, 2013). Significantly high levels of both IL-1α and IL-1β have been detected in patients with vitiligo (Kholmansik *et al*., 2010). Moreover, IL-1α is shown to significantly down-regulate MITF-M levels in NHM (Singh *et al.*, 2016). IL-1β seems to have a characteristic role in the down-regulation of MITF-M at the gene and protein levels (Arts *et al*., 2015).

Cytokines exhibit a complex network of auto and paracrine regulation of other cytokines and numerous studies have shown their inducible nature, for example IL-17A has been shown to extensively upregulate IL-6, IL-1β and TNF-α production in fibroblast and keratinocytes of the skin (Kotobuki *et al*., 2012). Kamaraju *et al*., (2002) have demonstrated the inhibition of melanogenesis in melanoma cells by IL-6 through the suppression of MITF and tyrosinase expression. Lack of a direct inhibition of melanin pigment synthesis, IFN-γ and IL-17A increased the synthesis of an anti-melanogenic cytokine IL-6 in NHM (Choi *et al*., 2005). NHM secreted IL-6 proteins in culture supernatants in response to IFN-γ or IL-17A treatment (Choi *et al.,* 2005). Melanogenesis of NHM is inhibited by IL-6 produced by keratinocytes through paracrine regulation (Imokawa, 2004; Choi *et al*., 2005). IFN-γ and IL-17A increased IL-6 production in epidermal keratinocytes (Fujisawa *et al*., 1997; Peric *et al*., 2008). Studies by Gutknecht *et al.,* (2015), have shown that IL-10 congruently inhibits the PI3K/Akt signaling and other pigmentary pathways through activation of MITF in dendritic cells, resulting in a tolerogenic phenotype. IL-4 directly inhibited melanogenesis in NHM and down-regulates both transcription and translation of melanin synthesis associated genes, such as *MITF* and *DCT* (Choi *et al.,* 2005). IL-4 treatment significantly down regulates the mRNA levels of *MITF, TYR, DCT,* melanoma antigen recognized by T cells *(MART)-1,* and Glycoprotein 100/ Premelanosome Protein *(gp100/PMEL17)* (Choi *et al.,* 2005).

Oxidative stress seems to have a crucial role in triggering onset of vitiligo (Shi *et al.,* 2015; Laddha *et al*., 2014 b). Shi *et al*., (2015) suggest that the activation of miR-25 under oxidative stress could suppress the antioxidant response through inhibiting MITF-APE1 pathway, which makes melanocytes more susceptible to oxidative stress-induced destruction. Studies by Liu *et al.,* (2008) and Jiménez-Cervantes *et al.,* (2001) also

suggest MITF-M down-regulation *via*. apurinic/apyrimidinic endonuclease (APE-1) under oxidative stress.

1.7 Role of cytokines in Endoplasmic Reticulum stress

ER stress-induced UPR signaling is also associated with the production of cytokines such as TNF-α, IL-6, IL-8, IL-1β, IL-23, Monocyte Chemoattractant Protein (MCP)-1, etc. (Li *et al.*, 2005; Garg *et al*., 2012). All three main branches of the UPR i.e., PERK, IRE1α, and ATF6 have been shown to mediate pro-inflammatory transcriptional programs, which are mainly governed by transcription factors such as NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-1 (Activator protein 1) (Verfaillie *et al*., 2010; Hotamisligil *et al.*, 2008; Zhang *et al*., 2008). NF-kB is one of the central mediators of pro-inflammatory pathways. Genes transcribed by NF-kB include those encoding crucial pro-inflammatory cytokines such as TNF-α, IFN-γ, IL-6, IL-8, IL-1β, IL-23, IL-17 (Li *et al*., 2005; Zhang *et al.*, 2008; Rius *et al*., 2008; Pahl *et al*., 1999). Toosi *et al*., (2012) have reported that vitiligo inducing phenols activate UPR in melanocytes and upregulate the expression of IL-6 and IL-8. In addition *XBP1* inhibitors have been shown to reduce IL-6 and IL-8 production induced by phenols (Toosi *et al*., 2012). We recently reported significantly increased transcript levels of CCAAT-Enhancer-Binding Protein (CHOP) and IL-23 in the skin of vitiligo patients providing an evidence of ER stress induced inflammation in vitiligo (Mansuri *et al*., 2016). Expression of IFN-γ (Watanabe *et al*., 2003), IL-1β (Akerfeldt *et al*., 2008, Gurzov *et al*., 2009), TNF-α, IL-6 (Zhang, *et al*., 2006), IL-17 by immune cells can generate further ER stress. Xin *et al*., (2005) have observed that TNF-α induces the UPR in a ROS-dependent fashion and leads to cell death. It has been reported that IL-17 mediates the production of other cytokines, including IL-1 and IL-6 and can potentiate other local inflammatory mediators like TNF-α (Kolls *et al*., 2004).

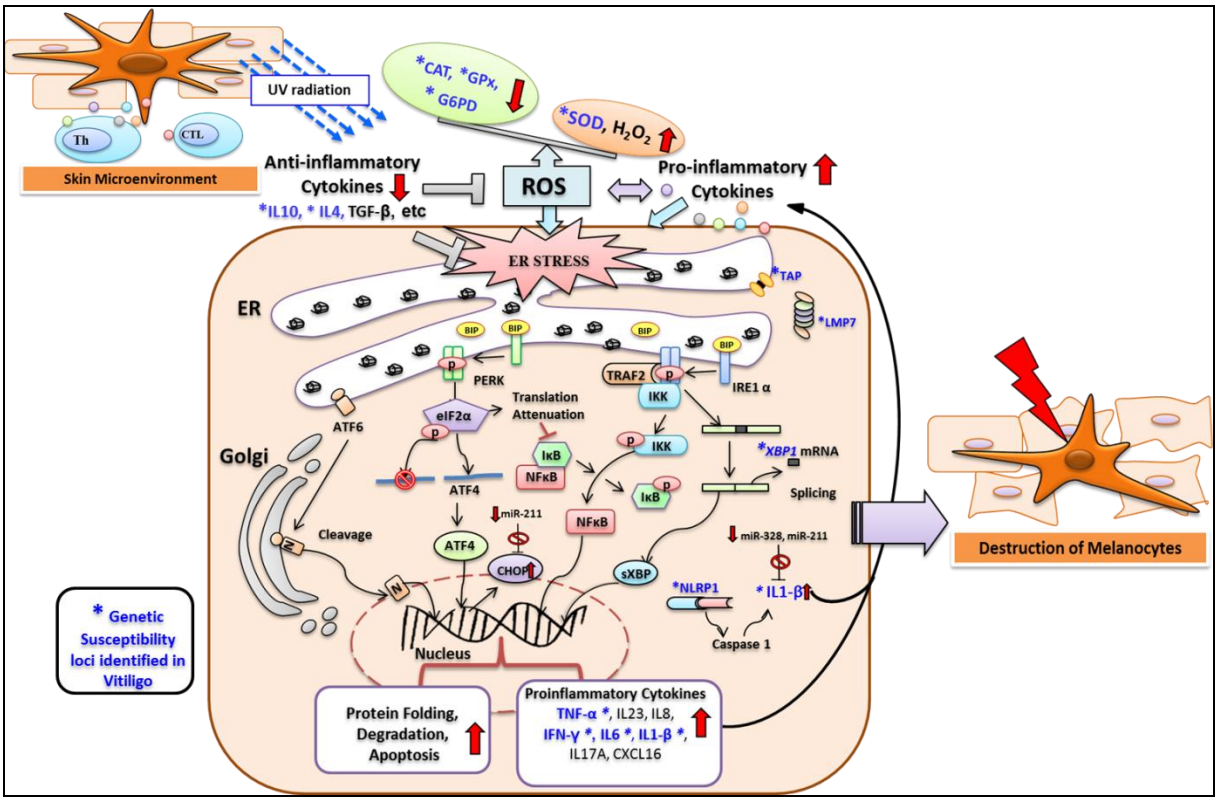


Figure 7: Interplay of oxidative stress, ER stress and inflammatory cytokines leading to melanocyte destruction in vitiligo

Various stressors in the skin microenvironment such as UV radiation, H_2O_2, pro-inflammatory cytokines secreted by immune cells and decreased levels of antioxidant and anti-inflammatory cytokines leads to generate oxidative stress and ER stress in the vitiliginous skin. ER stress activates unfolded protein response to resolve the stress however; prolonged ER stress leads to IRE1-α, PERK, and ATF6-mediated inflammatory transcriptional program through NFκB activation. NFκB induces expression of proinflammatory cytokines such as TNF-α, IL6, IL8, IL23, IL-1β, IL17A, etc which further generates ER stress and oxidative stress. Thus, altogether cross talk of oxidative stress, ER stress and immunity in addition to genetic predisposition and altered miRNA regulation leads to destruction of melanocytes in vitiligo.

1.8 Immunoregulatory role of miRNAs in Vitiligo

Micro-RNAs (miRNAs) are involved in the cellular regulatory pathways by affecting the expression of approximately 60% human genes at both post-transcriptional and translational levels (Sayed and Abdellatif, 2011). Atypical expression of several miRNAs in the skin and blood of patients with vitiligo has been demonstrated by several reports (Mansuri *et al*., 2014, 2016; Shi *et al*., 2013; Wang *et al*., 2015). Recently, Shahmatova *et al*., (2016) have reported up regulation of miR-155 in the lesional skin of vitiligo patients. Interestingly, miR-155 was also induced in response to TNF-α, IFN-α, IFN-γ, and IL-1β in melanocytes and keratinocytes. Upon over expression, miR-155 inhibited the expression of genes known to affect melanocyte differentiation and melanogenesis, such as *TYRP1, YWHAE, SDCBP* and *SOX10* in melanocytes (Shahmatova *et al*., 2016). Our recent study revealed that miR-1, miR-184, miR-328, miR-383, and miR-577 hold the similar pattern of expression in blood as that of skin (Mansuri *et al*., 2016). Our *in silico* results suggest that both miR-328 and miR-211 might target *IL1B* whereas; miR-1 and miR-211 might target IL1R1. Interestingly, our study suggests the down regulation of *PTPN22* might be influenced by miR-577 in skin as well as in circulation of vitiligo patients (Mansuri *et al*., 2016). The up-regulation of *IL1B*, *IL1R1*, and *IL23A* and, down-regulation of *PTPN22* in patients advocates its crucial role in the autoimmune pathogenesis of vitiligo. miR-211 also regulates *CHOP via* directly targeting the *CHOP* promoter (Chitnis *et al*., 2012). Further, CHOP regulates *IL23* expression and secretion from dendritic cells (Goodall *et al*., 2010). miR-211 also targets *IL23A* and is mainly produced by dendritic cells and macrophages, and in conjunction with IL6 and TGF-β1 plays a pivotal role in the induction of Th17 cells and secretion of IL17A (Bettelli *et al*., 2008). Our study showed significantly increased expression of *CHOP* and *IL23A* in lesional skin of vitiligo patients (Mansuri *et al*., 2016). Thus, the reports indicate that miRNAs have a collective role in oxidative stress, ER stress and autoimmunity in melanocyte destruction and further progression of the disease.

1.9 Poy(ADP-ribose) polymerase-1 (PARP-1) and vitiligo

Poly (ADP-ribose) polymerase-1 (PARP-1), a 116-kDa nuclear multitasking protein is involved in modulation of chromatin condensation leading to altered gene expression. It belongs to a family of ubiquitous nuclear enzymes that catalyzes NAD+ dependent addition of ADP- ribose polymers (PAR) to an array of acceptor proteins (D'Amours *et al*., 1999). In response to activation signals, it adds ADP-ribose units to various target proteins including itself, thus regulating various key cellular processes like DNA repair, cell death, transcription, mRNA splicing etc. (Jubin *et al*., 2016).

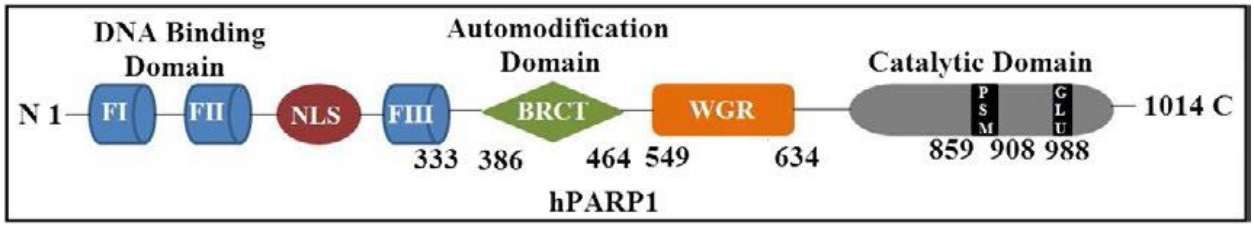


Figure 8: Structural organization of human PARP-1 (hPARP-1) It is characterized by FI, FII: Zinc finger motifs, FIII: Zinc ribbon domain (1-333 aa); NLS: Nuclear localization sequence; BRCT: BRCA1 C terminal motif (386-464 aa); WGR domain (549-634 aa) and the most conserved catalytic domain with PARP signature motif (PSM) between 859-908 aa and Glutamate (Glu) at 988 position (Jubin *et al*., 2016).

1.9.1 PARylation

Ubiquitous PARP activity has been found in organisms ranging from archae bacteria to mammals, apparently absent in yeast (Rolli *et al*., 2000). PARP-1's basal enzymatic activity is very low, but is stimulated dramatically in the presence of a variety of allosteric activators, including damaged DNA, some undamaged DNA structures, nucleosomes, and a variety of protein-binding partners (D'Amours *et al*., 1999; Oei and Shi 2001; Kun *et al*. 2002, 2004; Kim *et al*. 2004). PARP-1 binds to a variety of DNA structures, including single- and double-strand breaks, crossovers, cruciforms and supercoils as well as some specific double-stranded sequences (Lonskaya *et al*., 2005). PARP-1 mainly functions as a DNA damage sensor. In response to DNA damage, PARP-1 activity is rapidly increased upon binding to DNA strands containing nicks or breaks. On binding to the damaged DNA, PARP-1 forms homodimers and catalyzes the cleavage

of NAD^+ and converting it into nicotinamide and ADP-ribose. ADP-ribose formed is utilized in formation of PAR polymer which is covalently attached to the acceptor protein. The attachment of PAR to the protein is at Glutamate and Lysine residues through an ester linkage.

1.9.2 Functions of PARP-1

PARP-1 is involved in various biological and cellular processes like DNA damage detection and repair, chromatin modification, transcription, cell death pathways, insulator function, and mitotic apparatus function.

PARP-1 identifies the genomic lesions such as SSBs, DSBs, non-BDNA structures etc., gets activated, and recruits itself at the deformed site, forming homodimers and triggering the transfer of an ADP-ribosyl moiety from NAD+ to aspartate or glutamate residues of its target substrates in the chromatin, mainly linker histone H1. This leads to structural changes involving unwinding of the highly condensed chromatin that renders the DNA easily accessible to transcription and repair enzymes (Rouleau *et al.,* 2011; Tulin and Spradling, 2003).

1.9.3 PARP-1 in Cellular-Stress Responses

PARP-1 has been shown to be involved in cellular stress responses and cell death. From different studies, it became clear that PARP-1 also modulates the molecular biology and biochemistry of stress responses at multiple levels. It alters cellular stress responses through a series of regulatory processes that occur at the genomic, transcriptional, posttranscriptional, translational, and post-translational levels (Kim *et al*., 2005).

Depending on the type and level of stress, PARP-1 elucidates different responses. Levels of PARP-1 activation decide the cell fate (i.e. cell survival or death). PARP-1 activation by mild or moderate stresses leads to transcription and DNA repair responses that help to maintain genome stability and re-establish homeostasis (Kim *et al*., 2005; Gagne *et al*., 2006). In contrast, severe, or sustained stresses cause hyper-activation of PARP- 1 resulting in distinct cell death programs (severe responses), such as apoptosis or necrosis (Koh *et al*., 2005; David *et al*., 2009). Various PARP inhibitors like PJ-34, DPQ, ABT-888 etc. are under clinical trials for various kinds of cancer (Jubin *et al*., 2016).

The mechanisms by which PARP-1 activation leads to cell death are still under active debate in the literature. Several mechanisms have been proposed, including energy-failure-induced necrosis and apoptosis-inducing factor (AIF)-dependent apoptosis.

1.10 Treatment of Vitiligo

The goal of vitiligo treatment is to control the autoimmune damage to melanocytes and stimulate their migration from surrounding skin and adnexal reservoirs. Treatment may be divided into non-surgical, surgical and herbal products, which can sometimes be combined.

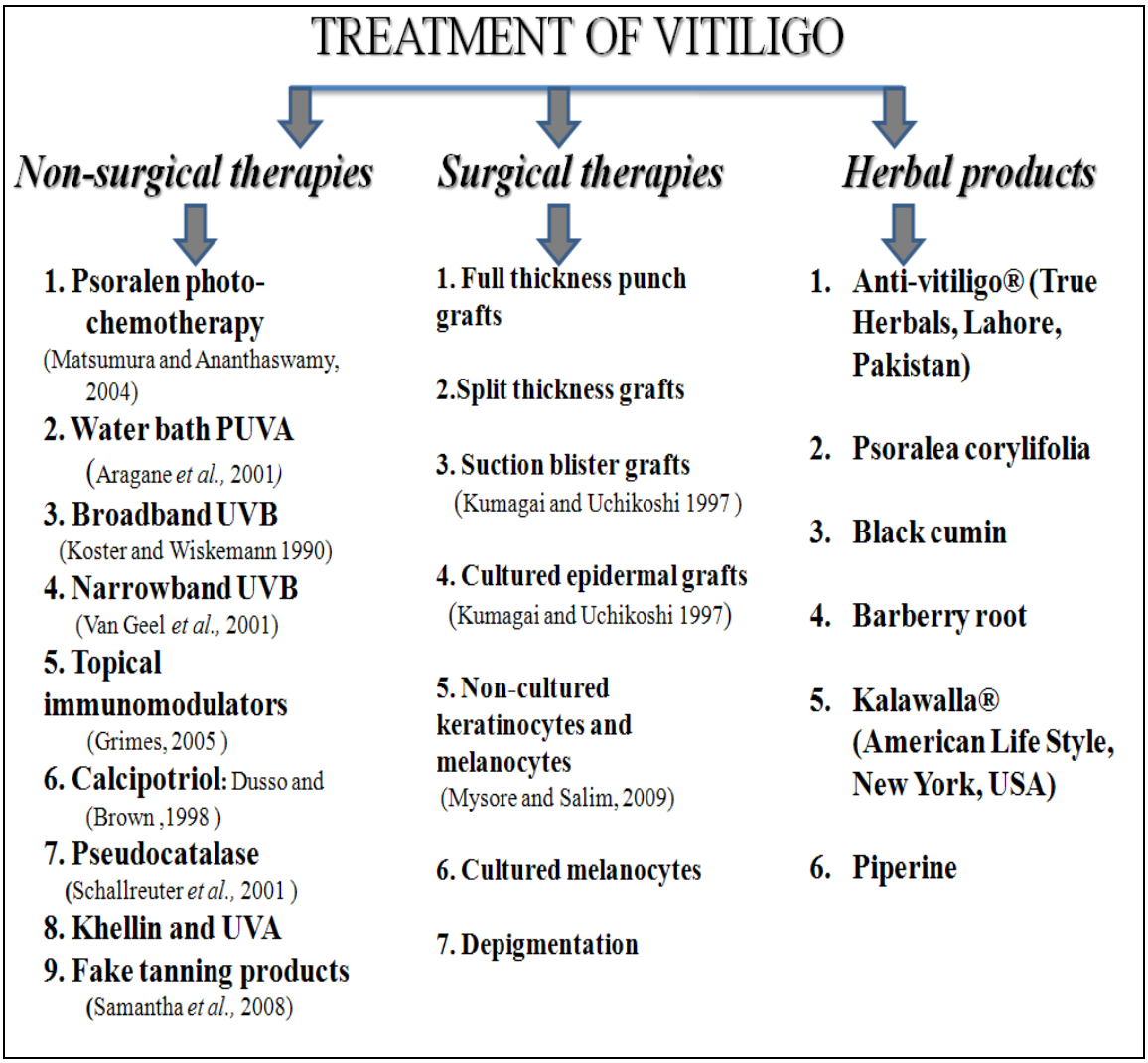


Figure 9: Developments in the direction of treatment are starting to become public, opening new approaches toward the full understanding of the genetic basis of complex diseases in general and especially of vitiligo.

1.10.1 Translational relevance of cytokines in Vitiligo

There is large body of evidence indicating imbalance of cytokines in Vitiligo suggesting Vitiligo could be an immune-mediated inflammatory disease. Not surprisingly, *biologics*, a new class of immune-modulators are being tested to assess their therapeutic potential in treating Vitiligo.

TNF-α inhibitors

Biologics of TNF-α antagonists are based on the observation that TNF-α protein expression and immunoreactivity is elevated in sera and lesional skin of Vitiligo patients. Till date five TNF-αantagonists (Table 5) have been studied in pilot clinical trials in treating pre-existing Vitiligo. Intriguingly, all of them have shown adverse reactions limiting their potential as therapeutic agents and warranting further research in the role of TNF-α in Vitiligo (Ramirez-Hernandez *et al*., 2005; Ismail *et al*., 2011; Posada *et al*., 2011; Smith and Heffernan, 2008).

Table 5: List of TNF-α antagonists used as biologics in clinical trials for Vitiligo therapy.

Biologics	**Type of biologic**
Infliximab, (Carvalho *et al*., 2014; Mattox *et al*., 2013)	Monoclonal antibody containing mouse variable region and a human IgG1 constant region.
Adalimumab, (Toissirot *et al*., 2013)	Monoclonal antibody containing human variable region and a human IgG1 constant region.
Golimumab	Monoclonal antibody containing human variable region and a human IgG1 constant region.
Etanercept (Rigopoulos *et al*., 2007)	Fusion protein of human TNFR2 and a truncated human IgG constant region
Certolizumab	Monoclonal antibody covalently attached to polyethylene glycol chains (PEGylated)

Infliximab, Adalimumab and Golimumab act as decoy receptors to bind TNF-α, hence it cannot bind and act through its true receptors. Notably, these TNF-α antagonists have proven successful in treating other autoimmune diseases namely psoriasis, psoriatic arthritis, ankylosing spondylitis, rheumatoid arthritis, and Crohn disease (Simon and Burgos-Vargas, 2008; Campanati *et al*., 2010; Tolaymat and Sluzevich, 2010, Maruthappu *et al*., 2013).

It is plausible that inhibiting TNF-α by antagonists promote depigmentation by decreasing production and activation of Tregs which facilitate T cell autoreactivity against melanocytes. These antagonists also create cytokine imbalance mainly of IFN-γ that plays a central role in Vitiligo by suppressing Treg function. Majority of the other side effects due to treatment with TNF-α antagonists will result in other autoimmune diseases such as Uveitis, Psoriasis, Hidradnetis supparativa, Crohns' disease, Sarcoidosis etc. that are all attributed to cytokine imbalance related to IL-23/Th-17 axis, IFN-γ and IFN-α.

Antagonists to IFN-γ have not been tested extensively in clinical trials. Nevertheless, in a small preclinical study, Vitiligo patients treated with polyclonal IFN-γ antibodies by intradermal injection followed by intramuscular injection showed some promise in repigmentation. Another biologic, Efalizumab a T-cell targeted recombinant antibody binding to the CD11a subunit of LFA-1 has shown some therapeutic value in long standing stable Vitiligo patient. This biologic blocks the interaction between LFA-1 and ICAM-1 that is expressed by perilesional melanocytes surrounding the Vitiligo patch (Skurkovich *et al*., 2002, Webb *et al*., 2015).

An emerging class of inhibitors of JAK-STAT (Janus kinase signal transducer and activator of transcription) pathway has shown promise as biologics for Vitiligo (Damsky and King, 2017). JAK-STAT pathway which is required for transmitting signals from the nucleus to the cell membrane is indispensable for immune functions and is mediated by signalling molecules including cytokines, interferons and interleukins. Amongst the cytokines IFN-γ which plays a crucial role in Vitiligo pathogenesis utilises JAK-STAT pathway, thus it is plausible that JAK inhibitors can be effective in Vitiligo treatment. Pilot clinical trials of JAK inhibitors, tofacitinib (Craiglow and King, 2015) and

ruxolitinib (Harris *et al*., 2016) on vitiligo patients showed significant repigmentation, however depigmentation recurred after discontinuing the treatment. Moreover, there is concern regarding continuous use of JAK inhibitors as they may increase risk of malignancy by reducing antitumor immune surveillance. Nevertheless, basic research and clinical trials to understand mechanism of JAK inhibitors and to test their potential in effective vitiligo treatment is warranted.

Plethora of scattred reports discussed above suggests the empirical need to address the expression levels and role of candidate cytokines in vitiligo pathogenesis. Therefore, for further experimentation we developed an ideal *in vitro* model system which is primary cultured normal human melanocytes in low serum, PMA and TPA free medium to investigate the role of cytokines in melanocyte biology. Furthermore, we aimed to monitor the expression levels of *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* in vitiliginous and control skin for screening of candidate cytokines. Important pro and anti-inflammtory cytokines TNF-α, IL-1, IL-6 and IL-10 screened from skin expression analysis were selected for further downstream effect of cytokines on melanocyte biology; particularly emphasizing their effect on receptor expression, genes involved in melanin synthesis and expression of other immune regulatory molecules. Moreover, we have also screened the genetic association of *IL10*, *IL1RN* and *IL6* polymorphisms, transcript levels as well as possible genotype-phenotype correlation analysis in vitiligo patients and controls from Gujarat population to access their effect on vitiligo suspectibility.Therefore, we have tried to monitor the effect of cytokine imbalance in vitiligo microenvironment, *in vitro* as well as population studies are also supplementing the above mentioned aspect to obtain a holistic figure of effect of cytokine imbalance in vitiligo pathogenesis.

1.11 References

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2.1 INTRODUCTION

Vitiligo is a hypopigmentary skin disorder caused due to selective destruction of melanocytes, and its etiology is poorly understood. Melanocytes are neural crest derived cells residing at the basal layer of the epidermis where they are surrounded by keratinocytes along with dendritic cells (DC), fibroblasts, antigen presenting cells (APC) etc. (Plonka *et al*., 2009). To develop a treatment modality for melanocyte related disorders such as vitiligo, melanoma etc., it is essential to understand the melanocyte biology, its various biochemical pathways, and factors affecting its different functions. Melanocytes are cells which synthesize melanin pigments by the activity of different melanogenic enzymes including tyrosinase (Hirobe, 1995).

Epidermal melanin unit is a structural and functional unit of epidermis where single melanocyte is surrounded by ~36 keratinocytes. Melanosomes, synthesized within melanocytes are transferred to surrounding keratinocytes *via* dendritic projections to impart skin coloration (Cichorek *et al*., 2013; Nordlund, 2011). The proliferation, differentiation, melanogenesis, and dendritogenesis of melanocytes in the epidermis and hair follicles of mammalian skin are primarily regulated by paracrine factors derived from keratinocytes. The paracrine regulation of melanocyte function by keratinocytes plays a key role in regulating the homeostasis of the epidermis and hair follicles (Hirobe, 2014). As keratinocytes secrete melanotropic molecules for regulating the growth and functioning of cells, melanocytes exhibit variations among different individuals as well as in different body regions of the same individual. The variation is attributed due to the size, number, composition and distribution of melanosomes as well as melanin synthesis capacity. Melanocytes are typically large, slow growing cells of ~19µm and their growth depend on the anatomical position of the source of cells as compared to other epidermal cells. The percentage of melanocytes in the epidermal layer is around 8- 10 % (Ivanova *et al*., 2006). Culture techniques for normal human melanocytes (NHM) have been developed over the last three decades. *In vitro* model for studying pigment-producing cells is expected to provide the knowledge of cell-to-cell and cell-to-matrix interactions, melanocyte and melanin biology, pathophysiology of pigment disorders, and malignant

melanomas (Lacour *et al*., 1992). However, melanocyte culturing and *in vitro* studies have become a challenge due to following reasons:

- Melanocytes undergo very little replication as compared to keratinocytes because they are rarely shed from the epidermis.
- Limitation for a good source of tissue, which contains melanocytes as the major component
- Slow growth and proliferation of melanocytes
- The presence of faster-growing cells in the skin such as keratinocytes and fibroblasts as compared to melanocytes.

In vitro studies of human melanocytes are complicated due to the presence of contaminating populations of rapidly multiplying cells other than melanocytes. This prevents sub culturing and long-term study of melanocytes in culture.

The imbalance between pro and anti-inflammatory cytokines play an important role in the pathogenesis of various autoimmune and inflammatory disorders. A complex melanogenic cytokine network between skins cells, which regulate melanocyte activity was demonstrated (Imokawa, 2004). Keratinocytes present in the vicinity of melanocytes secrete additional cytokines, such as IL-6 and TNF-α, which function as paracrine inhibitors of growth and proliferation of melanocytes (Swope *et al.*, 1991). Altered levels of keratinocyte derived mediators have been described in vitiligo epidermis, suggesting an important role of epidermal cytokines in vitiligo pathogenesis (Moretti *et al.*, 2002; Lee *et al.*, 2012). Though cytokine imbalance has been well documented in vitiligo patients, the exact mode of its action on melanocyte biology is still not well explored.

The proposed study aims to explore the role of cytokines in vitiligo pathogenesis and to address the response and downstream signaling in *in vitro* cultured melanocytes (established from human skin biopsies) exposed to various cytokines. This study will pave the way to identify the role of cytokines in the pathogenesis and progression of vitiligo. There is an imperative need for a proper *in vitro* model system to establish the role of different cytokines in melanocyte biology.

2.2 MATERIALS AND METHODS

2.2.1 Ethics statement

The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all subjects before performing the studies.

2.2.2 Culture establishment of primary normal human melanocytes (NHM)

Melanocytes were isolated from normal human skin biopsy samples and cultured successfully using the standard protocol with a few modifications (Hsu *et al*., 2005; Sungbin *et al*., 1992; Czajkowski *et al*., 2007). Briefly, the epidermis was separated from the dermis after an overnight incubation of skin biopsies in 0.25% Dispase II protease (Sigma Aldrich, USA) prepared in phosphate buffer saline (PBS at 4˚C. In order to separate epidermal cells, the epidermis was incubated at 37˚C for 10 min, in a solution of 1X trypsin phosphate versene glucose solution. Cellular suspension was centrifuged at 1,300 rpm for 5 min at room temperature to harvest cells. Melanocytes were selectively cultured in M254 medium with human melanocyte growth supplements (Gibco® life Technologies™, Cascade Biologics™, Portland, Oregon) and 1X antibiotic-antimycotic solution (Himedia, India). The cells were maintained in a humidified incubator with 5% CO_2 at 37˚C and media replenishment was given after every 2-3 days. Melanocyte-keratinocyte mixed population starts appearing around days 4-9. Melanocytes were purified from keratinocytes by differential trypsinisation, which is based on the more sturdy and adherent property of keratinocytes. In addition, melanocytes were given 100µg/ml G418 (Geneticin® Disulfate Salt, MP Biomedicals, France) treatment for gradual removal of fibroblasts to obtain primary human melanocytes and further were split at 1:3 ratio, when cell confluency reached 80%. Media was replenished after every 48-72 hrs; melanocytes cultured up to the fifth passage were used for the experiments.

2.2.3 Protocol for Cultivation of Normal Human Epidermal Melanocytes (Hsu *et al*., 2005)

- The skin samples were soaked in 70% ethanol for 1 min and transferred to the Petri dish containing HBSS to rinse off ethanol.

- For foreskin samples the skin-ring was cut opened and fat was trimmed off along with other tissue, with scissors. Skin samples were cut into pieces (approx 5 × 5 mm^2) using the surgical scalpel blade.
- The skin pieces were transferred into the tube containing epidermal isolation solution (M254 basal media and HMGS). Further, the tube was capped, inverted, and incubated in the refrigerator at 4°C for 18–24 hrs.
- The tube containing the sample was removed from the refrigerator and incubated at 37°C for 5 min.
- The tissue was poured into a Petri dish containing epidermal isolation solution. The epidermis (thin, brownish, translucent layer) was separated from the dermis (thick, white, opaque layer) with the help of the forceps. The dermal part of the skin piece was held with one pair of forceps, and the epidermal side with another forceps.
- The epidermis was gently teased apart from dermis. Immediately the dermis was discarded.
- The harvested epidermal sheets were transferred to an empty Petri dish, drops of Ca^{2+} and Mg^{2+}free HBSS was added to prevent the tissue sample from drying. The above described steps were repeated for each piece of tissue and further minced them into smaller pieces (approx. 2 × 2 mm^2) with the help of a surgical scalpel blade.
- The collected epidermal sheets were transferred from the Petri dish to the centrifuge tube containing 5 mL of cell-dispersal solution. The tube was incubated at 37°C for 5 min.
- The tube was shaken vigorously. The resulting single-cell suspension was washed once with 10 mL of Ca^{2+} and Mg^{2+} free HBSS.
- The suspension in was centrifuged for 5 min at 800g at room temperature.
- The supernatant was carefully aspirated, which may contain remaining stratum corneum. The pellet was re-suspended in 5 mL culture media. The resulting epidermal cell suspension was plated in a T25 cell-culture vessel and further incubated at 37°C in 5% CO_2 for 48–72 hrs without disturbance.

Antibiotic cocktail 1X (Penicillin, Gentamycin, Tetracycline, Streptomycin, Geneticin**)**

Human Melanocyte Growth Supplement (HMGS) is an ionically balanced supplement containing bovine pituitary extract (BPE), fetal bovine serum, bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin, and endothelin-1.

2.2.4 Confirmation of melanocytes by DOPA staining

The pure culture of melanocytes was confirmed by L-DOPA (L-3,4-dihydroxy phenylalanine, Hi-Media, India) staining (Iijima and Watanabe, 1957). NHM were trypsinized, plated on coverslips and cultured for ~3 days before detection of tyrosinase activity. For DOPA reaction, culture media was removed and cells were rinsed twice with PBS, fixed for 20 min in 4% formaldehyde solution in PBS, washed three times with PBS and then incubated at 37°C for 18 hrs in the dark with 10 mM L-DOPA. After incubation, the cells were rinsed with distilled water, dehydrated, mounted and the number of cells positive for tyrosinase activity was observed using light microscopy.

2.2.5 Human Melanocyte Cell lines

Immortalized human melanocyte cell lines PIG1 (derived from a healthy individual) and PIG3V (derived from vitiligo patient) (a gift from Dr. I.C. Le Poole, Loyola University, Chicago, Illinois) were cultured as described by Le Poole (Le Poole *et al*., 1997; 2000).

2.2.6 Melanoma derived cell lines

G361 and SK Mel28 melanoma derived cell lines were also included in our studies. The G361 is pigment producing malignant melanoma cell line which is derived from 31 years old Caucasian male and was cultured in McCoy's 5A Medium supplemented with 10% Fetal Bovine Serum (FBS) and 1X Antibiotic supplementation (Himedia, A002-20 ML, Antibiotic Antimycotic Solution 100X). Moreover, SK Mel 28 is also a malignant melanoma cell line derived from 51 years old male. However, it exhibits poor pigmentation phenotype and is cultured in Eagle's Minimum Essential Medium supplemented with 10% FBS and 1X Antibiotic supplementation.

2.3 RESULTS

2.3.1 Isolation and culturing of melanocytes from human skin biopsies: Melanocytes were successfully isolated and cultured from human skin biopsies (Figure 1). During initial three days, we observed ~ 5-10% of epidermal cell suspension got adhered to the culture flask and most of them were possessing dendrites. After media change around day 4-9, numerous polygonal and cobble shaped cells (keratinocytes) were observed surrounding dendritic melanocytes. In this mixed culture of the melanocytes and keratinocytes, the keratinocytes are more adherent as compared to melanocytes. With the help of two rounds of differential trypsinisation, we obtained keratinocyte free melanocyte culture. During the culturing of the melanocytes, Gentamicin was used to eradicate fast growing fibroblasts (Figure 1). The melanocytes were confirmed by DOPA staining which is based on Tyrosinase, a melanocyte specific enzyme which catalyzes the conversion of tyrosine to DOPA and DOPA to DOPAchrome (Figure 2).

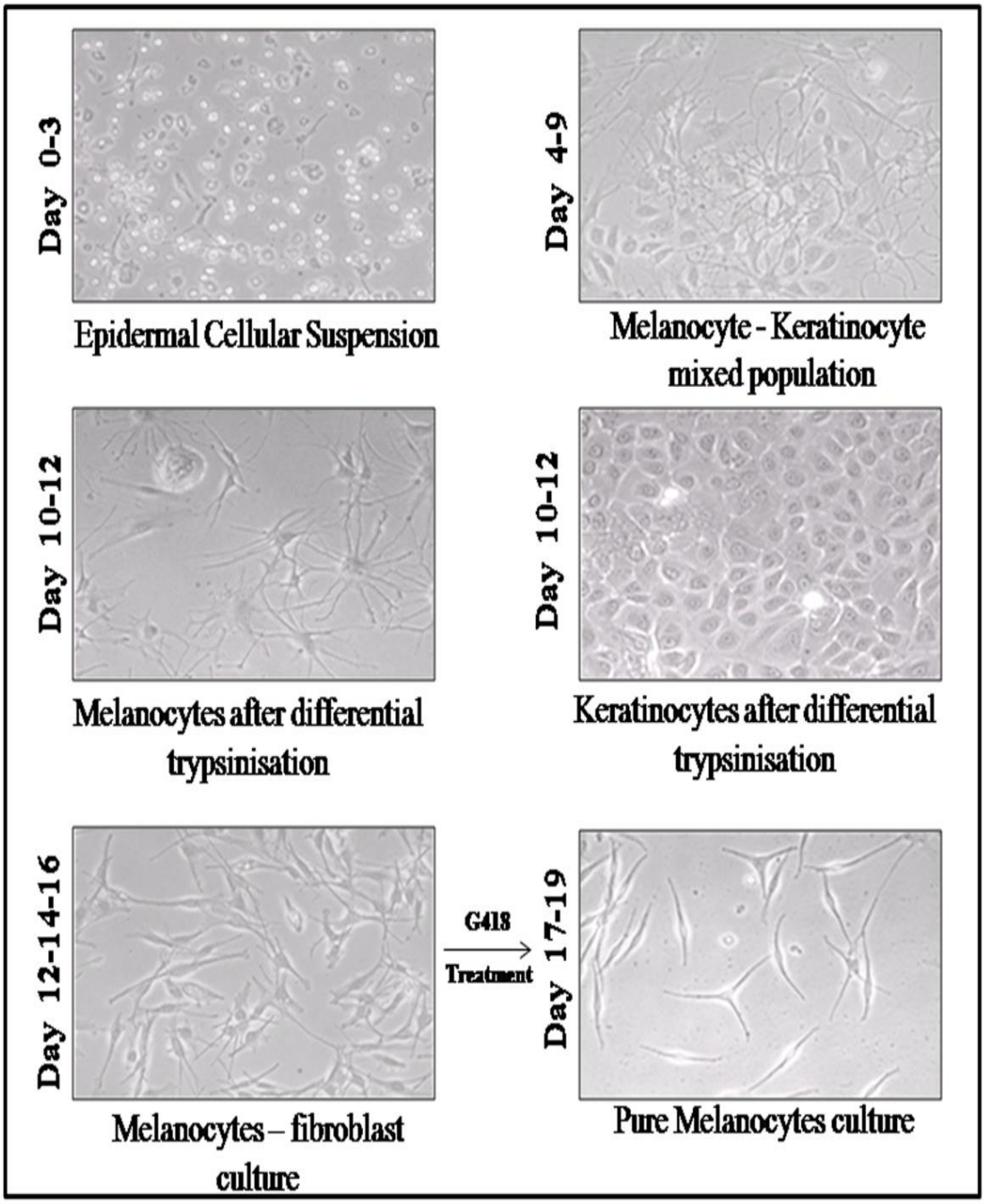

Figure 1: Establishment of primary cultured human skin melanocytes: Melanocytes were isolated and cultured successfully. Images were taken at different stages of differentiation under an inverted microscope (Magnification 10 X; scale 100µm).

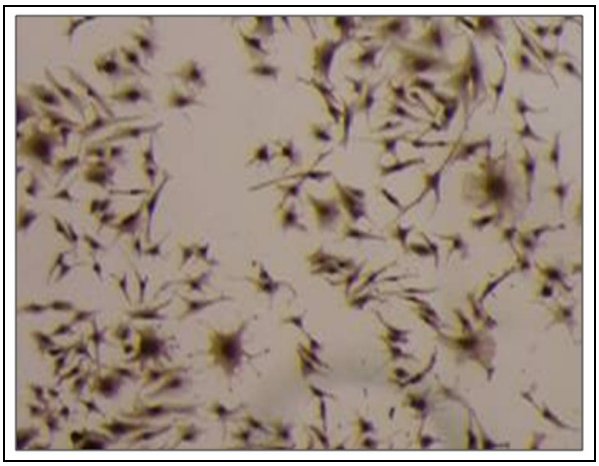

Figure 2: Confirmation of cultured melanocytes by DOPA staining as observed under an inverted microscope (Magnification 10 X; scale 100μm).

2.3.2 Melanocytes derived from different sources:

In addition to primary melanocytes, melanocyte culturing was also done from different sources. Viral transformed melanocytes derived from healthy persons PIG1 as well from vitiligo patients PIGV, (a gift from Dr. I.C. Le Poole, Loyola University, Chicago, Illinois) were also used for our preliminary experiments. PIG1 and PIGV are used as a substitute for melanocytes derived from healthy control and vitiligo patients due to the limitation of human samples. PIG3V vitiligo melanocytes showed a tendency to be more sensitive to 4-TBP as compared to control PIG1 melanocytes reflecting the restoration of intrinsic sensitivity of vitiligo subjects even after several times of subculturing (Kroll *et al*., 2005). Moreover, melanin producing and non-producing cell lines derived from malignant melanoma patients *viz* G361 and SKMel 28 cell lines respectively were also cultured (Figure 3). NHM showed higher melanin production as compared to PIG1 and G361 melanin positive cell line. Also, slender morphology and multiple dendrites were observed in NHM as compared to PIG1, PIGV, G361 and SK Mel 28 cell lines. On the other hand, the rest of the other cells exhibited bipolar dendrites while NHM exhibited multiple dendrites.

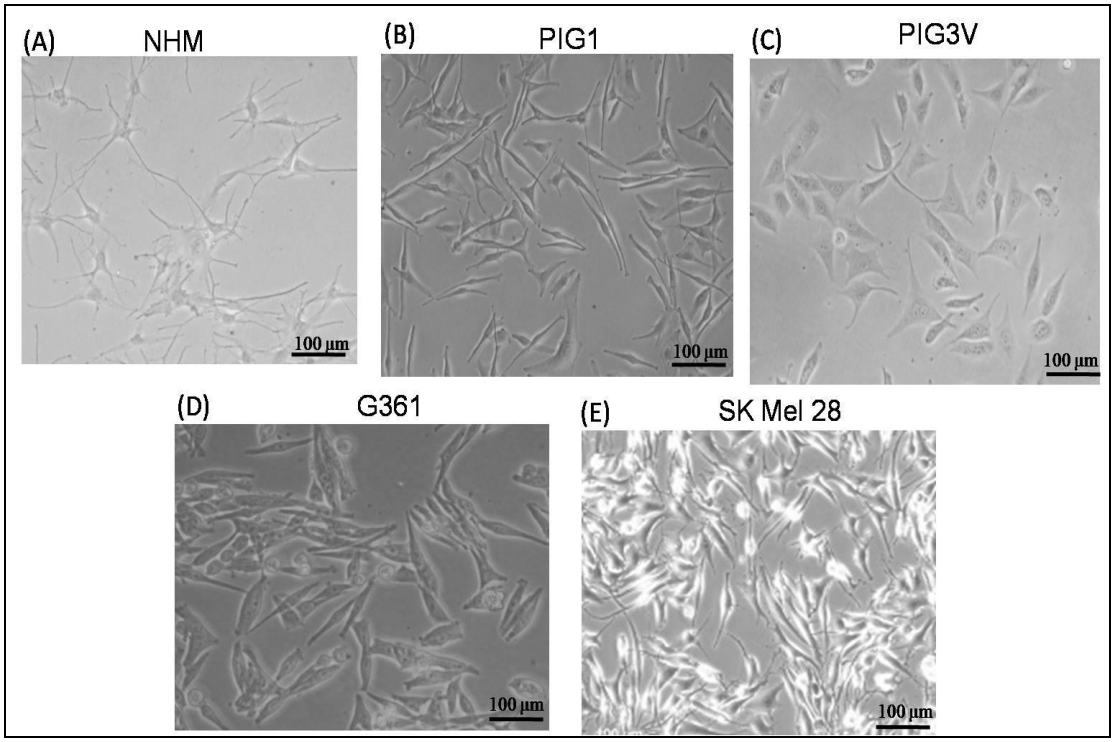

Figure 3: Differences in cell morphology were observed in the melanocyte culture obtained from different sources (A) NHM (B) PIG1 (C) PIGV (D) G361 (E) SK Mel 28 at 10 X magnification.

2.4 DISCUSSION

An important approach to study the molecular mechanisms of normal, diseased, and malignant cells is their growth profile under *in vitro* conditions. Due to ethical issues, and the scientific concern as well as limitation of human samples, it becomes difficult to investigate the molecular mechanisms of diseases on human subjects directly. Therefore, mice/animal models, primary cultures and cell lines (mostly cancerous, viral immortalized etc.) have been developed to overcome these constraints.

Cytokine imbalance is well reported in vitiligo patients, but their exact mode of action on melanocytes is still not well explored. Therefore, to study the effect of candidate cytokines on melanocyte biology, it is very essential to establish the *in vitro* system, as the *in vivo* or animal model system is not apt to study the role of cytokines *per se*.

Moreover, cytokines affect melanocyte biology by the activation of their respective receptors. To address melanocyte biology under cytokine imbalance it is essential to design of an ideal *in vitro* system.

Vitiligo is a multifactorial, polygenic and hypomelanotic skin disorder caused due to selective destruction of melanocytes of unknown etiology (Laddha *et al.*, 2013). As it involves melanocyte destruction, melanoma derived cell lines or viral transformed primary cell lines would not be the appropriate model system to investigate/validate hypothesis of our study. Hence, developing a proper *in vitro* model system is a prerequisite to study and understand the complex interplay of cytokines and oxidative stress in melanocyte biology.

Human epidermal melanocyte isolation and culturing has been attempted since 1957 (Hu *et al.*, 1957***;*** Yukio, 1976; Mayer, 1982; Wllkms and Szabo, 1981), but since 1982 only pure NHM cultures have been established to yield cells in adequate quantity for biochemical and molecular studies (Eisinger and Marko, 1982). Selective growth of the melanocytes, which comprises around 10% of epidermal cells, was initially attained by suppressing the growth of major populating cells (keratinocytes) and fast growing cells (fibroblasts) in epidermal cell suspensions. Previously, to suppress the growth of keratinocytes and fibroblasts tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) and the intracellular cyclic adenosine 3′, 5′ monophosphate (cAMP) enhancer cholera toxin were used respectively, both of which also act as melanocyte growth promoters. However, phorbol ester is metabolically stable and has prolonged effects on multiple cellular responses **(**Eisinger and Marko, 1982**)**. Therefore, in the present study, we established TPA, phorbol 12-myristate 13-acetate (PMA)-free culture medium for selective growth and long-term maintenance of human melanocytes. Based on the difference in the adherence properties of melanocytes and keratinocytes, and with the help of two rounds of differential trypsinization, we were able to get rid of keratinocytes from our melanocyte-keratinocyte co-culture. Also culture medium supplementation with gentamicin helped us to obtain a fibroblast free growth in our melanocyte culture. Melanocytes in the culture were easily distinguished from keratinocytes by their morphological characteristics. Keratinocytes had a round or polygonal shape while

melanocytes appeared bipolar or polydendritic. The detailed description of the present method will be useful in basic and applied biological research.

In our preliminary standardization experiments, we used melanoma derived cell lines: SKMel28 (low melanin producing Caucasian origin cell line) and G361 (melanin producing cell line). Also, viral transformed primary melanocytes PIG1, PIG3V derived from healthy individuals and vitiligo patients respectively (Le Poole *et al*., 1997). Our results showed that primary cultured normal human melanocytes exhibited increased sensitivity to exogenous stimulation of cytokines and oxidative stress as compared to melanoma and viral transformed cell lines. NHM exhibit close resemblance in terms of number of dendrites and melanin synthesis capacity to melanocytes residing in the skin as compared to melanoma and viral transformed cell lines. The difference could be attributed to the incorporation of mutations during the procedure of immortalization. Therefore, NHM appears to be a better model for exploring the effect of various immnoregulatory molecules on melanocyte biology. The NHM culture system used for our studies is free of PMA, TPA and serum which minimizes the artifact of cytokine mediated sensitive studies. Nevertheless, the NHM observed were multi dendritic during initial plating, however after subsequent sub-culturing most of the cells became bipolar and 10-15% of cells were tripolar. This is suggestive of the importance of keratinocyte & its derived factors for growth and functioning of melanocytes and this could also explain the decrease in melanin synthesis and growth properties of NHM with further passaging of cells.

In conclusion, isolation and culturing of primary melanocytes has been established in our lab which will facilitate research in melanocyte biology to understand the etiology of pigmentary disorders (vitiligo) and melanoma. The culture model developed is apt to study the effect of cytokines or immune modulators on melanocyte biology as the culture system is devoid of TPA and serum.

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3.1 INTRODUCTION

Vitiligo is an acquired, circumscribed hypomelanotic skin disorder, characterized by milky white patches caused due to loss of functional melanocytes from the epidermis. Generalized Vitiligo (GV) is considered to be an autoimmune disorder due its frequent association with other autoimmune diseases and presence of anti-melanocyte antibodies/autoreactive T cells in the circulation of vitiligo patients (Laddha *et al*., 2014). Cytokines have crucial functions in the development, differentiation and regulation of immune cells. As a result, dysregulation of cytokine production or their action is thought to have a central role in the development of autoimmunity (O'Shea, 2002). A large body of evidence suggests that epidermal melanocytes are an integral part of the skin immune system and can be considered as immunocompetent cells (Tam & Stępień, 2007). In addition to the photoprotective function, melanocytes also exhibit neuroendocrine activity and produce classical stress neurotransmitters, neuropeptides, cytokines and hormones (Slominski *et al*., 2009). Moreover in the epidermal melanin unit the surrounding keratinocytes synthesize cytokines such as IL-1α and IL-6, which are paracrine inhibitors of melanocyte proliferation and melanogenesis. This association with surrounding cells is of prime importance for melanocyte survival and differentiation as keratinocyte-derived cytokines act on melanocytes *via.* specific receptors (Moretti *et al.,* 2002). One of the emerging aspects of autoimmunity is epidermal cytokine imbalance which might pave the way for better understanding of vitiligo pathomechanisms.

Our results on selected candidate genes conferring oxidative stress and autoimmunity suggests that *HLA-A*33:01, HLA-A*02:01, HLA-B*44:03, HLADRB1* 07:01* and studied polymorphisms in *IL4, CTLA4, SOD2, SOD3, GPX1, NALP1, MYG1, TNFA, TNFB, IFNG* and *IL10* genes are strongly associated with vitiligo susceptibility, whereas the polymorphisms in *PTPN22, MBL2, ACE, CAT, G6PD* and *SOD1* genes are not found to be significantly associated with vitiligo in Gujarat population (Begum *et al.,* 2014). Moreover, several reports including our studies have implicated cytokine imbalance in vitiligo pathogenesis (Mansuri *et al.,* 2014). Various reports as shown in Figure 1 have demonstrated association of cytokine and immune regulatory genes with vitiligo susceptibility (Laddha *et al*., 2014; Pehlivan *et al*., 2009; Lee *et al*., 1995; Imran *et al*.,

2012; Aydıngoz *et al*., 2015; Abanmi *et al*., 2008; Yun *et al*., 2010; Dwivedi *et al*., 2015; Namian *et al*., 2009, Alkhateeb *et al*., 2010).

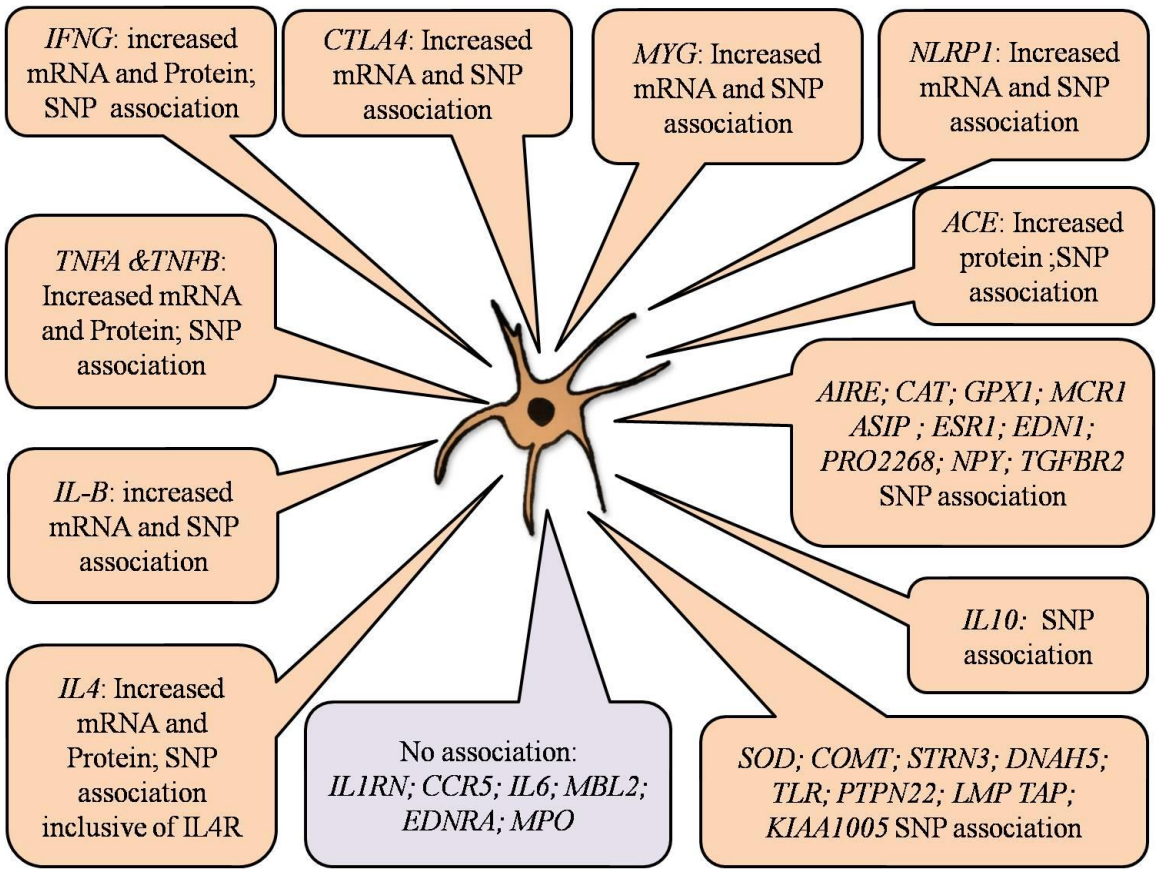

Figure 1: Illustration of SNPs association of candidate genes with vitiligo.

However, at present there is paucity of studies regarding cytokine expression in the skin samples derived from vitiligo patients. Therefore, we aim to monitor the expression profile of pro- and anti-inflammatory cytokines in lesional and non-lesional skin of vitiligo patients as compared to healthy controls. This study will provide better insights to understand the role of epidermal cytokine imbalance in the microenvironment of vitiliginous skin.

Various reports suggest that genetic predisposition of candidate genes which are involved in vitiligo susceptibility may affect the expression or function of respective genes (Figure 1). The target genes for the study were pro and anti-inflammatory cytokines: Tumor Necrosis Factor-α (*TNFA*), Interferon-γ (*IFNG*), Interleukin 1 Receptor 1 (*IL1R1*),

Interleukin-1α (*IL1A*), Interleukin-1β (*IL1B*), Interleukin-1 Receptor Antagonist (*IL1RN*), Interleukin-4 (*IL4*), Interleukin-6 (*IL6*) and Interleukin-10 (*IL10*). Results obtained from the proposed study might aid in understanding the role of epidermal cytokine imbalance in vitiligo pathogenesis.

3.2 MATERIALS AND METHODS

3.2.1 **Study Subjects**

Vitiligo patients who had not received systemic immunosuppressive treatment or PUVA/UVB, for at least 1 month, and topical therapy for at least 2 weeks before skin biopsy excision were recruited. Skin biopsies were taken from lesional and non-lesional skin of patients with vitiligo (n= 12) and from non-inflamed, non-irritated skin of healthy individuals (n= 12). Both vitiligo patients and healthy controls were of native Gujarat origin (Table 1). The importance of the study was explained to all participants and written consent was taken from controls and patients. The details regarding age, age of onset of vitiligo, duration of disease, family history, type of vitiligo, etc. of patients were filled in vitiligo questionnaire. The skin samples were procured by punch biopsy or from the left over skin after surgery. At the time of excision, the skin sample was collected in a sterile container with 20 ml of normal skin transporting medium containing Ca^{++} and Mg^{++} free Hank's balanced salt solution (HBSS) supplemented with 4X cocktail of penicillin, streptomycin and amphotericin B and kept at 4^0C while carrying it to cell culture laboratory. Immediately one part of the skin (from vitiligo patients and controls) was stored in RNA*later*® solution (Ambion, USA) for RNA isolation and gene expression studies. Samples obtained by methods other than punch biopsy were used for melanocyte culture.The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India and conducted according to the Declaration of Helsinki's principles.

Table 1: Demographic characteristics of vitiligo patients and unaffected controls recruited for skin sample collection.

	Vitiligo Patients		**Controls**
	(n = 12)		(n = 12)
Average age (mean age ± SD)	29.38± 4.480		28.33± 3.240
Sex: Male	4		6
Female	8		6
Age of onset (mean age ± SD)	27.83 ± 6.05 yr		NA
Duration of disease (mean ± SD)	6.83 ± 2.36 yr		NA
Type of vitiligo			
Active vitiligo	7		NA
Stable vitiligo	5		NA

3.2.2 RNA isolation and 1st strand cDNA synthesis from skin samples

Skin samples stored in RNA *later*® solution (Ambion, USA) were used for RNA isolation. Skin samples were gradually thawed from -80^0 C to 4^0 C. The skin sample was homogenized in TriZol® solution under cooling conditions. RNA was isolated by using Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction method. RNA purity and integrity were assessed by the ratio of absorbance at 260 to 280 nm to be >1.95 and by 1.5% agarose gel electrophoresis/ethidium bromide staining, respectively. 1µg of RNA was reverse transcribed and converted to first strand cDNA using verso cDNA kit (Thermo scientific, USA) and cDNA was stored at -20^{0}C for gene expression studies.

Table 2: Details of primers used for expression of various candidate genes.

Primer	Sequence (5' to 3')	Amplicon size(bp)	Annealing Temperature (^{0}C)
TNFA FP	GCCCCCAGAGGGAAGAGTTCCCCA	124	70
TNFA RP	GCTTGAGGGTTTGCTACAACATGGGC		
IL1A FP	GCTGCTGAAGGAGATGCCTG	143	66
IL1A RP	CTACGCCTGGTTTTCCAGT		
IL1B FP	AGATGAAGTGCTCCTTCCAGG	153	65
IL1B RP	TGGTCGGAGATTCGTAGCTG		
IL4 FP	GCCTCCAAGAACACAACTGAGAAGG	212	68
IL4 RP	TCACAGGACAGGAATTCAAGCCCG		
IL6 FP	AAATTCGGTACATCCTCGACGGCA	88	61
IL6 RP	AGTGCCTCTTTGCTGCTTTCACAC		
IL10 FP	ACCTGGGTTGCCAAGCCTT	189	62
IL10 RP	CCACGGCCTTGCTCTTGTT		
IFNG FP	TTGGAAAGAGGAGAGTGACAG	212	65
IFNG RP	GGACATTCAAGTCAGTTACCGA		
IL1R1 FP	GGAGGCTGATAAATGCAAGG	195	62
IL1R1 RP	GTAAGATGAATTTACCACGC		
IL1RN FP	GCTGGAGGCAGTTAACATC	165	65
IL1RN RP	CTACTCGTCCTCCTGGAAG		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65
GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

3.2.3 Cytokine gene expression studies:

The expressions of candidate genes were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene

was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using Light Cycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification. The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out to check the specificity of the products formed. The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value. Cp values greater than 40 were excluded from the analysis. mRNA expression data was normalized with *GAPDH*.

3.2.4 Data analysis

ΔCp values were compared between different groups and data was analyzed by non-parametric unpaired t-test using Prism 4 software (Graph Pad Software, USA, 2003) to determine its statistical significance. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software (Faul *et al.*, 2007). *p*-values less than 0.05 were considered as statistically significant.

3.3 RESULTS

3.3.1 Gene expression studies of *TNFA* in control and vitiliginous human skin.

We performed a comparative analysis for *TNFA* expression in lesional and non-lesional skin from vitiligo patients and healthy skin from controls. Our results showed that lesional as well as non-lesional skin of vitiligo patients exhibit significant increase in expression of *TNFA* compared to control skin (p=0.024, p=0.0467 respectively). However, there was no significant difference in *TNFA* levels between lesional and non-lesional skin of vitiligo patients (p=0.6749; Figure 2).

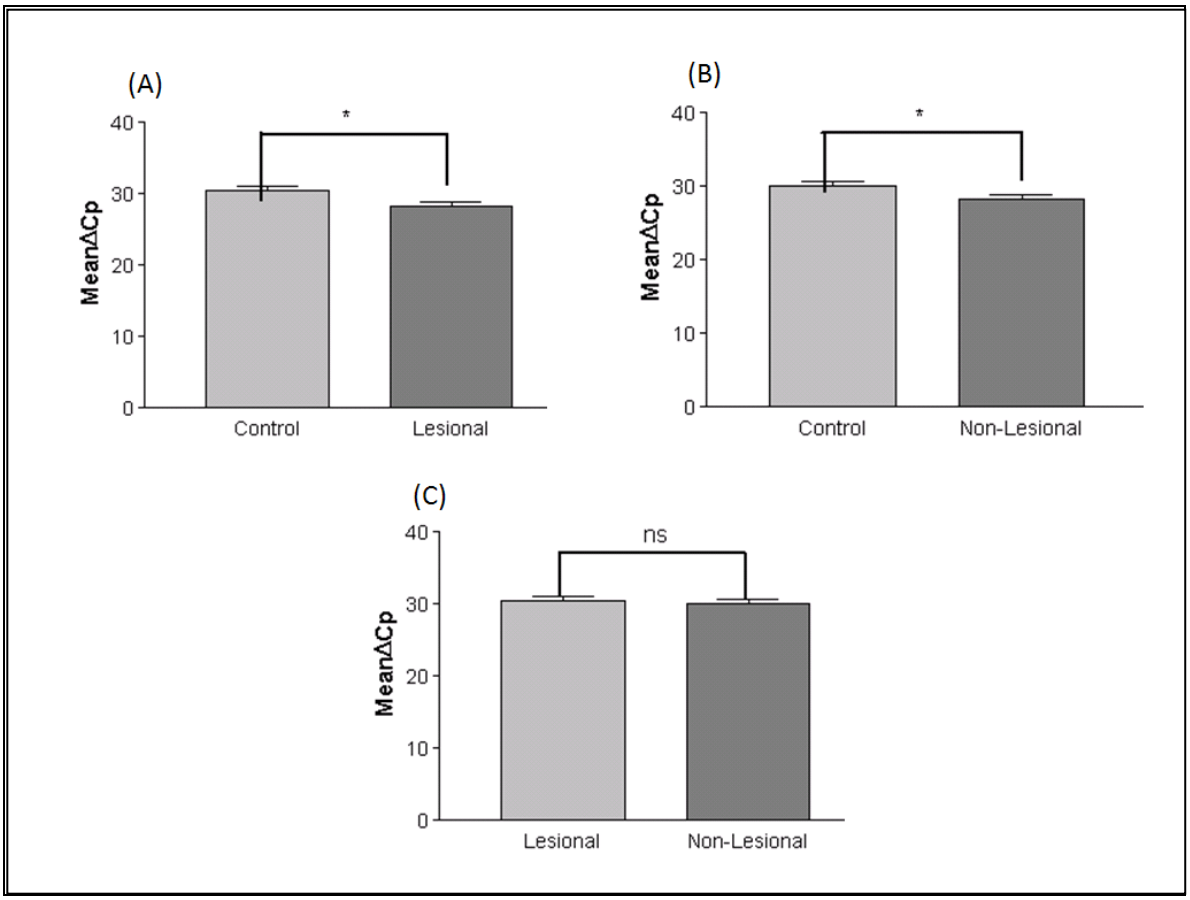

Figure 2: *TNFA* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) Lesional skin from vitiligo patient showed significant increase in expression of *TNFA* compared to control skin (p=0.0246).

(B) Non-lesional skin from vitiligo patient showed significant increase in expression of *TNFA* compared to control skin (p=0.0467).

(C) No significant difference was seen in *TNFA* levels between lesional and non-lesional skin of vitiligo patients (p=0.6749).

3.3.2 Gene expression studies of *IL1A* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1A* compared to control skin (p=0.6760

and p=0.4004, respectively). Also, there was no significant difference in *IL1A* levels between lesional and non-lesional skin of vitiligo patients (p=0.2886; Figure 3).

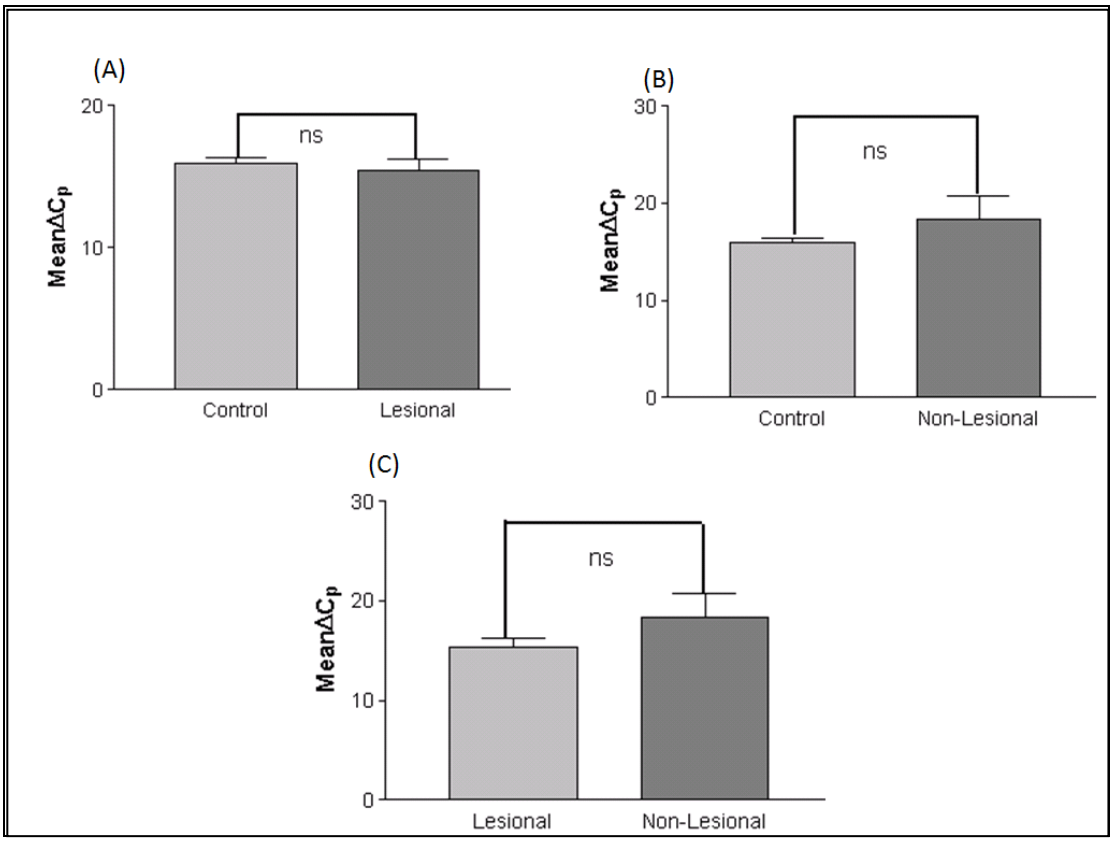

Figure 3: *IL1A* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1A* levels between lesional and control skin (p=0. 6000).

(B) No significant difference was seen in *IL1A* levels between non-lesional and control skin (p=0. 4004).

(C) No significant difference was seen in *IL1A* levels between lesional and non-lesional skin of vitiligo patients (p=0.2886).

3.3.3 Gene expression studies of *IL1B* in control and vitiliginous human skin.

Our results showed that lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1B* compared to control skin (p=0.5085). However, there

was a significant increase in *IL1B* levels in non-lesional compared to control skin (p=0.0290). Also significant increase in the expression of *IL1B* in non- lesional skin was observed compared to lesional skin of vitiligo patients (p=0.0021; Figure 4).

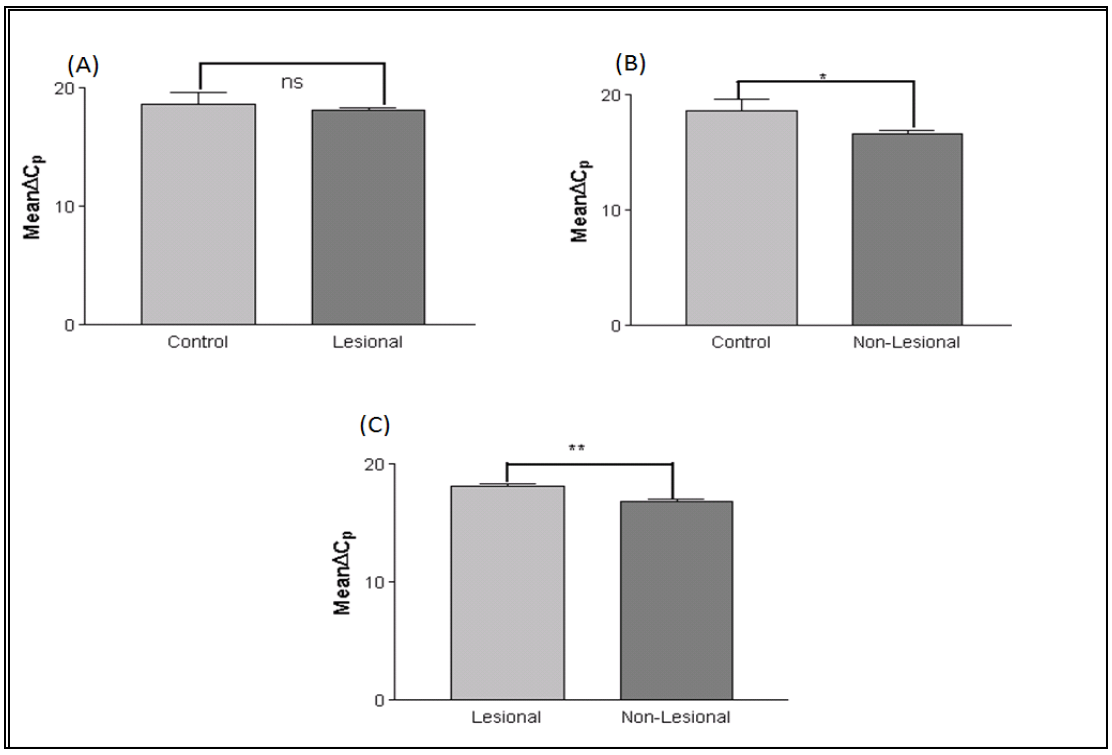

Figure 4: *IL1B* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1B* levels between lesional and control skin (p=0. 5085).

(B) Non-lesional skin from vitiligo patient showed significant increase in the expression of *IL1B* compared to control skin (p=0.0290).

(C) Significant increase was observed in *IL1B* levels in non-lesional compared to lesional skin of vitiligo patients (p=0.0021).

3.3.4 Gene expression studies of *IL4* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in the expression of *IL4* compared to control skin (p=0.2886

and p=0.2152, respectively). Further, there was no significant difference in *IL4* levels between lesional and non-lesional skin of vitiligo patients (p=0.9669; Figure 5).

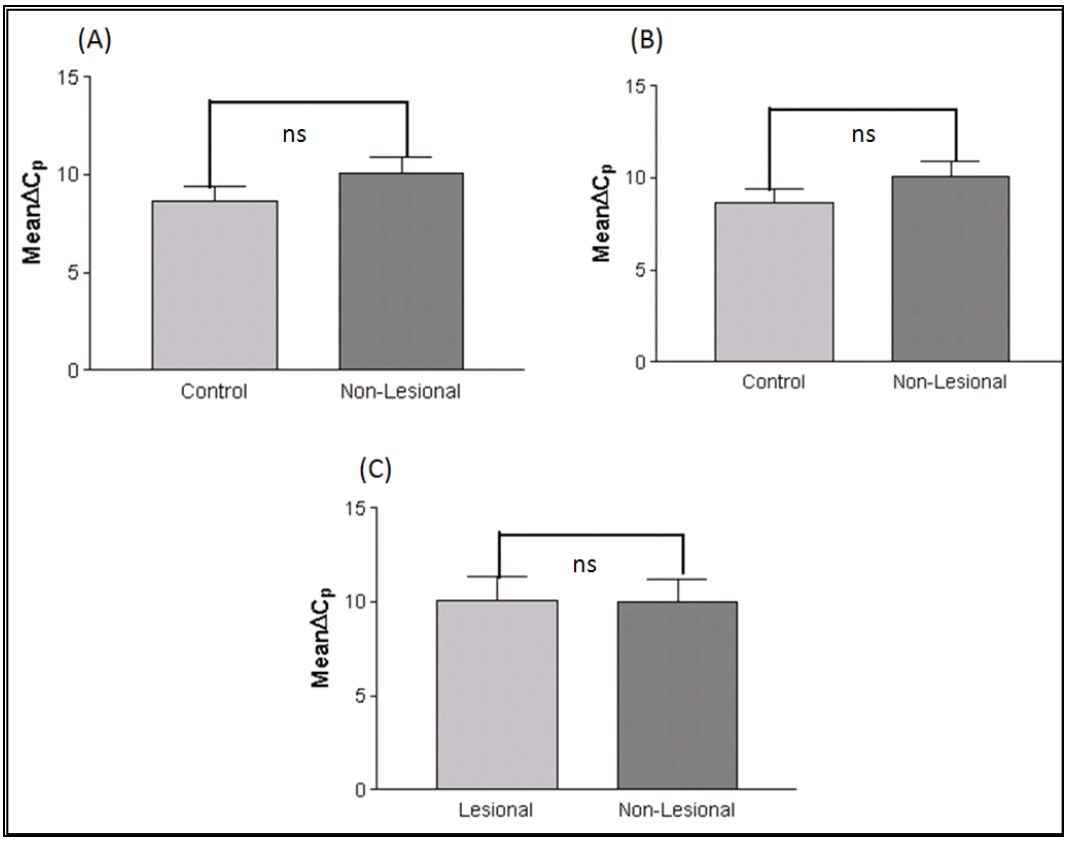

Figure 5: *IL4* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL4* levels between lesional and control skin (p=0. 2886).

(B) No significant difference was seen in *IL4* levels between non-lesional and control skin (p=0. 2152).

(C) No significant difference was observed in *IL4* levels between lesional and non-lesional skin of vitiligo patients (p=0.9669).

3.3.5 Gene expression studies of *IL6* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL6* compared to control skin (p=0.5473 and p=0.5697, respectively). Further, there was no significant difference in *IL6* levels between lesional and non-lesional skin of vitiligo patients (p=0.9511; Figure 6).

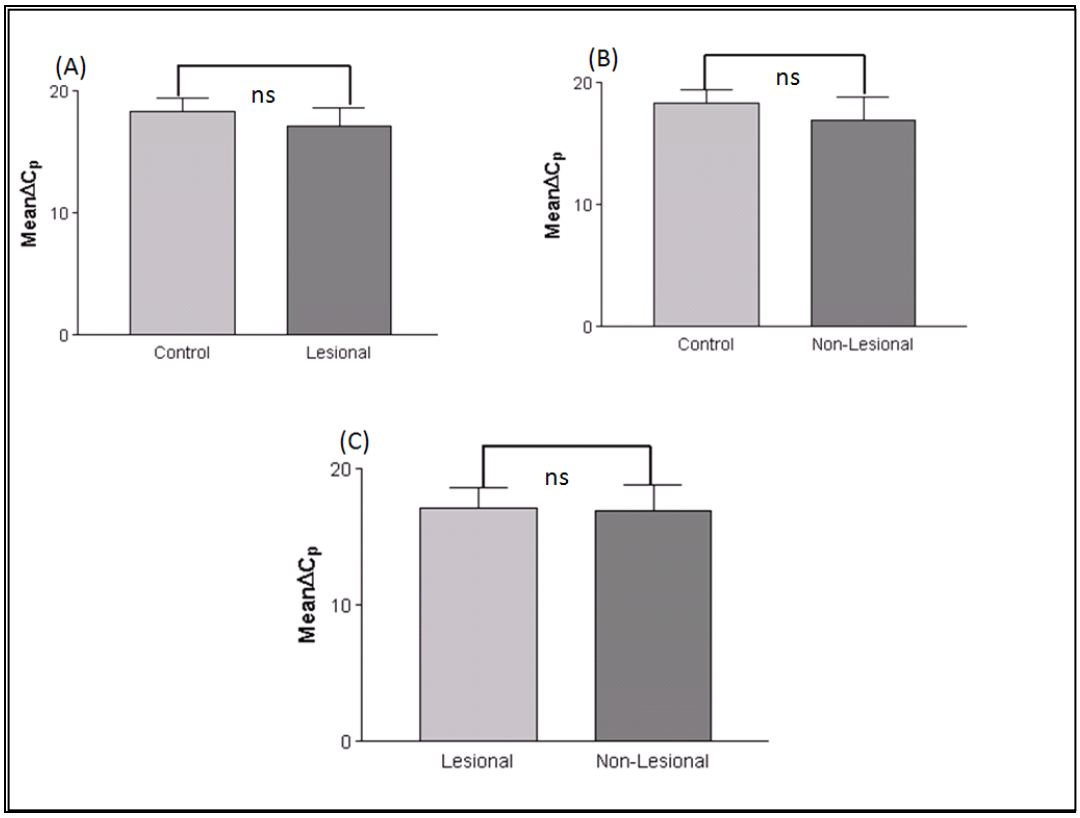

Figure 6: *IL6* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL6* levels between lesional and control skin (p=0.5473).

(B) No significant difference was seen in *IL6* levels between non-lesional and control skin (p=0. 5697).

(C) No significant difference was observed in *IL6* levels between lesional and non-lesional skin of vitiligo patients (p=0. 9511).

3.3.6 Gene expression studies of *IL10* in control and vitiliginous human skin.

IL10 levels were found to be reduced significantly in lesional skin of vitiligo patients compared to control skin (p=0.0357) while no significant difference was found in *IL10* levels between control and non-lesional skin (p=0.5565). Further, there was no significant difference in *IL10* levels between lesional and non-lesional skin of vitiligo patients (p=0.0980; Figure 7).

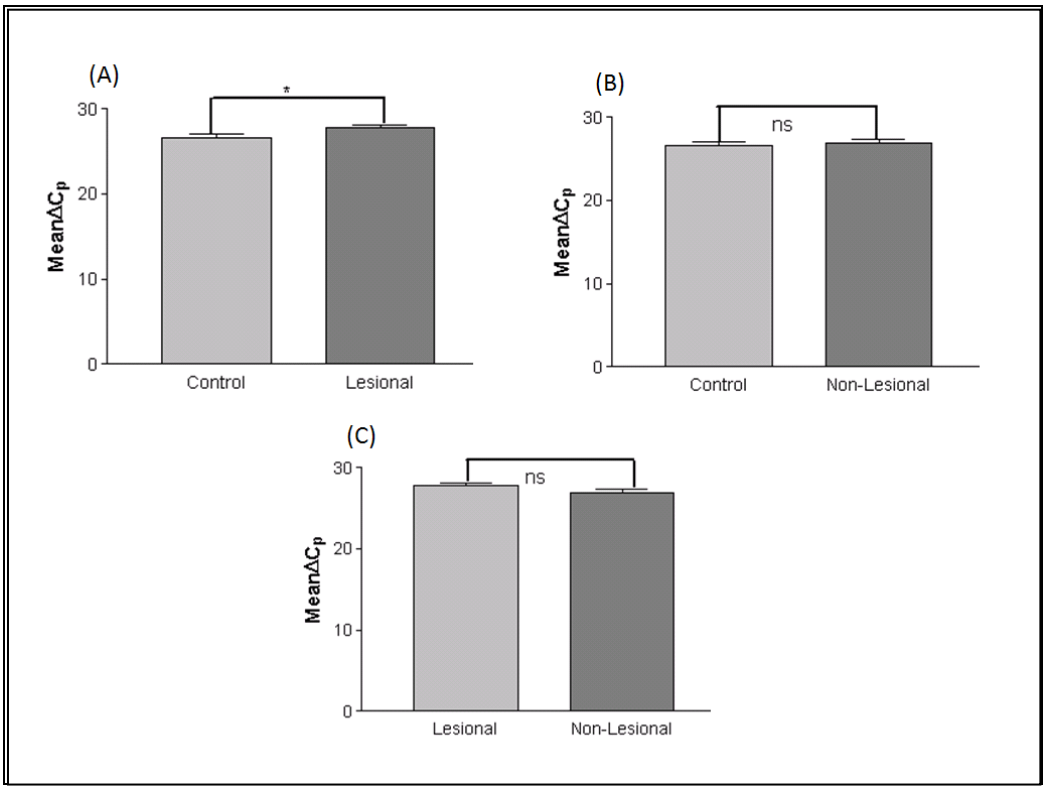

Figure 7: *IL10* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) Lesional skin from vitiligo patient showed significant decrease in the expression of *IL10* compared to control skin (p=0.0357).

(B) No significant difference was seen in *IL10 l*evels between non-lesional and control skin (p=0.5565).

(C) No significant difference was seen in *IL10* levels between lesional and non lesional skin of vitiligo patients (p=0.0980).

3.3.7 Gene expression studies of *IFNG* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients exhibited significant increase in expression of *IFNG* compared to control skin (p=0.0231 and p=0.0138, respectively). Further, there was no significant difference in *IFNG* levels between lesional and non-lesional skin of vitiligo patients (p=0.0808; Figure 8).

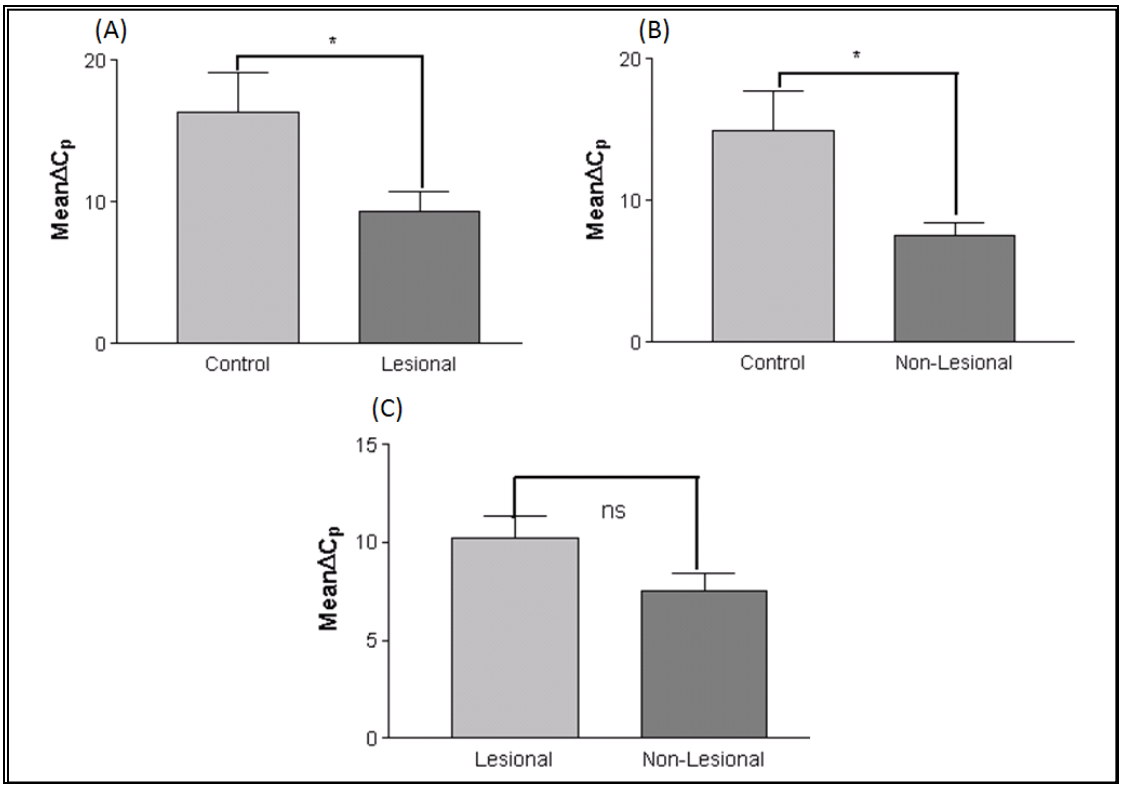

Figure 8: *IFNG* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) Lesional skin from vitiligo patient showed significant increase in the expression of *IFNG* compared to control skin (p=0.0231).

(B) Non-lesional skin from vitiligo patient showed significant increase in the expression of *IFNG* compared to control skin (p=0.0138).

(C) No significant difference was observed in *IFNG* levels between lesional and non-lesional skin of vitiligo patients (p=0.0808).

3.3.8 Gene expression studies of *IL1R1* in control and vitiliginous human skin.

We performed a comparative analysis for *IL1R1* expression between lesional and non-lesional skin from vitiligo patients and healthy skin from controls. Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1R1* compared to control skin (p=0.8186, p=0.2418 respectively). Further, there was no significant difference in *IL1R1* levels between lesional and non-lesional skin of vitiligo patients (p=0.8180; Figure 9).

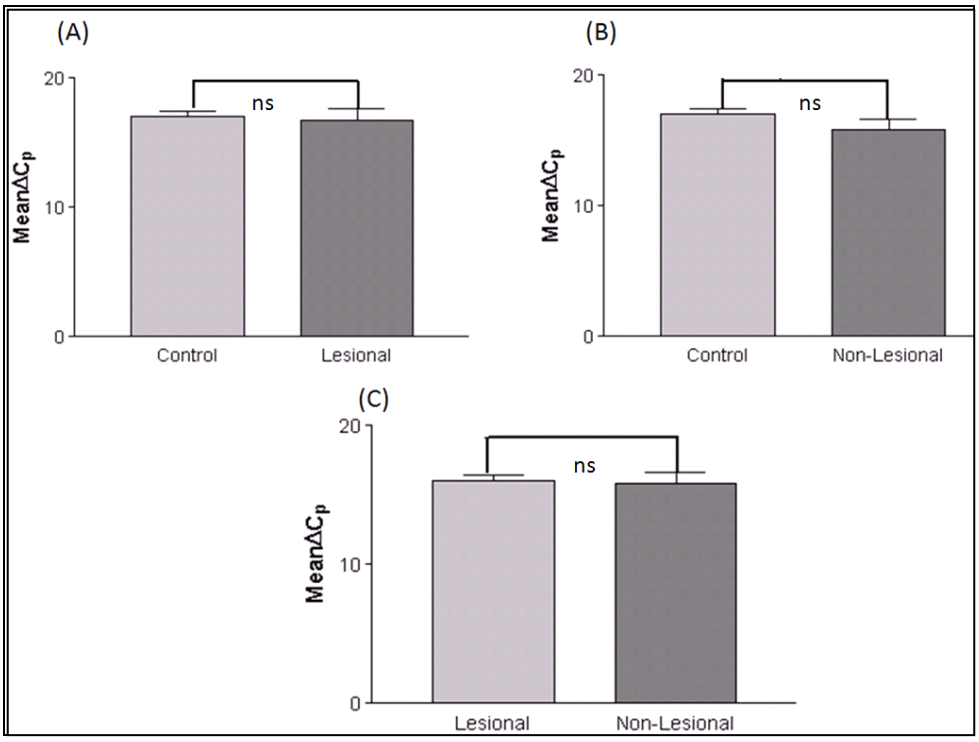

Figure 9: *IL1R1* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1R1* levels between lesional and control skin (p=0.8186).

(B) No significant difference was seen in *IL1R1 l*evels between non-lesional and control skin (p=0. 2418).

(C) No significant difference was seen in *IL1R1* levels between lesional and non-lesional skin of vitiligo patients (p=0.8180).

3.3.9 Gene expression studies of *IL1RN* in control and vitiliginous human skin.

Our results showed that lesional as well as non-lesional skin of vitiligo patients did not exhibit significant difference in expression of *IL1RN* compared to control skin (p=0.2147 and p=0.8604, respectively). Further, there was no significant difference in *IL1RN* levels between lesional and non-lesional skin of vitiligo patients (p=0.4080; Figure 10).

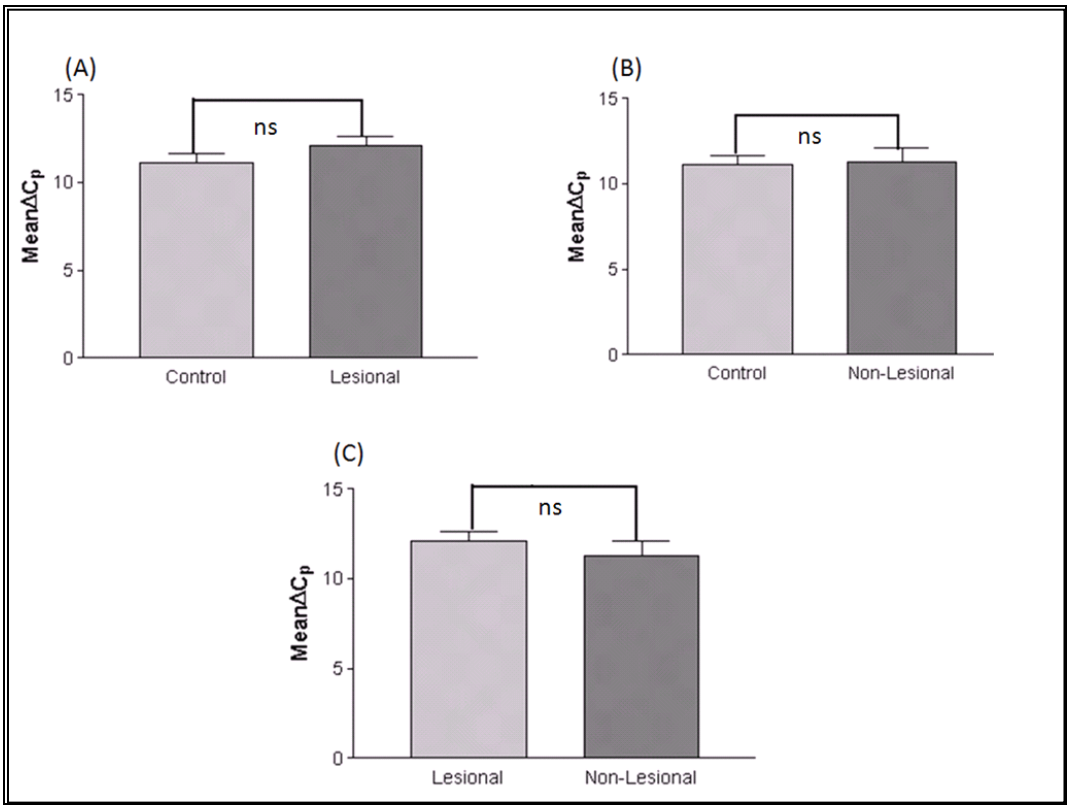

Figure 10: *IL1RN* transcript levels in skin of vitiligo patients (n=12) and controls (n=12).

(A) No significant difference was seen in *IL1RN* levels between lesional and control skin (p=0.2147).

(B) No significant difference was seen in *IL1RN* levels between non-lesional and control skin (p=0. 8604).

(C) No significant difference was observed in *IL1RN* levels between lesional and non-lesional skin of vitiligo patients (p=0. 4080).

Table 3: Analysis of transcripts levels of candidate genes in vitiligo patients and controls (n=12)

Gene	Lesional skin vs. Control (*p* value)	Non-lesional skin vs. Control (*p* value)	Non-lesional vs. lesional skin (*p* value)
TNFA	(0.0246) ↑	(0.0467) ↑	(0.6749) ns
IL1A	(0.6000) ns	(0.4004) ns	(0.2886) ns
IL1B	(0.5085) ns	(0.0290) ↑	(0.0021) ↑
IL4	(0.2886) ns	(0.2152) ns	(0.9669) ns
IL6	(0.5473) ns	(0.5697) ns	(0.9511) ns
IL10	(0.0357) ↓	(0.5565) ns	(0.0980) ns
IL1R1	(0.8186) ns	(0.2418) ns	(0.8180) ns
IL1RN	(0.2147) ns	(0.8604) ns	(0.4080) ns
IFNG	(0.0231) ↑	(0.0138) ↑	(0.0808) ns

3.4 DISCUSSION

Vitiligo is a multifactorial, polygenic skin disorder with involvement of various genes important for immune system, oxidative stress etc. (Laddha *et al.*, 2013). Immune responses in the skin involve an armamentarium of immune-competent cells and biological response modifiers including cytokines (Salmon *et al.*, 1994). Melanocytes are pigmentary cells residing at the basal layer of epidermis and contribute to cutaneous immune system. Melanocytes secrete various cytokines and express adhesion molecules upon exposure to different stimuli for e.g. cytokines, H_2O_2 etc. (Dwivedi *et al.*, 2013).

Moreover, in the epidermal melanin unit neighboring keratinocytes secrete several growth factors affecting melanocyte proliferation and melanin synthesis (Abdel-Naser *et al*., 1999; Abdel-Naser *et al*., 2005). Our previous lab results have demonstrated the increased levels of *TNFA*, *TNFB*, *IFNG*, and *IL4* in the circulation of vitiligo patients (Laddha *et al.*, 2012; Laddha *et al.*, 2013; Dwivedi *et al.*, 2013; Imran *et al.*, 2012).

Albeit, dissimilar outcomes are reported for few cytokines and candidate genes, with respect to its expression levels and genetic association with vitiligo, suggesting the involvement of other factors and role of ethnicity in disease precipitation (Figure 1). Thus, it is pertinent to study the levels of key candidate genes in the same set of skin samples. Overall, the above results indicate the importance of assessing the transcript levels of these cytokines in the microenvironment of skin of vitiligo patients. IFN-γ plays an important role in maintaining the homeostasis of melanocyte biology and its levels are important for maintaining cytokine balance. IFN-γ and IFN-γ-induced chemokines, specifically, CXCL10 and its cognate receptor C-X-C Motif Chemokine Receptor (CXCR)-3 are found to be increased in lesional skin and serum samples of patients with vitiligo, and also seen to be upregulated in autoreactive T cells in the blood and skin of patients with vitiligo (Rashighi *et al.*, 2014; Ezzedine *et al.*, 2015; Harris, 2015)**.** We have also found significantly increased transcript levels of *IFNG* in lesional as well as non-lesional skin of vitiligo patients (Figure 8). Increased levels of TNF-α have been reported in the lesional skin (Moretti *et al.*, 2002; Grimes *et al.*, 2004; Birol *et al.*, 2006) which are in accordance with our results, where we have found increased levels of *TNFA* in lesional and non-lesional skin of vitiligo patients (Figure 2). Interleukin 1 regulation is important for immune homeostasis whereas increased levels of IL-1α and IL-1β were reported in vitiligo (Birol *et al.*, 2006; Tu *et al.*, 2003; Dani *et al.*, 2017). Increased levels of IL-6 in skin (Moretti *et al.*, 2009) have also been reported in vitiligo patients. Unlike other studies, we have found decreased levels of *IL10* in skin samples of vitiligo patients (Figure 7). On the contrary, Grimes *et al.*, (2004) and Aydingoz *et al.*, (2015) have reported increased levels of *IL10* in skin and serum samples of vitiligo patients. After topical application of tacrolimus, Grimes *et al.*, (2004) found no significant difference in the *IL10* transcript levels, warranting the need for further investigation of genetic variants and levels of *IL10* in vitiligo patients.

It has been shown that human keratinocytes constitutively express inflammasome proteins together with pro-IL-1α and β and pro-IL-18 (Feldmeyer *et al.,* 2007). IL-1β activation in human epidermis can also occur *via.* an alternative mechanism involving stratum corneum chymotryptic enzyme (kallikrein 7), a serine protease specifically expressed in keratinizing squamous epithelia (Lundqvist *et al.,* 1997). Our results showed increased *IL1B* expression levels in non-lesional skin as compared to lesional skin (Figure 4), substantiating its important role in disease progression and are in accordance with studies of Wang *et al.,* (2011). They have also found increased level of *IL1B* in skin samples of vitiligo patients. IL-1β acts on lymphocytes and up regulates IL-2 receptor expression, prolonging the survival of T-cells, enhancing the antibody production by B-cells and increasing B-cell proliferation. IL-1β also helps in differentiation and proliferation of Th17 cells. Thus, IL-1β amplifies T and B-cell responses that might lead to destruction of melanocytes in the lesional skin (Marie *et al.,* 2014). Non significant changes in the expression levels of *IL1A, IL4, IL6, IL1R1* and *IL1RN* were observed in lesional and non-lesional skin of vitiligo patients compared to controls. A previous report showed that the IL-4 levels were decreased in peri-lesional skin, when compared to non-lesional and lesional skin (Wang *et al.,* 2011). However, we did not find difference in the expression levels of *IL4*. Overall, our studies provide evidence of cytokine imbalance in the microenvironment of skin of vitiligo patients (Table 3).

In conclusion, we have established the existence of epidermal cytokine imbalance between pro and anti–inflammatory cytokines in the skin microenvironment of vitiligo patients. We have found that *TNFA, IL1B, IFNG* and *IL10* are potential genes and might contribute to autoimmune mediated pathogenesis of vitiligo.

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4.1 INTRODUCTION

Cytokines are low molecular weight glycoproteins produced by myriad cell types, predominantly leukocytes, which regulate various physiological and pathological functions including innate and adaptive immunity in addition to plethora of inflammatory responses. Redundancy, synergy, and pleiotropism are the characteristics of cytokine action and provide accountability for the effectiveness of these proteins in regulating immune response (Kuby, Immunology, 6th Ed., 2007). Vitiligo is a hypo-pigmentary skin disorder caused due to selective destruction of melanocytes of poorly understood etiology. There is growing evidence that cytokines play an important role in the depigmentation process of vitiligo (Moretti *et al*., 2002; Moretti *et al*., 2009). Cytokines such as IL1, IFN-γ or TNF-α released by lymphocytes and keratinocytes can initiate apoptosis of melanocytes. Also, TNF-α, IL1-α, IL6, and TGF-β are potent inhibitors of melanocyte growth (Swope *et al*., 1991; Moretti *et al*., 2002). Previous studies, including ours, have shown increased levels of TNF-α, TNF-β, IFN-γ, IL4, IL1-β and ICAM1 in vitiligo patients (Laddha *et al*., 2012; Laddha *et al*., 2013; Dwivedi *et al*., 2013; Imran *et al*., 2012; Laddha *et al*., 2014; Tu *et al*., 2003; Badri *et al*., 1993). Therefore, it becomes pertinent to investigate the effect of these cytokines in melanocyte biology. The modulation of inflammatory phenomena and immune response including restoration of the keratinocyte-melanocyte axis with specific low dose of interleukins, antibodies and basic fibroblast growth factor represent an innovative therapeutic approach for vitiligo treatment. Exploring this avenue will substantiate the basis of 'low dose cytokine therapy' for vitiligo (Lotti, 2015). To develop effective treatment modality for melanocyte related disorders such as vitiligo, melanoma etc. it is essential to understand the biology of melanocyte, especially its primary function i.e., melanin synthesis. Hence, we aimed to explore the effect of TNF-α, IL-6, IL-1α and IL-10 on the melanocyte biology by monitoring cell viability, melanogenesis [Tyrosinase *(TYR)* and Microphthalmia Associated Transcription Factor *(MITF)-M*] and *ICAM1* expression levels upon exogenous stimulation of primary cultured normal human melanocytes (NHM) with different cytokines.

4.2 MATERIALS AND METHODS

4.2.1 Ethics statement It is stated in Chapter II.

4.2.2 Study subjects (Histopathological studies)

This case–control study was carried out on 6 vitiligo patients and controls. Vitiligo patients were selected from the Dermatology Unit of Seth Vadilal Sarabhai Hospital, Ahmedabad. The disease duration in patients was ranging from 6 months to 50 years. The control group consisted healthy individuals, without any history of auto-immune or skin related diseases with an age range from 20 to 50 years old. Vitiligo was clinically classified according to Taieb and Picardo, 2007. The importance of the study was explained to all participants and written consent was obtained. Demographic details of the subjects recruited and sample details are provided in Table 1.

Table 1: Demographic details of control and vitiligo patients (Histopathological studies)

	Control (n=6)	**Patient (n = 6)**
Sex (Men: Women)	2:4	1:5
Age, mean (range), yrs	25.86 ± 4.728	34.43 ± 5.173
Duration of lesions	NA	13.0 ± 6.633 months
Size of lesions	2-10 cm in diameter	2-10 cm in diameter
Type of Vitiligo	NA	
Segmental Vitiligo	-	3
Non-segmental Vitiligo	-	3
Sites	Breast, Abdomen and Foreskin	Head and neck, trunk and the extremities.

4.2.3 Skin biopsies

From each patient, 5-mm punch biopsy collected after applying 2% lignocaine (a local anesthesthetic agent). In vitiligo patients, skin biopsies were taken from three regions: non-lesional, peri-lesional and lesional. Here non-lesional is the normal appearing skin of vitiligo patient, peri-lesional is the active border of the depigmented patch and lesional is the depigmented patch of the skin of vitiligo patient. All specimens were fixed in 10% neutral-buffered formalin and subjected to routine tissue processing that ended with paraffin-embedded blocks ready for sectioning. Hematoxylin and Eosin (H and E)-stained sections were examined microscopically to evaluate pathological changes in epidermal and dermal region.

4.2.4 Epidermal thickness and rete ridges pattern

The sections were randomly examined and photographed with Nikon D100 digital camera at low magnification (20X) under a microscope (Olympus, Tokyo, Japan). Viable epidermal thicknesses were measured at 10 different points including the thickest and the thinnest areas of each section with the exception of follicles using a computerized image analyzer (Image-J software). The viable epidermis was defined from the top of the stratum granulosum or from the top of the stratum lucidum in the case of palms and soles to the base of the stratum basale. The pattern of rete ridges was observed between non-lesional, peri-lesional, lesional and control skin under the microscope.

4.2.5 Hematoxylin and Eosin stain (Baker, 1962)

Hematoxylin and eosin staining were performed for 6 vitiligo patients and 6 controls. Skin biopsies were fixed in a 10% buffered formalin solution, processed, and embedded in paraffin. Paraffin blocks were cut into 4- to 5-micron sections on a microtome and placed on glass slides coated with Poly-L-Lysine. Sections were deparaffinized by giving 2 changes of xylene, 10 mins each. Sections were hydrated once in absolute alcohol for 5 mins followed with 95% alcohol for 2 mins and 70% alcohol for 2 mins followed by brief wash with distilled water.

The slides were then washed briefly in distilled water and stained with Harris hematoxylin (BHI, Hi-Media, India) solution for 8 minutes and then wash in running tap water for 5 minutes. The differentiation step was carried out in 1% acid alcohol for 30 seconds followed by a wash under running tap water for 1 minute. Subsequently, the bluing of the sections was done in 0.2% ammonia water or saturated lithium carbonate solution or under running tap water for 30 seconds to 1 minute. The slides were washed under running tap water for 5 mins and rinsed in 95% alcohol, 3mins/10dips. Eosin-phloxine (BHI, Hi-Media, India) solution was used to counter stain for 30 seconds to 1 minute. Lastly the slides were dehydrated through 95% and, absolute alcohol, 5 minutes each and clear in 2 changes of xylene, 5 minutes each. The sections were mounted with Xylene/DPX based mounting medium. The sections were examined randomly and photographed with Nikon D100 digital camera at low magnification (20X) under a microscope (Olympus, Tokyo, Japan).

4.2.6 Immunohistochemical studies

Skin biopsies were fixed in 10% buffered formalin solution, processed, and embedded in paraffin. Paraffin blocks were cut into 4 to 5micron sections using a microtome and placed on glass slides coated with Poly-L-Lysine. Deparaffinization of the sections was done in xylene and rehydration in a graded series of alcohol was carried out. The sections were then washed in PBS for 5 mins and then permeabilization of the sections was done with PBST (0.1% triton X / tween 20) for 15 mins followed by wash by PBS for 5 mins.

Antigen retrieval for demasking of antigens was carried out by boiling the slide in citrate buffer (pH 6.0)/Tris-EDTA (pH 9.0) using a microwave oven for three cycles, 5 mins each with a 1-min break between each cycle. After cooling to room temperature, the slides were washed twice with PBS-buffer (pH 7.4). The blocking of non-specific interactions was accomplished by incubating the samples with blocking serum (5% BSA) for 30 min at room temperature. For immunofluorescence studies, the sections were probed overnight at 4^0C with anti-MITF mouse monoclonal antibody (Abcam, ab19139, Cambridge, MA) at a dilution of 1:400. After thorough washes in PBS for three times, 5 mins each, the sections were incubated with their respective Cy3 conjugated anti-mouse IgG secondary antibodies (1:200) (Invitrogen, Carlsbad, CA, United States) for 1 hr, and wash with PBS for three

times, 5 mins each. 30-40µL of the diluted DAPI solution was added to each well and incubated for 10-15 mins at room temperature. DAPI binds to DNA and is a convenient nuclear counter stain. It has an absorption maximum at 358 nm and fluoresces blue at an emission maximum of 461 nm. The sections were mounted using cover slip with anti-fading medium and the confocal imaging was carried out on a FV1000-D (Olympus, Tokyo, Japan) confocal laser 0020 scanning microscope. Excitation and detection of the samples were carried out in sequential modes to avoid overlapping of signals. The sections were scanned simultaneously at both wavelengths (555/580 nm) with appropriate laser intensity, confocal aperture, and gain. The Black-level setting was kept constant for all the samples**.**

4.2.7 Ethics statement & Culture establishment of primary normal human melanocytes (NHM) These aspects are described in Chapter II.

4.2.8 MTT assay

The number of viable cells was recorded using MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium salts] (Mosmann, 1983). NHM were seeded in 96-well plates at an initial density of 5×10^3 cells per well and after overnight incubation, the cells were treated with human recombinant cytokines: TNF-α, IL-1α, IL-6, and IL10 in a dose dependent manner (0, 10, 50, 100 ng/ml). After 48 hrs of treatment, MTT (Molecular probes® by Life Technologies™, China) was added to each well and the cells were incubated at 37°C for 4 hrs. The medium was removed and dimethyl sulfoxide (DMSO) (Sisco Research Laboratories, Mumbai) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using ELISA plate reader (Thermo Scientific Multiskan EX, Shanghai, China).

4.2.9 Melanin content assay (Hu, 2008)

NHM were cultured in 6-well plates at a concentration of 1×10^5 cells/well overnight and then treated with TNF-α for 48 hrs. Then the cells were trypsinized and counted. The same number (1×10^5) of cells were collected and the pellets were dissolved in 1 M NaOH at

80°C for 1 hr. Melanin concentrations were measured by absorbance at 470 nm. The results are the compilation of three independent experiments.

4.2.10 Analysis of complex-I activity (Prajapati *et al*., 2015)

Complex-I activity of electron transport chain was determined by Spectrophotometry. The activity of complex-I was determined using decyl ubiquinone as the electron acceptor and NADH as the donor. Briefly, cells were harvested and washed with cold DPBS. Cells were subjected to freeze-thawing two or three times in Freeze–Thaw complete solution (0.25 M Sucrose, 20 mM Tris–HCl (pH 7.4), 40 mM KCl, 2 mM EDTA supplemented with 1 mg/ml fatty acid-free BSA, 0.01% Digitonin and 10% Percoll). After completion of the freeze-thawing process, the cells were washed with Freeze–Thaw solution devoid of digitonin to remove the residual digitonin as it interferes with the complex-I enzyme assay. Cells were suspended in complex-I assay buffer (35 mM Potassium Phosphate (pH7.4), 1 mM EDTA. 2.5 mM NaN_3, 1 mg/ml BSA, 2 µg/ml Antimycin A and 5mM NADH). Complex-I activity was measured for 3 mins by following the decrease in absorbance at 340 nm after the addition of 2.5 mM decyl ubiquinone indicating the oxidation of NADH.

4.2.11 Cellular and Mitochondrial reactive oxygen species (ROS) estimation

In order to observe the formation of reactive oxygen species, a fluorescent dye 2',7' dichlorodihydrofluorescein diacetate (H_2DCFDA-AM) was used. Oxidation of H_2DCFDA by ROS converts the molecule to 2',7' dichlorodihydrofluorescein (DCF), which is highly fluorescent. Upon stimulation, the resultant production of ROS causes an increase in fluorescence over time. $1x10^6$ cells were harvested and washed with 1X PBS twice. DCFDA (50 nM) was added to the cells and was incubated for 15 min at 37^0C with shaking, followed by two washes with 1X PBS. Fluorescence was measured by flow cytometry (BD Biosciences FACS-ARIA III) using 200 µl sample diluted 5 times using 1X PBS. Excitation (λex) and emission (λem) wavelengths used for fluorimetric studies were 480 and 525 nm respectively (Esposti, 2002). Similarly, 5µM MITOSOX dye which specifically measures mitochondrial ROS levels with an excitation wavelength of 510 nm and an emission wavelength of 580 nm was used**.**

4.2.12. Monitoring MITF-M levels upon cytokine stimulation by immunofluorescence

MITF-M antibody at a concentration of 0.5 µg/100µl (1:200, Abcam 80651) and anti-mouse IgG (whole molecule) TRITC conjugate (Sigma) at a dilution of 1:400 were used to study MITF-M expression upon cytokine stimulation. 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, cytokine treatment was given along with media replacement and the cells were further incubated for 48 hrs. After treatment, cells were washed once with phosphate buffered saline (PBS) pH 7.4, fixed in 70% chilled methanol for 10 min at -20°C and then washed with blocking solution (1.5% BSA with 0.05% Tween 20 in PBS), incubated for 8 hrs in primary antibody. After incubation, the cells were washed 2-3 times with blocking solution and further incubated for 1 hr with TRITC labeled secondary antibody. Followed by 2 PBS washes, cells were observed for fluorescence which was monitored under 63X by Zeiss confocal laser scan fluorescence-inverted microscope (LSM 710; Carl Zeiss). Data were analyzed using Image Pro plus software to calculate mean density of fluorescence and graphs were plotted using Graph Pad prism software.

4.2.13 Western Blot analysis

To detect alterations in protein levels, NHM were treated with or without TNF-α. Total cell lysates were obtained by Laemmli Buffer (10% SDS, 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, and 5% 2-beta-mercaptoethanol) extraction. Thirty µg of total protein was subjected to electrophoresis in 12.5% SDS gel under reducing conditions and subsequently transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were incubated with the following antibodies: TNFR1, MITF, caspase 3, LCI-II and β-actin. Protein bands were detected by enhanced chemiluminescence system ECL (Amersham Pharmacia Biotech) and densitometric analysis was carried out with β-actin as a loading control.

4.2.14 RNA extraction and cDNA synthesis

Total RNA from primary NHM was isolated and purified using TriZol® reagent (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by 1.5% agarose gel

electrophoresis and 260/280 absorbance ratio. RNA was treated with DNase I (Ambion Inc., Austin, TX, USA) before cDNA synthesis, to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed using the Verso cDNA Synthesis Kit (Thermo Scientific, US) according to the manufacturer's instructions in the G Palm Thermal Cycler (Model PTC-200, Watertown, MA, USA).

4.2.15 Relative gene expression analysis

First strand cDNA was used as a template with gene-specific primers to synthesize second strand DNA by conventional PCR. Expression kinetics of *IL1R1*, *IL1RN*, *IL1A*, *IL1B*, *IL6*, *TNFA*, *ICAM1*, *MITF-M*, *IL8*, *TYR* and *TYRP1* genes were analysed. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* was used as internal control (Table 2). Reactions were performed according to the manufacturer's instructions (Fermentas, Burlington, ON, Canada). DNA fragments were amplified for 39 cycles and signal intensities were analysed on 3.5% agarose gel stained with ethidium bromide. Densitometric analysis was performed using Alpha Imager software, and mean density for respective genes with respect to *GAPDH*, was plotted.

Table 2: Primer sequences used for gene expression analysis.

Primer	**Sequence (5' to 3')**	**Amplicon size (bp)**	**Annealing Temperature (^{0}C)**
IL1R1 FP	GGAGGCTGATAAATGCAAGG	195	62
IL1R1 RP	GTAAGATGAATTTACCACGC		
IL1RN FP	GCTGGAGGCAGTTAACATC	165	65
IL1RN RP	CTACTCGTCCTCCTGGAAG		
IL1A FP	GCTGCTGAAGGAGATGCCTG	143	66

IL1A RP	CTACGCCTGGTTTTCCAGT		
IL1B FP	AGATGAAGTGCTCCTTCCAGG	153	65
IL1B RP	TGGTCGGAGATTCGTAGCTG		
IL6 FP	AAATTCGGTACATCCTCGACGGCA	88	62
IL6 RP	AGTGCCTCTTTGCTGCTTTCACAC		
TNFA FP	GCCCCCAGAGGGAAGAGTTCCCCA	124	70
TNFA RP	GCTTGAGGGTTTGCTACAACATGGGC		
ICAM1FP	TCTGTTCCCAGGACCTGGCAATG	282	65
ICAM1RP	GGAGTCCAGTACACGGTGAGGAAG		
MITFM FP	CAAATGATCCAGACATGCGCTGG	180	61
MITFM RP	CTCGAGCCTGCATTTCAAGTTCC		
IL8FP	GATCCACAAGTCCTTGTTCCA	110	56
IL8RP	GCTTCCACATGTCCTCACAA		
TYRFP	AGCACCCCACAAATCCTAACTTAC	92	63
TYRRP	ATGGCTGTTGTACTCCTCCAATC		
TYRP1FP	TTTGTAACAGCACCGAGGATG	192	62
TYRP1RP	TGGGGTCACTGTAACCTTCCAC		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65
GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

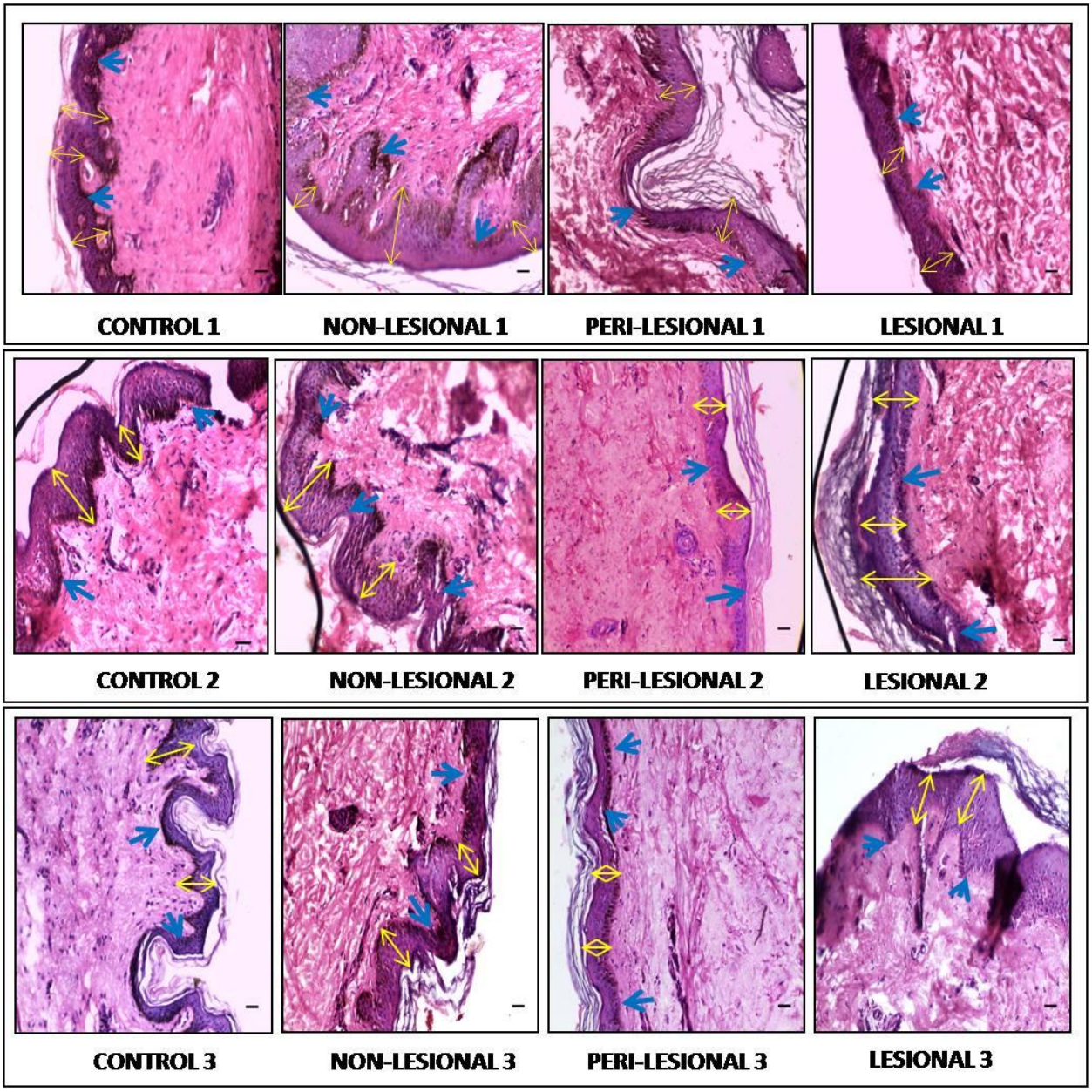
4.2.16 Statistical analyses

All the experiments were performed at least three times in triplicates independently using different batches of cells. Data are presented as the mean ± standard deviation (SD) and the statistical difference between two groups was analyzed by Student's t-test using GraphPad Prism version 3.02 software (GraphPad Software Inc. San Diego, CA, USA). *p*-values less than 0.05 were considered statistically significant. Histopathological studies: for measuring the epidermal thickness was performed using the Image J software (Stat Soft, Inc., USA). Descriptive statistics of quantitative traits was presented as mean. The Correlation between the length of epidermal thickness between patients and control was analyzed in GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California, USA) where unpaired t-test was used for comparison of unrelated groups between NL, PL, L compared to control. In testing hypotheses, the differences were considered statistically significant at $p< 0.05$.

4.3 RESULTS

4.3.1 Histopathological analysis of skin biopsies (A) Viable epidermal thickness and rete-ridges pattern:

To examine the difference in skin architecture of vitiligo patients and controls, Hematoxylin and Eosin (H&E) staining was performed to assess epidermal hyperplasia/ epidermal thickening, viable epidermis thickness and the pattern of rete-ridges in skin biopsies derived from lesional (L), peri-lesional (PL), non-lesional (NL) and healthy matched controls. Our results showed significant increase in viable epidermal thickness in lesional compared to control (p=0.0100) as well as in non-lesional compared to lesional skin (p=0.0304) (Figure 1). Also, significant increase in the epidermal thickness between control and lesional skin was observed (p=0.0082). Similarly, significant difference in epidermal thickness between non-lesional and lesional skin was observed (p = 0.0442).



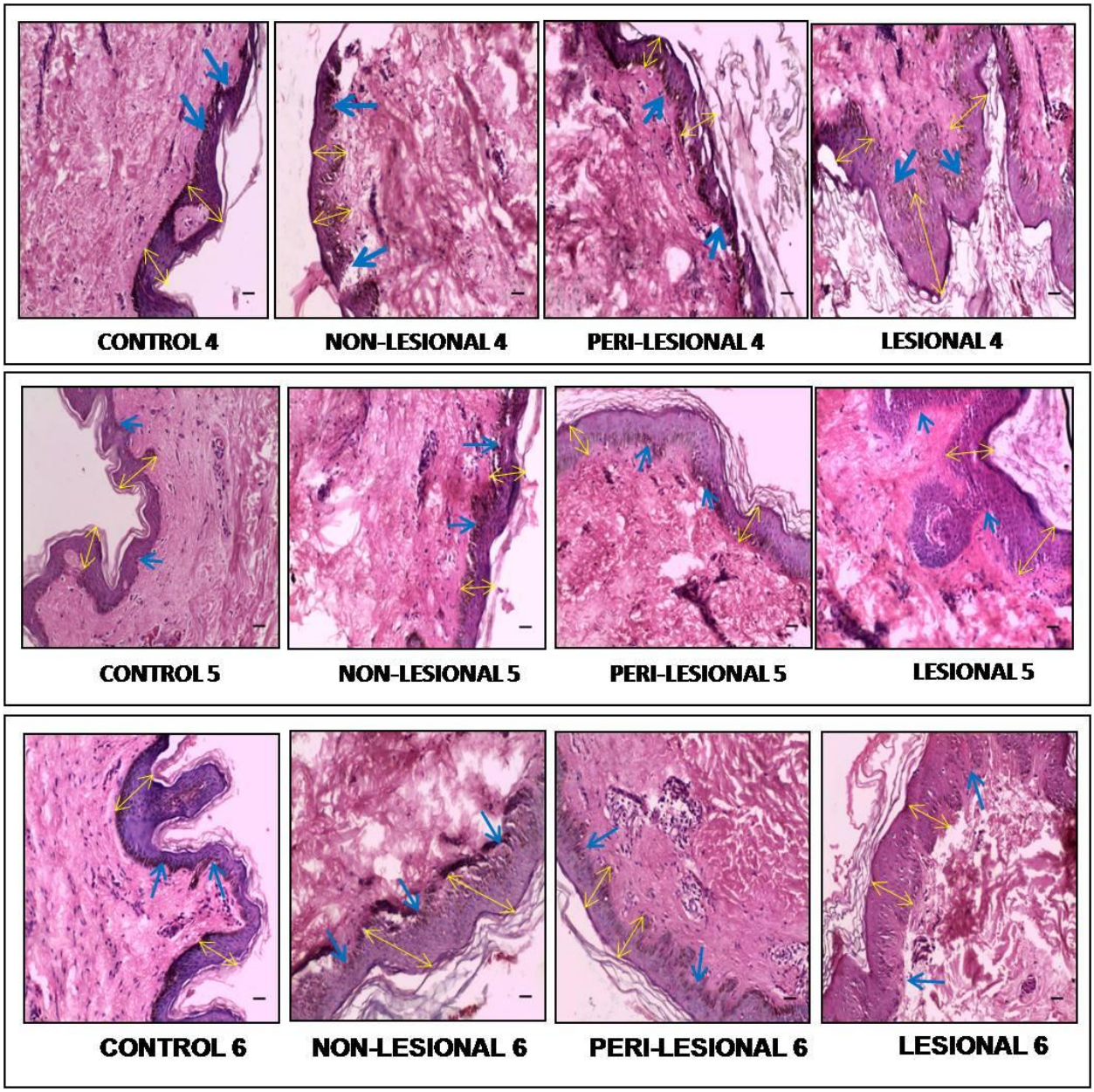


Figure 1: H & E stained tissue samples from vitiligo patients and healthy controls: Change in length of viable epidermis and pattern of rete-ridges was observed at magnification 20X. Yellow arrows represent epidermal thickness (µm); blue arrows represent the pattern of rete-ridges between vitiligenous skin and control skin (n=6). Scale: 10µm.

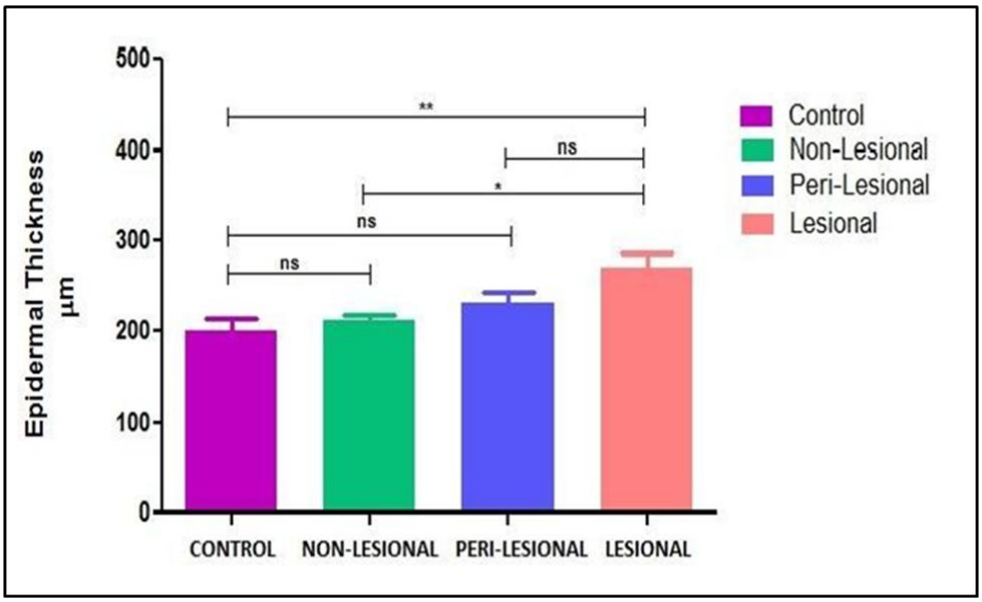


Figure 2: Viable epidermal thickness: Significant increase in viable epidermal thickness was observed in lesional (L) compared to control (p =0.0100) as well as in non-lesional compared to lesional (p=0.0304) skin.

(B) MITF-M expression in skin biopsies of control and vitiligo patients: In order to observe the expression levels of MITF-M in skin biopsies of vitiligo patients and controls, we performed immunofluorescence studies (Figure 3). Our results showed decreased levels of MITF-M in lesional and non-lesional skin of vitiligo patients as compared to controls (p=0.0282 and p=0.0039 respectively) (Figure 4; n=3).

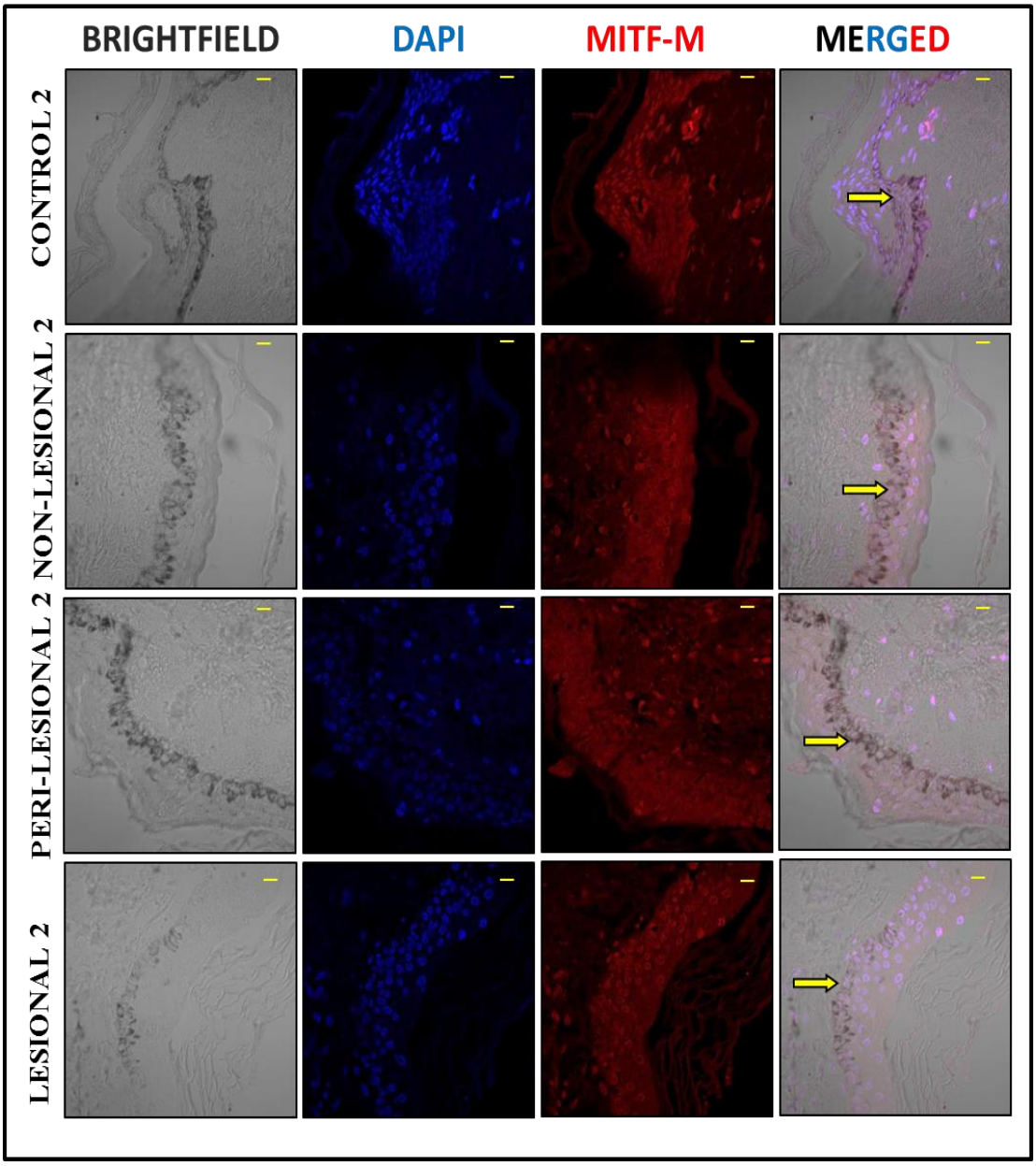


Figure 3: MITF-M expression in control, lesional, non-lesional and peri-lesional skin by Immunofluorescence (Magnification: 63 X; Scale: 100 μm).

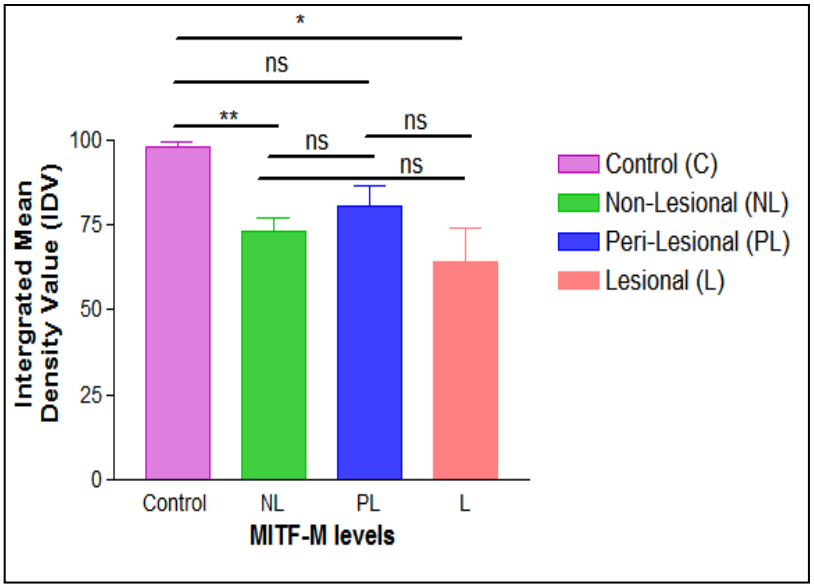


Figure 4: Densitometric analysis of *in situ* MITF-M levels monitored by immunofluorescence: MITF-M levels were found to be significantly decreased in NL vs. C skin (p= 0.0039) and L vs. C skin (p= 0.0282; n=3).

4.3.2 Effect of TNF-α on melanocyte biology:

4.3.2.1 Dose dependent effect of TNF-α on melanocyte cell death:

Primary melanocytes were treated with different doses of TNF-α (0, 10, 50, 100 ng/ml) for 24 and 48 hrs (n=3). No significant difference was observed in morphology and cell death up to 24 hrs (data not shown). However, after 48 hrs of treatment, there was a significant increase in cell death of melanocytes in a dose dependent manner of TNF-α treatment (10, 20, 50 and 100 ng/ml) (Figure 5A and B; p<0.005).

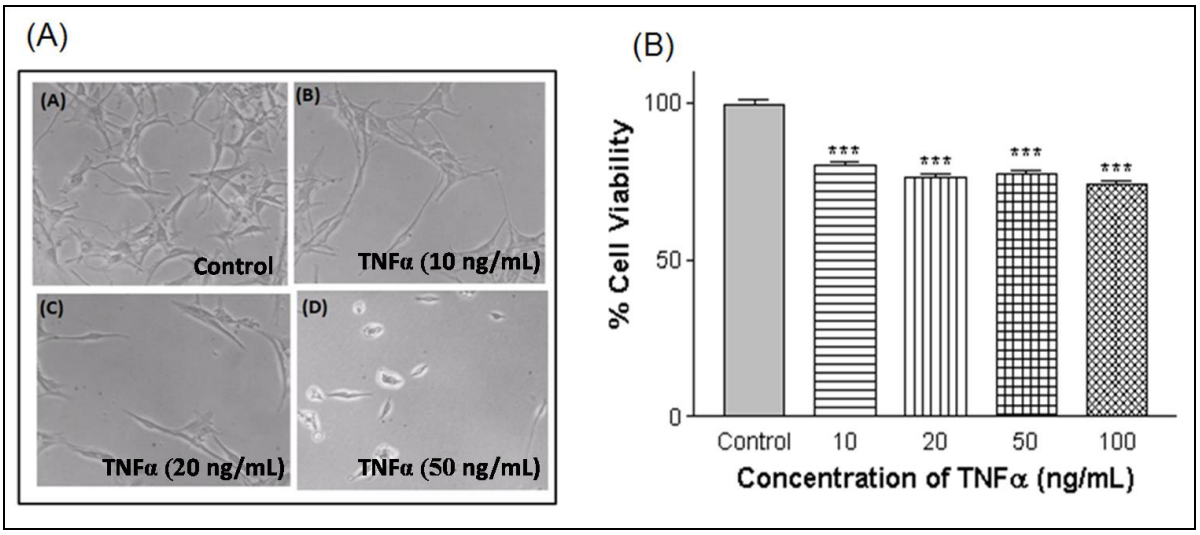


Figure 5: Effect of TNF-α on NHM death: (A) Morphological observation of the effect of TNF-α treatment (a) Control b) 10 ng/ml c) 20 ng/ml d) 50 ng/ml) on NHM as compared to untreated cells (Magnification: 10X; Scale 100μm). (B) Dose dependent effect of exogenous stimulation by TNF-α on melanocytes (0-100 ng/ml TNF-α, 48 hrs).

4.3.2.2 Effect of TNF-α on cellular ROS:

For further experiments, 10ng/ml and 48 hrs of TNF-α dose were selected as it is the minimum concentration with significant effect on the melanocyte viability. We found significant increase in the cellular ROS levels (p=0.0290; n=4; Figures 6A and 6B) in NHM upon TNF-α treatment as compared to untreated cells.

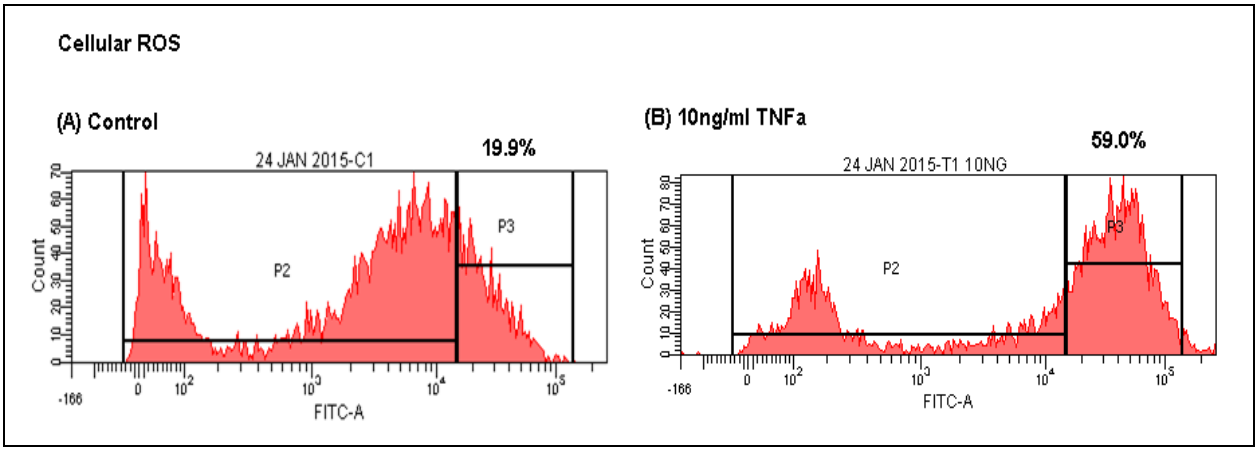


Figure 6: Estimation of cellular ROS levels at 10 ng/ml TNF-α at 48 hrs in NHM. Analysis of ROS levels by FACS in NHM treated with TNF-α and control revealed significant increase in ROS levels upon TNF-α treated treatment (n=4).

4.3.2.3 Effect of TNF-α on mitochondrial ROS:

Further, we monitored the effect of TNF-α on mitochondrial ROS levels and found significant increase in mitochondrial ROS levels (p=0.0033; n=3; Figures 7A and 7B) in NHM upon TNF-α treatment as compared to untreated cells.

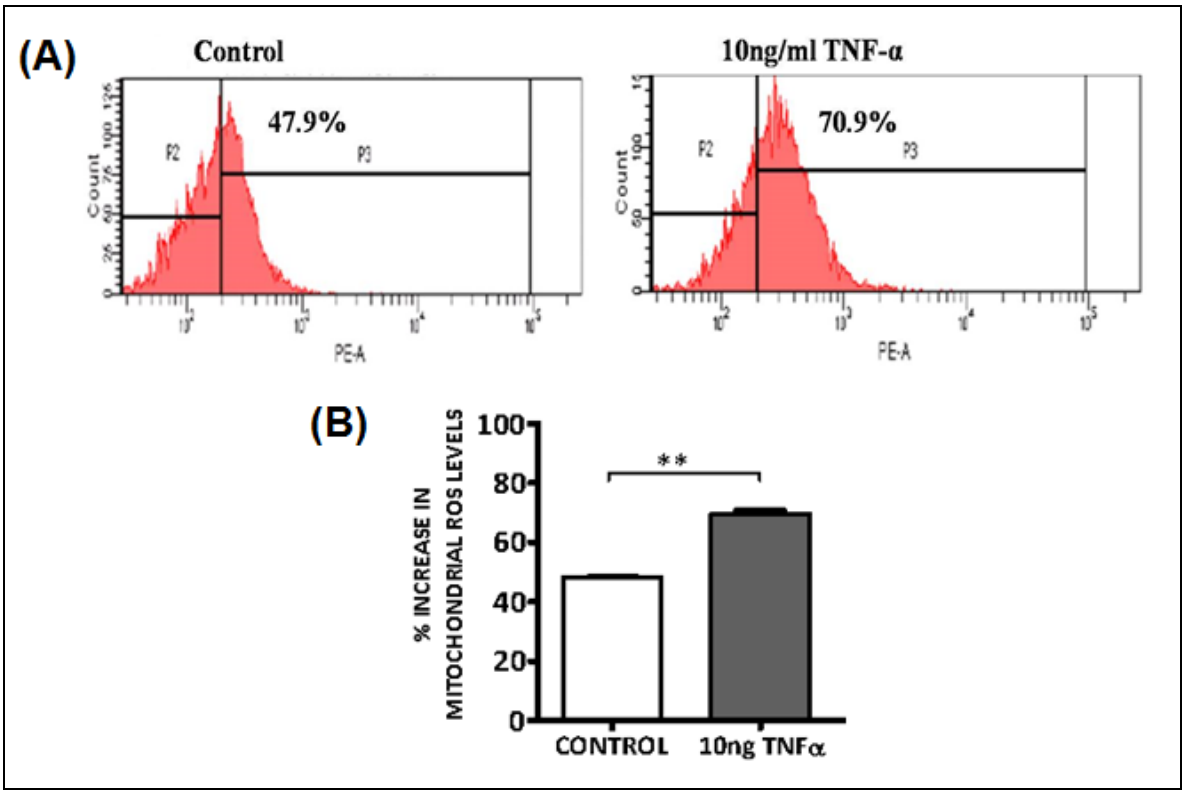


Figure 7: Analysis of Mitochondrial ROS levels in TNF-α treated as compared to untreated NHM: (A) Analysis of mitochondrial ROS levels by FACS in NHM treated with TNF-α and control. (B) Significant increase was found in mitochondrial ROS at 10 ng/ml TNF-α compared to control (48 hrs; *p*=0.0033; n=3).

4.3.2.4 Effect of TNF-α on Complex 1 activity:

In the electron transport chain (ETC), complex 1 is one of the major sites of mitochondrial ROS generation; therefore we aimed to monitor the complex 1 activity in TNF-α treated NHM as compared to untreated cells. Interestingly, we found ~20% decrease in complex 1 activity in TNF-α treated NHM compared to untreated NHM.

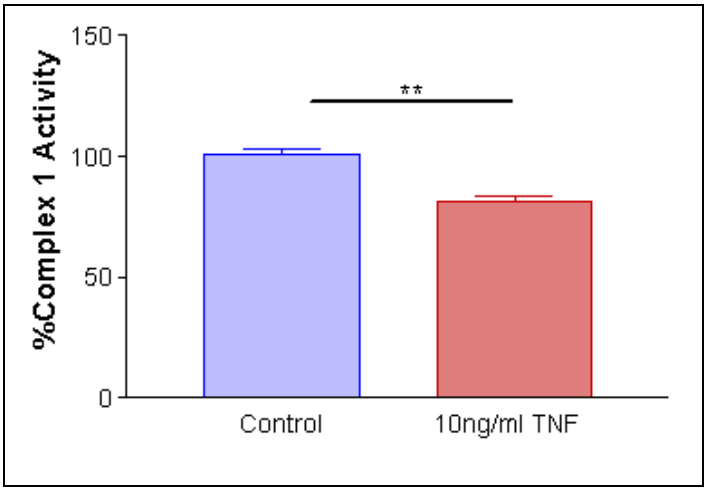


Figure 8: Effect of TNF-α on Complex 1 activity: Complex 1 analysis showed ~20% decrease in complex 1 activity upon TNF-α treatment as compared to controls (48 hrs; n=3).

4.3.2.5 Effect of TNF-α stimulation on AIF expression in NHM:

As we found increased mitochondrial ROS levels with concomitant decrease in complex 1 activity, we monitored the effect of TNF-α on AIF levels. It has been suggested that AIF plays a vital role in mitochondrial respiration and redox metabolism. AIF may interact with complex I and complex III of ETC and helps in structural maintenance (Sevrioukova, 2011). However, our results showed no significant difference in AIF levels upon TNF-α treatment.

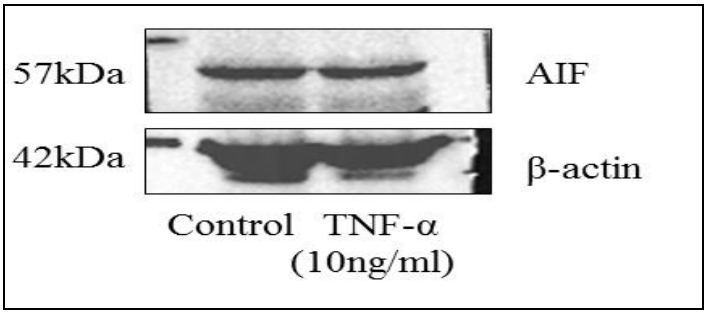


Figure 9: Effect of TNF-α on AIF levels: No significant difference was observed in AIF levels after 48 hrs of TNF-α treatment to NHM as compared to untreated cells (n=3).

4.3.2.6 Effect of TNF-α on Melanogenesis and *IL6* and *ICAM1* expression: NHM were treated with 10ng/ml TNF-α for 48 hrs and analyzed for transcript levels of melanin synthesis genes (*MITF-M, TYR*) melanin content along with *IL6* and *ICAM1* expression levels. Our results showed significant decrease in the expression of melanin synthesis genes *MITF-M* and *TYR* (p=0.0055 and p=0.0055 respectively) with concomitant decrease in melanin content (Figure 10. A, B and C). Moreover, TNF-α treatment resulted in significant increase in the expression of *IL6* as well as *ICAM1* (p=0.0044 and p=0.0072 respectively) (Figures 10. D and E). Furthermore, we demonstrated significant decrease in the protein expression of MITF-M upon 10ng/ml TNF-α treatment on NHM for 48 hrs (Figure 11; p=0.0004; n=3).

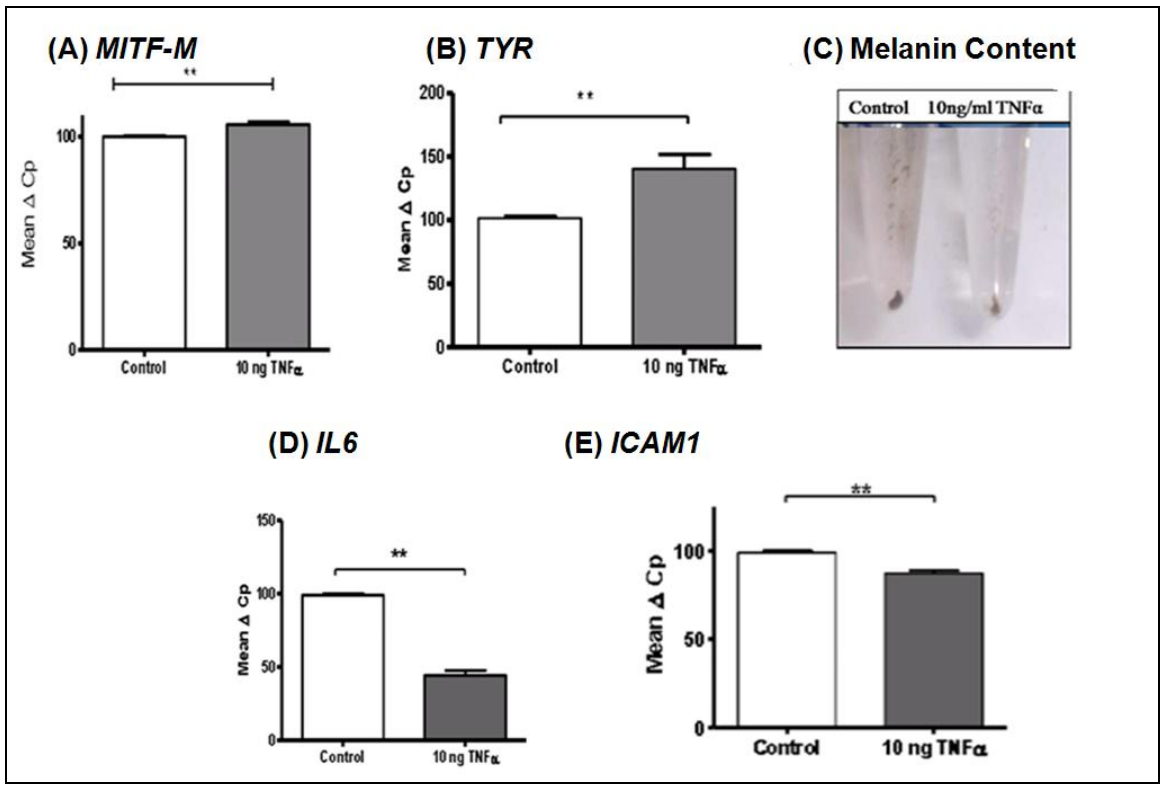


Figure 10: Effect of TNF-α on melanogenesis and, *IL6* and *ICAM1* expression: Exogenous stimulation of TNF-α (10 ng/ml; 48 hrs) on NHM caused (A) significant decrease in *MITF-M* levels (p=0.0055), (B) significant decrease in *TYR* levels (p=0.0055) and (C) significant decrease in Melanin content whereas (D) significant increase in *IL6*

(p=0.0044) and (E) significant increase in *ICAM1* levels (p=0.0072) were observed in NHM upon TNF-α stimulation (n = 3).

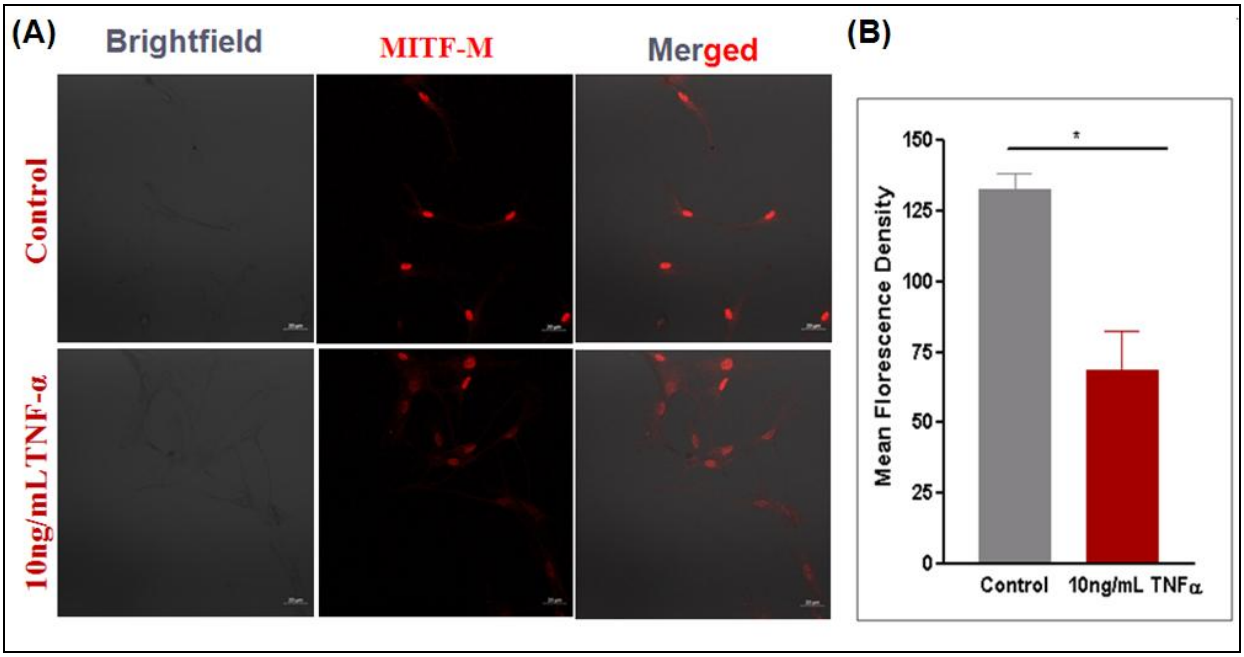


Figure 11: Analysis of MITF-M expression by Immunofluorescence upon exogenous stimulation of TNF-α (10ng/ml; 48hrs) on NHM: (A) MITF-M expression analysis by confocal microscopy at (Magnification: 63X; Scale 20μm) (B) Mean fluorescence densitometric analysis showed significant reduction in the expression of MITF-M in TNF-α induced NHM compared to control (p=0.0004; n=3).

4.3.2.7 Effect of TNF-α on Autophagy induction: NHM were treated with TNF-α in a time dependent manner for 12hrs, 24hrs and 48 hrs and observed for autophagy induction by LC3-I to LC3-II conversion. Our results showed induction of autophagy in NHM around 12 hrs of treatment which was confirmed by LC3-I to LC3-II conversion whereas at 24 and 48 hrs, no significant difference was observed for LC3-I to LC3-II conversion.

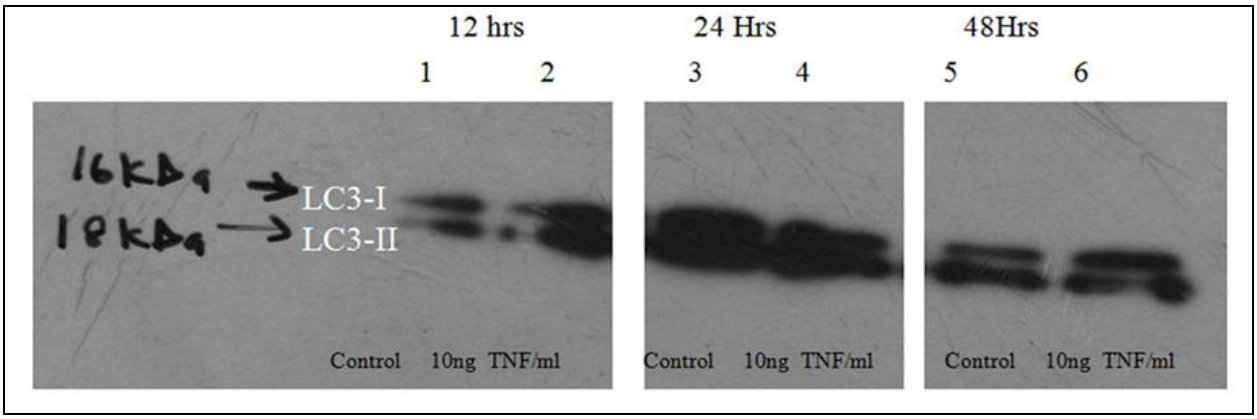


Figure 12: Effect of TNF-α on Autophagy induction: Exogenous stimulation of TNF-α (10ng/ml; 48 hrs) induced autophagy in NHM around 12 hrs of treatment which was confirmed by western blot analysis of LC3-I to LC3-II conversion, whereas at 24 and 48 hrs, no significant difference was observed for LC3-I to LC3-II conversion.

4.3.2.8 Effect of TNF-α on NHM Apoptosis:

In order to elucidate the mode of cell death mediated by TNF-α, we have monitored caspase 8 cleavage. TNF-α binds to cell surface receptors and in turn induces the activation and cleavage of the initiator procaspase 8. After 48 hrs of TNF-α treatment, we observed 18 kDa and 42 kDa cleaved fragments of procaspase 8. NF-κB has been reported to be involved in downstream signaling of various inflammatory and cell death pathways. However, we did not find any significant difference for NF-κB p65 levels in TNF-α treated NHM as compared to untreated NHM.

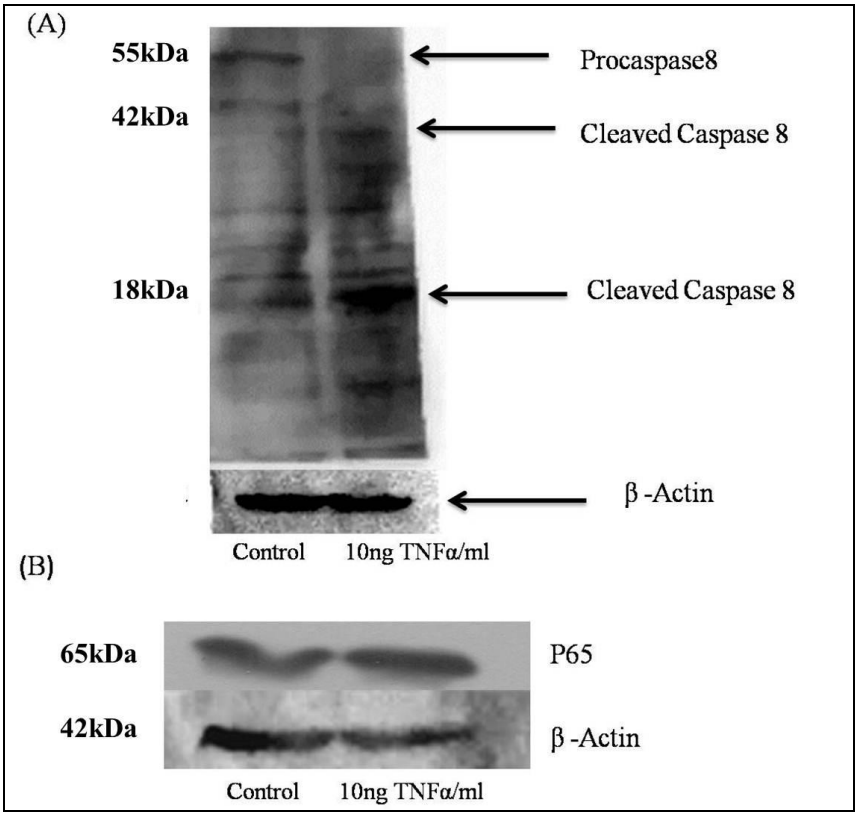


Figure 13: Effect of TNF-α on apoptosis of NHM by western blot: (A) TNF-α (10 ng/ml; 48 hrs) induced apoptotic cell death evidenced by caspase 8 cleavage (B) No difference was observed in the NF-κB p65 levels post TNF-α treatment (n=3).

4.3.3 Role of IL-6 in melanocyte biology:

4.3.3.1 Dose dependent effect of IL-6 on melanocyte cell death:

Primary melanocytes were treated with IL-6 in a dose dependent manner (0, 10, 50, 100 ng/ml) for 24 hrs and 48 hrs (n=3). No significant difference was observed in the cell morphology and cell death up to 24 hrs (data not shown). However, after 48 hrs of treatment, there was a significant increase in cell death in a dose dependent manner of IL-6 treatment (10, 20, 50 and 100 ng/ml) (Figure 14 A and B; $p<0.005$).

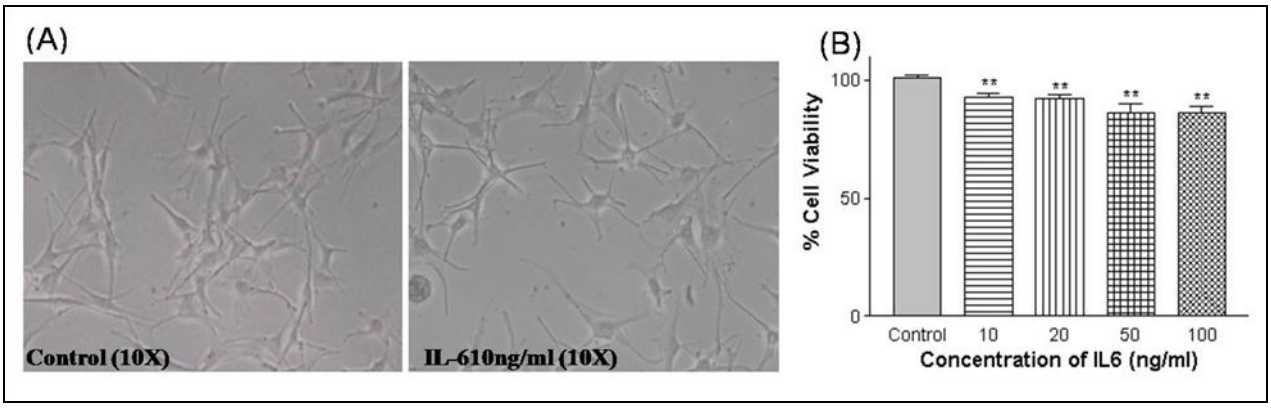


Figure 14: Effect of IL-6 on melanocyte cell death: (A) No morphological effect of IL-6 was found on the proliferation of NHM as compared to untreated NHM (Magnification: 10X Scale:100µm. (B) Significant increase in cell death of melanocytes was observed upon exogenous stimulation of IL-6 in a dose dependent manner (0-100 ng/ml IL-6, 48 hrs) (n=3).

4.3.3.2 Effect of IL-6 on *MITF-M, TYR* and *ICAM1* transcript levels in NHM:

Exogenous stimulation by IL-6 (50 ng/ml; 48 hrs) on NHM showed significant decrease in *MITF-M* expression in a dose dependent manner (10, 20 and 50 ng/ml; 48 hrs) upon IL-6 treatment (p= 0.0332; p=0.0117 and p=0.0050 respectively) (Figure 15 A). However, *TYR* expression was unaffected at 10 and 20 ng/ml of IL6 for 48 hrs while higher concentration (50 ng/ml; 48 hrs) resulted in a significant down regulation of *TYR* expression (p=0.0465; Figure 15 B). *ICAM1* was up regulated by IL6 at 20 and 50 ng/ml; 48 hrs treatment (p=0.0276 and p=0.0170 respectively); whereas at 10 ng/ml IL6 treatment for 48 hrs there was no difference in *ICAM1* expression (p=0.2487; Figure 15 C).

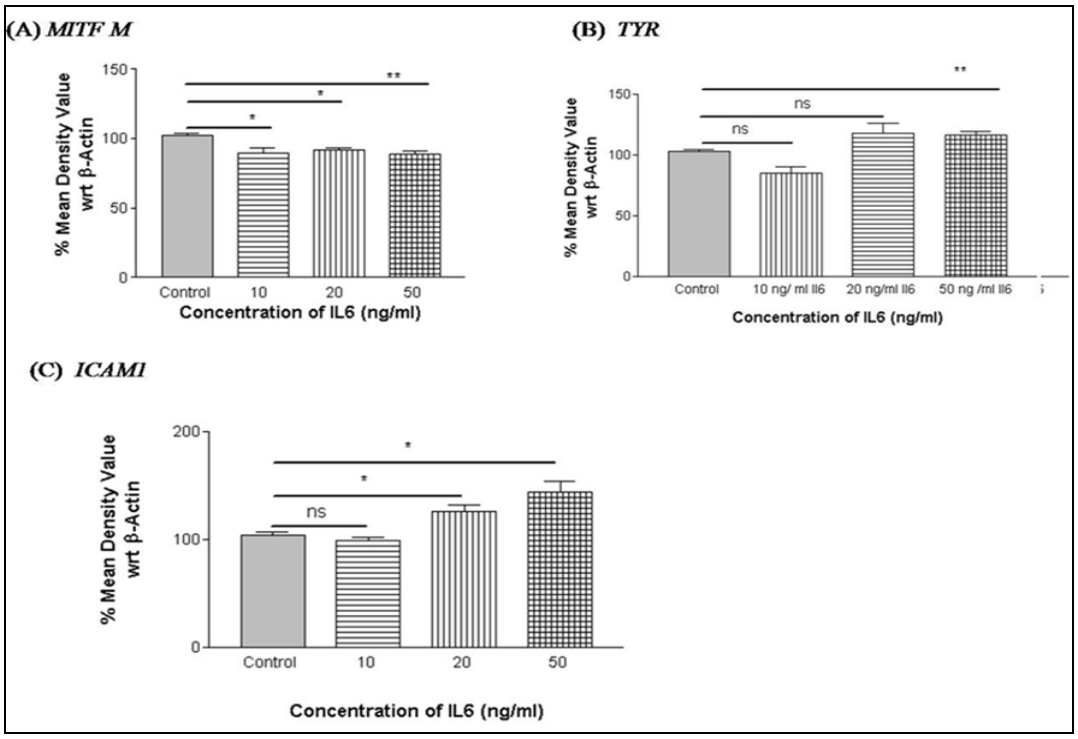


Figure 15: Effect of IL-6 on *MITF-M*, *IL6R* and *ICAM1* expression in NHM: Semi quantitative relative gene expression of *MITF M*, *TYR*, *IL6R* and *ICAM1* genes upon 10, 20 and 50 ng/ml IL-6 stimulation in NHM, with respect to *GAPDH* as a reference gene. (A) Significant decrease in *MITF-M* was observed in a dose dependent manner (10, 20 and 50ng/ml; 48hrs) upon IL-6 treatment (p= 0.0332; p=0.0117 and p=0.0050 respectively). (B) 50 ng/ml IL-6 at 48 hrs resulted in significant down regulation of *TYR* expression (p=0.0465). However, no significant difference was seen in *IL6*R transcript levels at the lower concentration of IL-6. (C) *ICAM1* was up regulated by IL-6 (20 and 50 ng/ml; 48 hrs) treatment (p=0.0276 and p=0.0170) respectively whereas at 10 ng/ml IL-6 treatment (48 hrs) caused no difference in *ICAM1* expression (p=0.2487). All the experiments were performed in triplicate and means ± S.D. have been shown in figures [*p<0.05, **p<0.01, ***p<0.001, p>0.05, ns (non significant)].

4.3.3.3 Effect of IL-6 on MITF-M expression: As we observed decreased transcript levels of *MITF-M* upon Il-6 treatment, we were further interested to monitor the effect of IL-6 on

protein expression of MITF-M on NHM. However, our results showed no significant difference in MITF-M protein expression in NHM (p = 0.2258; n=3).

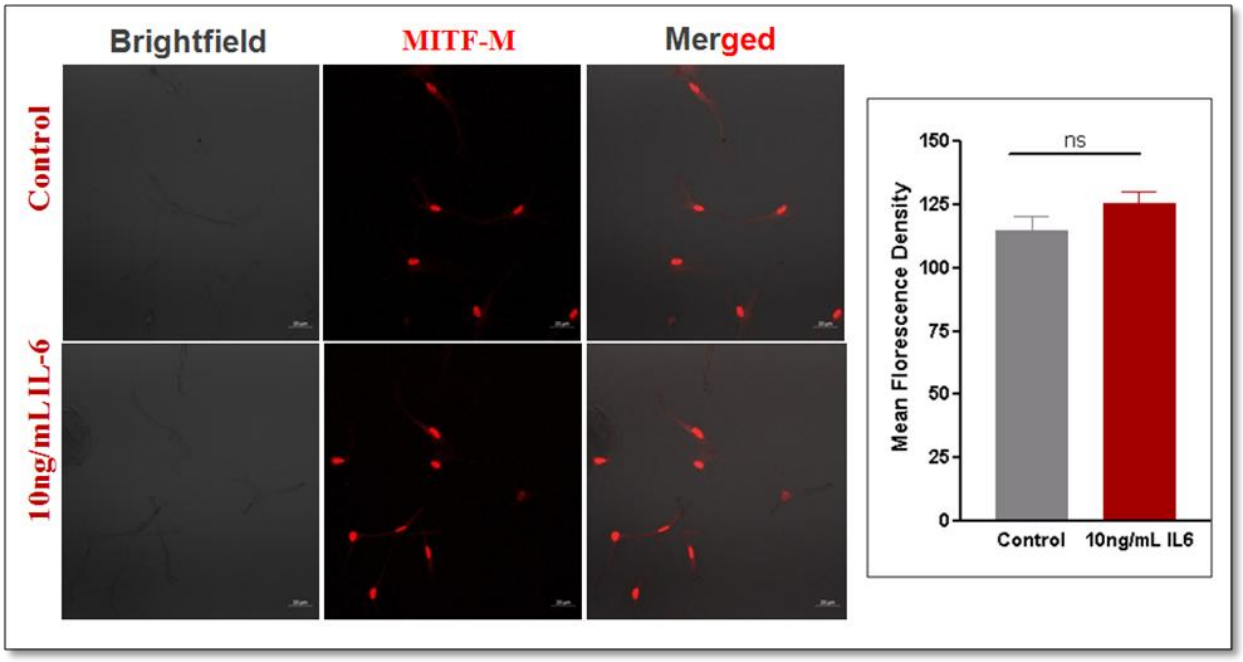


Figure 16: Analysis of MITF-M levels in NHM upon IL-6 treatment by Immunofluorescence: Treatment with IL-6 (10 ng/mL) did not cause any significant change in MITF-M protein expression levels. (n=3; treatment duration 48 hrs; Magnification 63X: scale bar 20µ; p=0.2258).

4.3.4 Role of IL-1α on melanocyte biology:

4.3.4.1 Dose dependent effect of IL-1α on melanocytes cell death:

Primary melanocytes were treated with the different doses of IL-1α (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for the cell morphology and viability (n=3). The NHM did not show any difference in morphology and viability up to 24 hrs (data not shown). Also, there was no significant morphological change observed in NHM even after 48 hrs of IL-1α treatment (Figure 17 A). However, 100 ng/ml of IL-1α treated NHM showed significant decrease in cell viability (p=0.0210) after 48 hrs of treatment, as compared to untreated NHM (Figure 17 B). However, 10 & 50 ng/ml doses of IL-1α did not show any significant difference (p=0.6658 and p=0.9301 respectively) in cell viability.

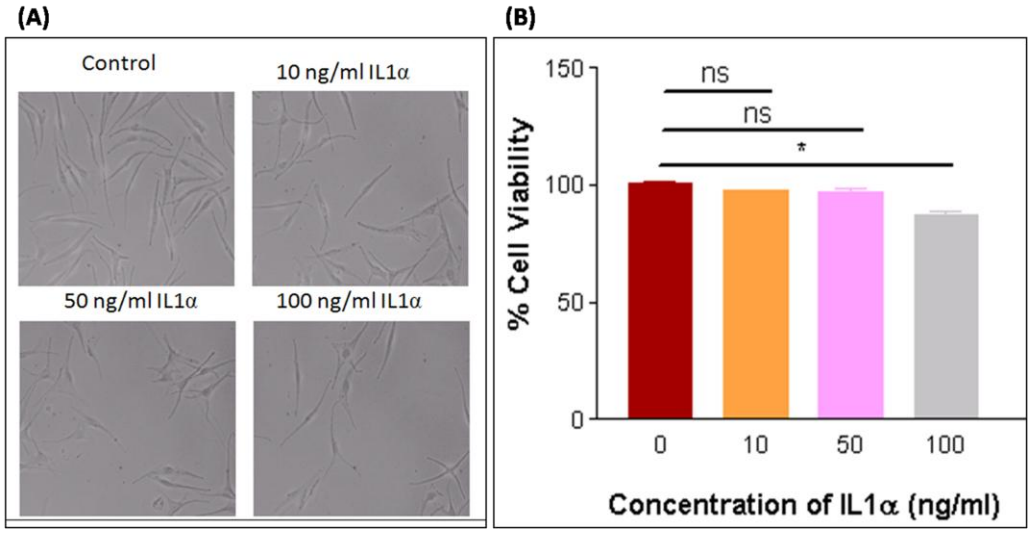


Figure 17: Dose dependent effect of exogenous stimulation of IL-1α on melanocytes cell death (0-100 ng/ml IL-1α, 48 hrs): (A) NHM showed no cell morphological changes after 48 hrs of IL-1α treatment (Magnification: 10X; Scale: 100μm). (B) Mean % cell viability on 100 ng/ml IL-1α treatment showed significant decrease in cell viability after 48 hrs (n=3, p<0.0210). However, 10 & 50 ng/ml doses of IL-1α did not show any significant difference in cell viability.

4.3.4.2 IL-1α regulates melanogenesis and other immunomodulatory genes:

IL-1α (10 and 100 ng/ml) treated NHM were used for expression analysis of *IL1RN*, *IL1A*, *IL1B*, *IL6*, *TNFA*, *ICAM1*, *MITF-M*, *IL8*, *TYR* and *TYRP1* genes. Our results showed significant decrease in expression of *MITF-M* in 100 ng/ml IL-1α treated NHM as compared to untreated NHM (p=0.0066; Fig. 18). However, there was no difference at 10 ng/ml IL-1α treatment (p=0.0694). Also, *TYR* and *TYRP1* genes of melanin synthesis pathway exhibited no significant difference (p=0.4532, p=0.5659; p=0.4395, p=0.8858) upon IL-1α treatment (10 and 100 ng/ml respectively). Interestingly, the expression levels of *IL1RN*, *IL1A*, *IL1B*, *IL6*, *TNFA* and *ICAM1* were significantly up regulated in 100 ng/ml IL-1α treated NHM as compared to untreated NHM (p=0.0005, p=0.0063, p<0.0001, p<0.0001, p=0.0034 and p=0.0017 respectively). Also, *IL1RN*, *IL1B*, *TNFA* and *ICAM1* were found to be significantly up regulated in 10 ng/ml IL-1α treated NHM as compared to untreated NHM (p=0.0360, p=0.0385, p=0.0007, and p=0.0421 respectively). However, no

significant difference was observed for *IL1A* and *IL6* in 10 ng/ml IL-1α treated NHM as compared to untreated NHM (p=0.9001 and p=0.0841 respectively).While, transcript levels of *IL8* remained unaltered upon 10 ng/ml IL-1α (p=0.6838,) as well as 100 ng/ml IL-1α stimulation (p=0.7091; Fig. 18).

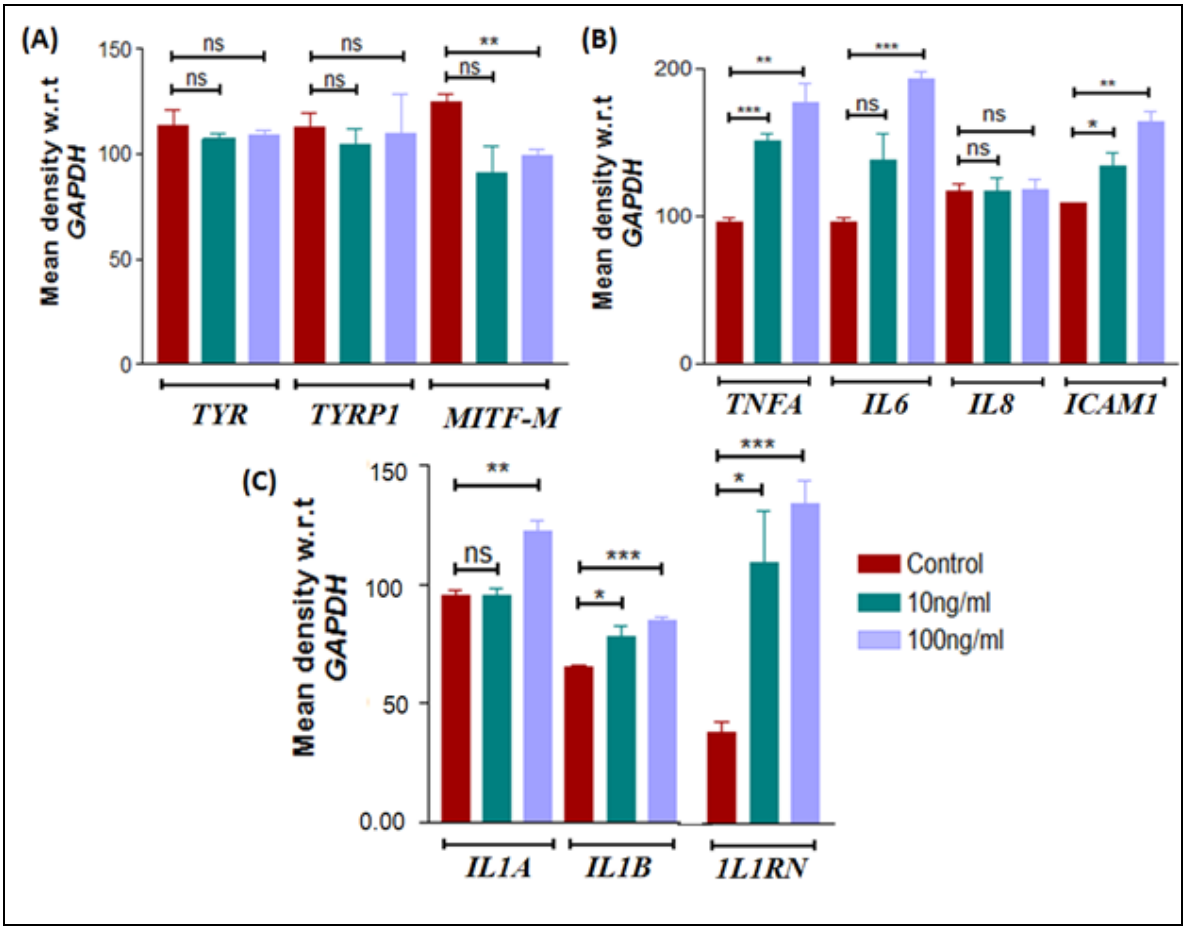


Figure 18: Effect of IL-1α on the genes involved in melanogenesis and other immuno-modulatory molecules in NHM. Semi quantitative relative gene expression analysis of *IL1RN, IL1A, IL1B, IL6, TNFA, ICAM1, MITF-M, IL8, TYR, TYRP1* genes with respect to *GAPDH* in NHM upon stimulation with 10 and 100 ng/ml IL-1α. (A) 100 ng/ml of IL-1α stimulation on NHM showed a significant decrease in *MITF-M* expression (p=0.0066) as compared to untreated NHM. However, no significant difference was observed for *TYR* and *TYRP1* expression upon IL-1α treatment (p>0.05). (B) 100 ng/ml IL-1α stimulation to NHM showed a significant increase in *TNFA* (p=0.0034), *IL6* (p<0.0001), *ICAM1* (p=0.0017) as compared to untreated NHM. Also, *TNFA* and *ICAM1* showed significantly

increased expression upon 10 ng/ml IL-1α stimulation to NHM. However, there was no difference in expression of *IL8* upon 10 ng/ml and 100 ng/ml IL-1α stimulation to NHM (p=0.6838 and p=0.7091 respectively). (C) 100 ng/ml IL-1α treated NHM showed significant increase in *IL1A* (p=0.0063), *IL1B* (p<0.0001) and *IL1RN* (p=0.0005) expression. Also, *IL1B* (p=0.0385) and *IL1RN* (p=0.0360) transcripts showed significant increase upon 10 ng/ml IL-1α stimulation to NHM. All the experiments were performed in triplicate and mean ± S.D. was shown in figures [*p<0.05, **p<0.01, ***p<0.001, p>0.05, ns (non significant)].

4.3.4.3 Effect of IL-1α on MITF-M expression: As we observed decreased transcript levels of *MITF-M* upon IL-1α treatment, we were further interested to monitor its effect on MITF-M protein expression. However, our results showed no significant difference in MITF-M protein expression in NHM upon IL-1α treatment (p = 0.1476; n=3).

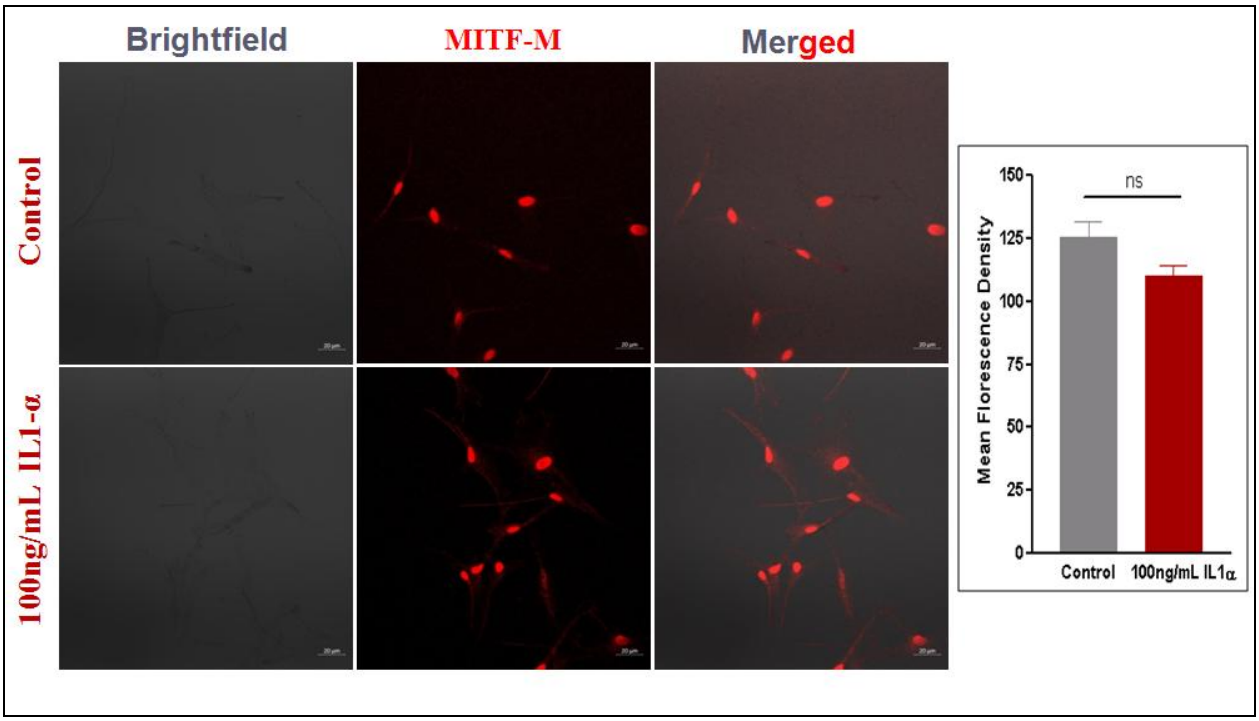


Figure 19: Analysis of MITF-M levels in NHM upon IL-1α treatment by Immunofluorescence: Treatment with IL-1α (100ng/mL) did not cause any significant change in MITF-M protein expression levels (treatment duration 48 hrs; scale bar 20µ; p = 0.1476; n=3).

4.3.5 Role of IL-10 in melanocyte biology:

4.3.5.1 Dose dependent effect of IL-10 on melanocyte cell death:

IL-10 did not show any significant effect on growth and viability of melanocytes in a dose dependent manner (0.25-100 ng/ml) (Figures 20 A and B). Further we have studied the effect of IL-10 on NHM (10ng/ml ;48 hrs) on melanin synthesis genes *MITF-M*, *TYR* and *ICAM-1*, and found no significant effect on *MITF-M* (p= 0.2401), *TYR* (p = 0.2934) and *ICAM1*(p=0.3608) transcript levels (n=3) (Figure 21). Interestingly, we observed significant increased expression of MITF-M protein levels on IL-10 (10ng/ml) treatment to NHM (p= 0.0152; 48 hrs; n=3) (Figure 22).

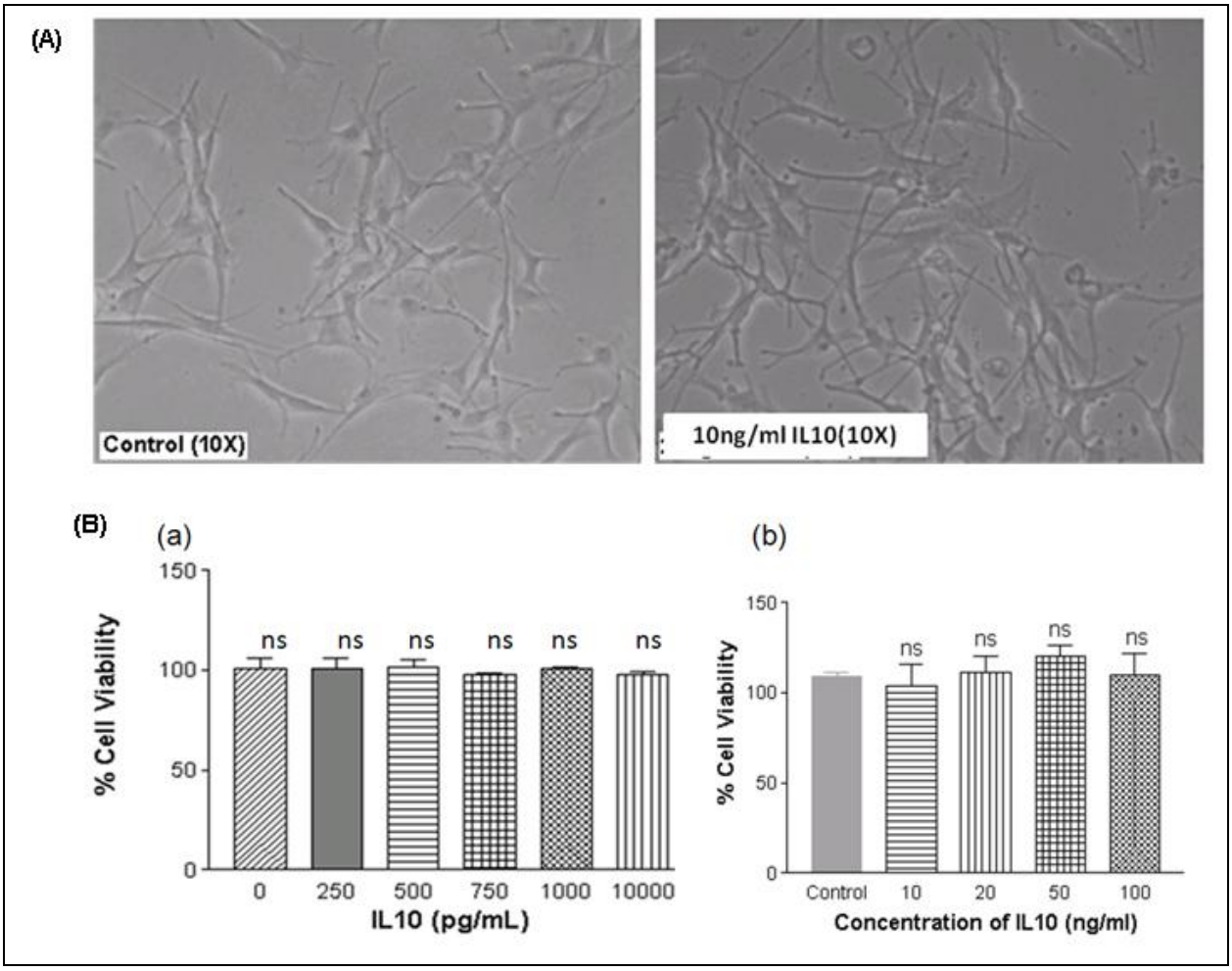


Figure 20: Effect of IL-10 on cell death of NHM: Effect of IL-10 stimulation on NHM morphology (Magnification: 10X; Scale: 100µm).The NHM cells were treated with IL-10

(0.25-10ng/ml) for 48 hrs and Mean % cell viability was shown at 48 hrs. IL-10 did not affect growth and viability of NHM (n=3, *p*=0.075).

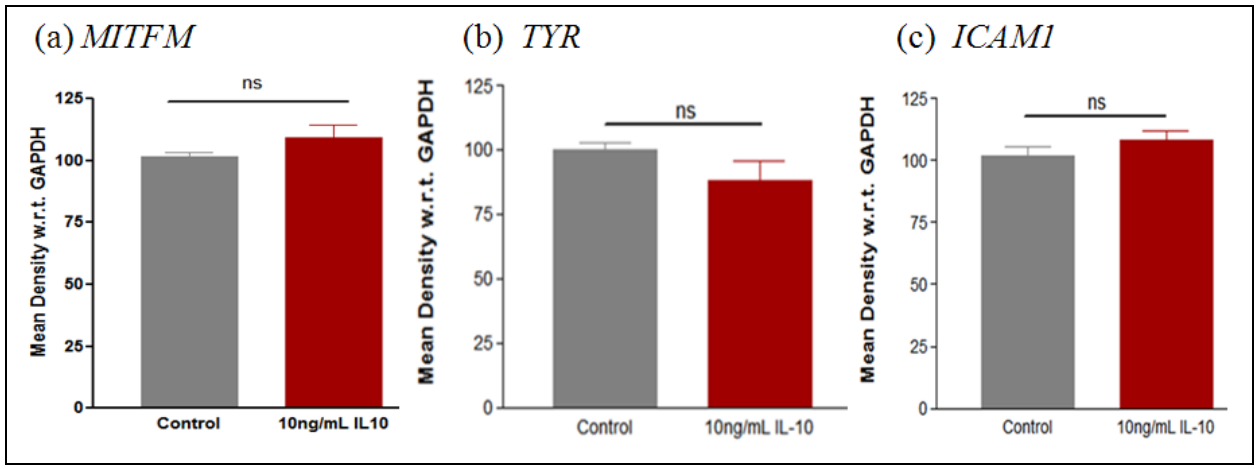


Figure 21: Effect of IL10 on *MITF-M, TYR* and *ICAM1* transcript levels in NHM: No significant effect was observed for *MITF-M* (*p*= 0.2401), *TYR* (*p* = 0.2934) and *ICAM1* (*p* = 0.3608) transcript levels upon IL-10 treatment on NHM (n=3, 48 hrs treatment).

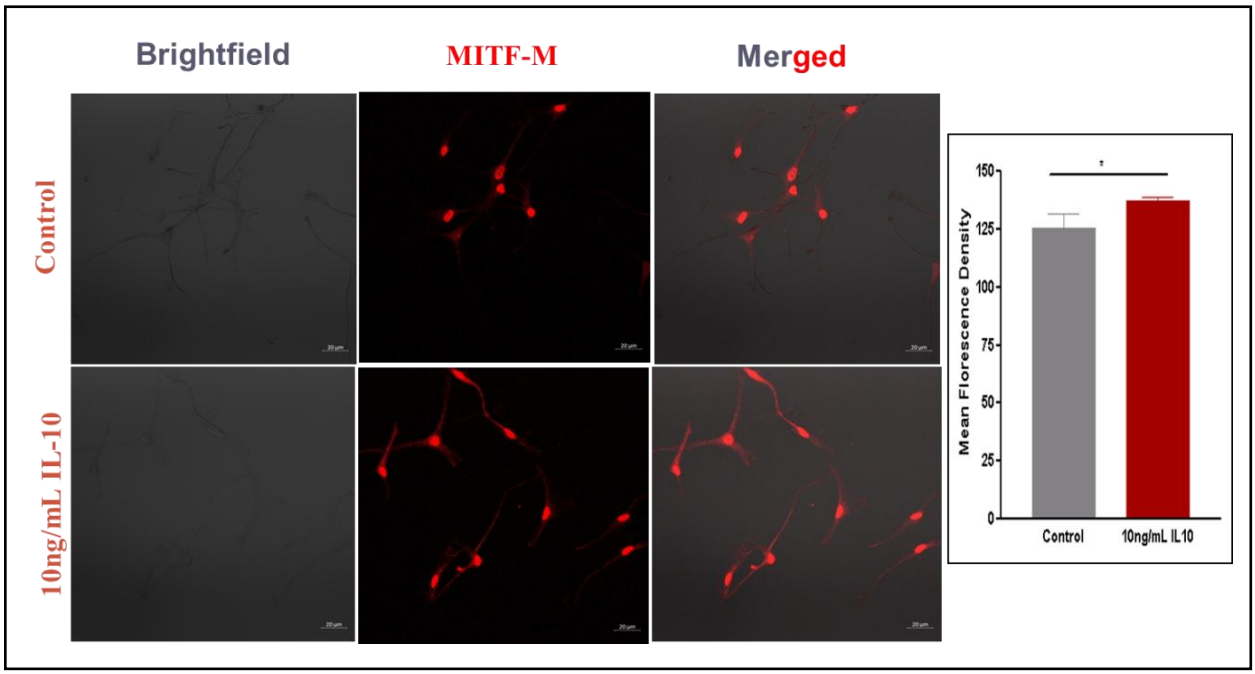


Figure 22: Analysis of MITF-M levels in NHM upon IL-10 treatment by Immunofluorescence: Treatment with IL-10 (10ng/mL) caused significant increase (*p*= 0.0152) in MITF-M protein expression levels (n=3).

4.3.6 Combinatorial effect of cytokines on NHM viability:

TNF-α, IL-1α, and IL-6 caused a dose dependent decrease in cell viability of cultured melanocytes. However, IL-10 did not show any significant alteration in growth and viability of cultured melanocytes. All cytokines in synergism with high H_2O_2 environment may lead to enhanced compromised state of melanocytes, affecting overall survival and homeostasis of melanocytes. Hence, we have treated NHM with various combinations of cytokines.

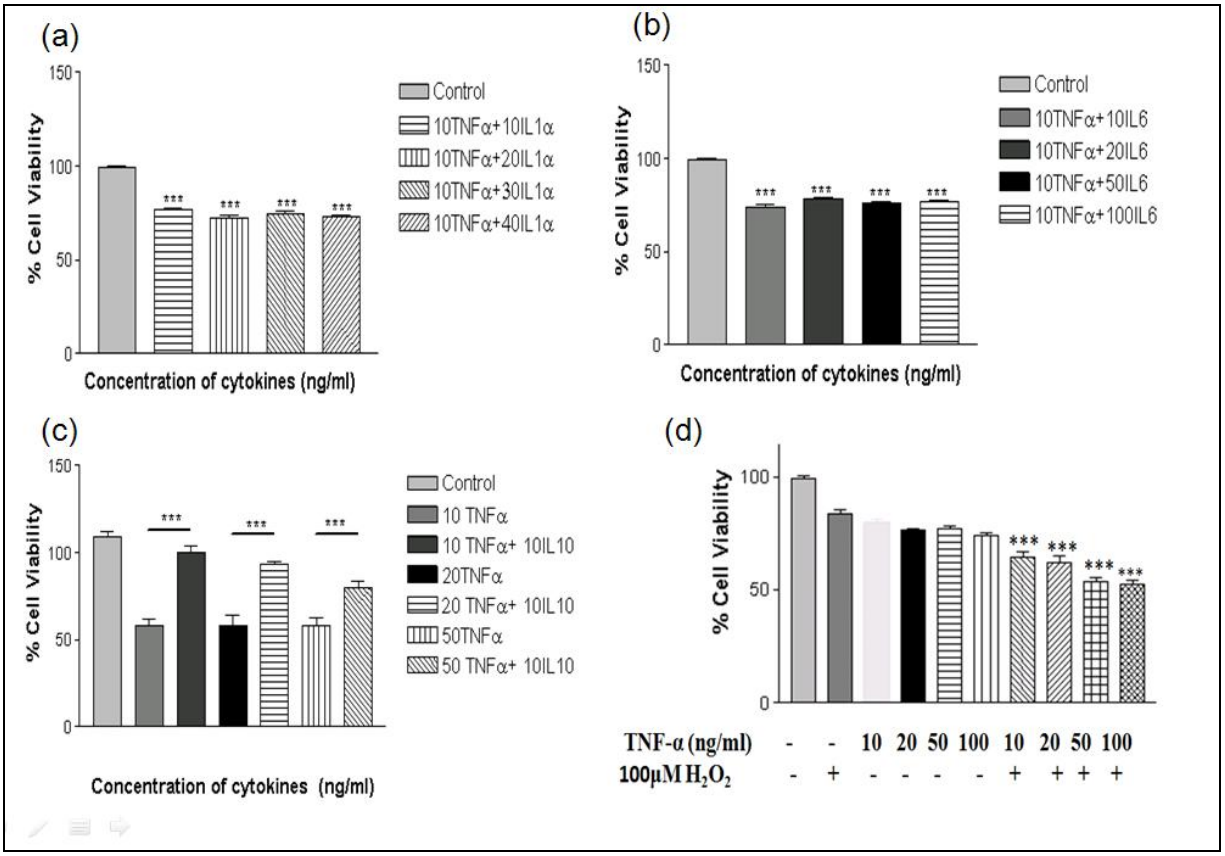


Figure 23: Combinatorial effect of cytokines on NHM viability: (a, b) Synergistic effect of TNF-α along with IL-1α, IL-6 and (c) antagonistic effect of IL-10 on NHM viability in a dose dependent manner was observed. (d) Also, synergistic effect of TNF-α and H_2O_2 on NHM viability was observed.

4.4 DISCUSSION

Melanocytes are neural crest derived cells residing at the basal layer of the epidermis. Melanocyte survival and proliferation seem to be strongly correlated with expression of various factors such as cytokines secreted by keratinocytes, α-MSH, SCF etc. that govern the signaling pathways mediating differentiation and proliferation of melanocytes. Vitiligo is an acquired, progressive, hypomelanotic skin disorder manifested by selective destruction of melanocytes. Melanin pigment is a potent UV absorbent with antioxidant and radical scavenging properties and thus epidermal pigmentation is a major protective factor against genotoxic damage by UV radiation (Lin and Fischer, 2007).

As the top-most layer of the skin is mainly made up of dead cells, an increase in the thickness of the epidermis may occur as a combative measure against UV penetrance. Due to epidermal thickening, the skin acts as a barrier for epidermal somatic cell protection from the mutagenic effects of UV radiation. Earlier studies postulate development of epidermal hyperplasia, such as hyperkeratosis and acanthosis as a means of compensation for the absence of pigmentation in vitiligo skin (Jung *et al.*, 2015; El-Khateeb *et al.*, 2011; Awad, 2012). Inflammatory changes in the disturbed skin environment are more profound and intense and reflected in degenerative changes in melanocytes, vacuolar changes of basal cells, epidermal infiltration of lymphocytes, dermal infiltration of lymphocytes, and melanophages in the upper dermis (Hann *et al.*, 1992). Diminished melanocyte proliferation and melanogenesis can be correlated to the loss of rete-ridges in the basal skin layer where the melanocytes reside and proliferate. In our study, we found significant increase in the thickening of the viable epidermis in lesional skin of patients compared to control skin (Figs 1 and 2) which are in accordance with the previous reports (Jung *et al.*, 2015; El-Khateeb *et al.*, 2011; Awad, 2012). An interesting finding of our present study is the significant thickening of lesional skin as compared to non-lesional and control skin, postulating importance of the microenvironment of thickened skin in the hypomelanotic patches of skin. Vitiligo patients showed reduced expression of *MITF-M* gene in perilesional *vs.* lesional and, lesional as well as non lesional skin when compared to controls (Kingo *et al.*, 2008). This is in accordance with our results where we found

significant decrease in MITF-M protein expression in lesional and non-lesional skin when compared to control skin samples (Figs 3 and 4).

Dysregulation of production of cytokines or their action plays a central role in the development of autoimmunity (O'Shea, 2002). In the epidermal melanin unit, the keratinocyte derived cytokines TNF-α, IL1-α, IL-6 act as paracrine inhibitors of melanocyte proliferation and melanogenesis (Swope *et al*., 1991). The epidermal as well as systemic cytokine imbalance have been reported in vitiligo patients including higher TNF-α, IL-6, IL-1α and IL1-β levels (Moretti *et al*., 2002, Laddha *et al*., 2012, Laddha *et al*., 2014, Tu *et al*., 2003, Birol *et al*., 2006, Singh *et al*., 2012). Our results showed significant decrease in melanocyte viability upon treatment with TNF-α, IL-6, IL-1α in a dose dependent manner (Figs 5, 14 and 17). However, IL-10 was having non-significant effect on melanocyte viability. Interestingly, we found ameliorative effect of IL-10 on TNF-induced melanocyte viability; on the contrary TNF-α was exerting synergism with IL-6, IL-1α, and oxidative stress. All these conditions of increased oxidative stress, as well as cytokine imbalance were well evident in circulation and skin microenvironment of vitiligo patients. MITF is a master transcriptional regulator of melanogenesis and melanocyte survival and exerts its mechanism by regulating TYR, TYRP1, DCT etc. Our results showed a significant decrease in MITF-M levels on NHM upon TNF-α, IL-6, IL-1α treatment. Interestingly, we found IL-10 significantly increases MITF-M expression on NHM, suggesting an imbalance of pro and anti-inflammatory cytokines in the regulation of master transcriptional regulator of melanogenesis. Various studies reported down regulation of MITF-M and melanogenesis in melanocytes by treatment with IL-17A, IL-1β, IL-6, and TNF-α (Kamaraju *et al*., 2002; Kotobuki *et al*., 2012; Choi *et al*., 2005) which are in the line of our findings. TYR and TYRP1 are the target genes of MITF-M and catalyse the rate limiting steps of melanin synthesis and we found down regulation of *TYR* by TNF-α and IL-6 treatment. IL-1α ($3x10^{-14}$ M) was found to decrease *MITF-M* expression and showed an inhibitory effect on tyrosinase activity in melanocytes (Swope *et al*.,1991). The MITF transcriptional activity is also dependent on its phosphorylation state (Hemesath *et al*., 1998). Therefore, the inhibitory effect of IL-1α might regulate tyrosinase function rather than its transcription. *TYRP1* gene encodes a melanosomal enzyme that belongs to the tyrosinase family and plays an important role in the melanin biosynthetic

pathway (Cui *et al*., 2015). No significant difference was observed in the *TYRP1* levels similar to tyrosinase, upon IL-1α stimulation of NHM.

Both IL-1α and IL1-β can self-regulate their expression which serves as a positive-feedback loop that amplifies the IL1 response in an autocrine or paracrine manner (Hu *et al*., 2003). In contrast, IL1-β produced by different cell types including tissue macrophages and skin dendritic cells was reported to activate complement components and other cytokines including IL1 itself (Dinarello *et al*., 2011). Interestingly, in the present study, both *IL1A* and *IL1B* were found to be significantly upregulated upon IL1 stimulation in NHM (Figure 18). IL1RN is a specific inhibitor of the activity of both IL-1α and IL1-β. IL-1RN binds tightly to IL1RI and blocks the activity of either IL-1α or IL1-β. Cell activation by IL1 is 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 merely as a 'decoy receptor' (Martin *et al*., 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 *IL1RN* transcript levels upon IL-1α stimulation in NHM. In addition, IL-1α is reported to stimulate *IL1*, *TNF-A*, and *IL-6* expression in human cardiac myofibroblasts (Turner *et al*., 2007). We observed upregulation of *IL1RN*, *IL1A*, *IL1B*, *IL6*, *TNFA* and *ICAM1* and down regulation of *MITF-M* upon exogenous IL-1α stimulation in NHM (Figure 18), suggesting its regulatory role in melanocyte homeostasis in vitiligo. Both TNF-α and IL1-β were reported to stimulate IL8 release from melanocytes (Miniati *et al*., 2014), whereas in the present study we did not find any effect of IL-1α on IL8 production in NHM (Figure 18). IL8 is an important chemokine produced by monocytes, mast cells, fibroblasts, endothelial cells, dendritic cells and keratinocytes in inflammatory skin diseases. IL8 is chemotactic for neutrophils, T-lymphocytes, basophils, and keratinocytes (Luger *et al*., 1990). ICAM1 is important for activating T-cells and recruiting leukocytes (Hedley *et al*., 1998). Beyond its classically described function as an adhesion and viral entry molecule, ICAM1 on the surface of T-cells participates in signal transduction affecting several T-cell functions, including activation, proliferation, cytotoxicity, and cytokine production and thereby plays an important role in modulating autoimmune diseases (Stanciu *et al*., 1998). Earlier our lab reported increased expression of *ICAM1*, *IFNG* and *TNFB*, along with decreased cytotoxic

T-lymphocyte associated antigen-4 (*CTLA4*) and decreased T reg cells in vitiligo patients (Laddha *et al*., 2012, Laddha *et al*., 2013, Dwivedi *et al*., 2013, Dwivedi *et al*., 2011, Dwivedi *et al*., 2013). Increased IFN-γ levels in vitiligo patients lead to increased *ICAM1* expression which can be a probable link between cytokines and T-cell involvement in the pathogenesis of vitiligo (Dwivedi *et al*., 2013). TNF-α is reported to induce IL1-α, IL1-β and IL-6 synthesis in glioma cells and cardiac fibroblasts (Turner *et al*., 2007). In addition, TNF-α in the presence of IFN-γ modulates IL6 and its receptor in human monocytic cells (Sanceau *et al*., 1991). Interestingly, we found IL-1α to induce IL6 expression in NHM at 100 ng/ml (Figure 18).

IL-1α was reported to be cytostatic rather than cytotoxic on NHM (Swope *et al*., 1991). The present study also showed a significant decrease in cell viability of NHM with reduced growth upon exogenous IL-1α stimulation suggesting the role of IL-1α in melanocyte destruction in vitiligo. The blockade of IL1 by Anakinra, a recombinant IL1RN which affects the biological activity of naturally occurring IL1 by competitively inhibiting the binding of IL1 to IL1R1 has been implicated to control various autoimmune disorders including inflammation and rheumatoid arthritis, juvenile idiopathic arthritis (Mertens *et al*., 2009, Pascual *et al*., 2005). Protective effect of IL1-β monoclonal antibody in insulin-producing beta cells is reported in type 2 diabetic patients (Donath *et al*., 2008). TNF-α, IL-1α and IL-6 induce ICAM-1 expression in melanocytes, thus promoting melanocyte-leukocyte attachment. This might explain the selective destruction of vitiliginous melanocytes (Yohn *et al*., 1990). Previously, we reported increased *ICAM-1* expression in vitiligo patients (Dwivedi *et al*., 2013). Interestingly, we also found induction of *ICAM1* expression upon IL-6 treatment in melanocytes. Toosi *et al*., (2012) have reported that exposure of vitiligo inducing phenols (4-tertiary butyl phenol and mono-benzyl ether of hydroquinone) to NHM increased the expression of *XBP1*, further activating IL6 and IL8 production by melanocytes.

Our results indicated increased levels of *TNFA* in vitiligo skin (lesional and non lesional). Exogenous stimulation of TNF-α on melanocytes caused significant decrease in viability, significant increase in cellular and mitochondrial ROS levels, ~20% decrease in mitochondrial complex1 activity, decrease in melanin content *via* shedding of dendrites and

down regulation of *MITF-M, TYR* and increased *TNFR1, IL6, ICAM1* expression while *TNFR2* levels remained unaltered (Fig 10). Upon TNF-α stimulation, LC3I-II conversion at 12 hrs and caspase-8 activation at 48 hrs were observed, which disappeared at 48 hrs and 24 hrs respectively (Figs 12 and 13). All these studies advocate the crucial role of TNF-α in melanocyte homeostasis and autoimmune pathogenesis of vitiligo. On the contrary to pro-inflammatory cytokines, IL-10 showed non-significant effect on NHM viability, *TYR, MITF-M* and *ICAM-1* transcript levels, but increased the MITF-M protein expression and ameliorates TNF-α induced cytotoxicity.

In conclusion, candidate cytokines addressed in the present study i.e., TNF-α, IL-1α, IL-6 and IL-10 found to regulate melanogenesis and proliferation of NHM. Overall, the present study along with other existing reports advocate the therapeutic potential of low cytokine dose therapy in amelioration of vitiligo. Thus, this will pave the way for better understating and designing of personalized treatment modality for vitiligo.

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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.

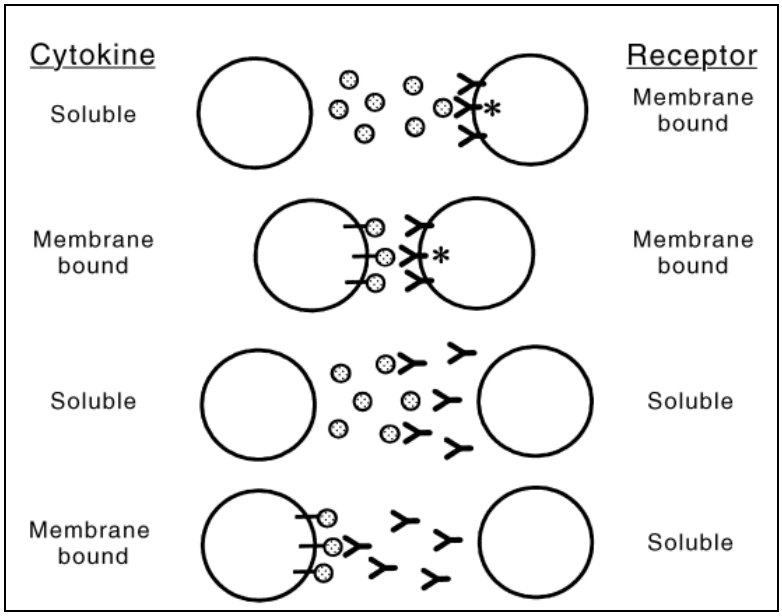

Figure 1: Cytokine-receptor interactions

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.

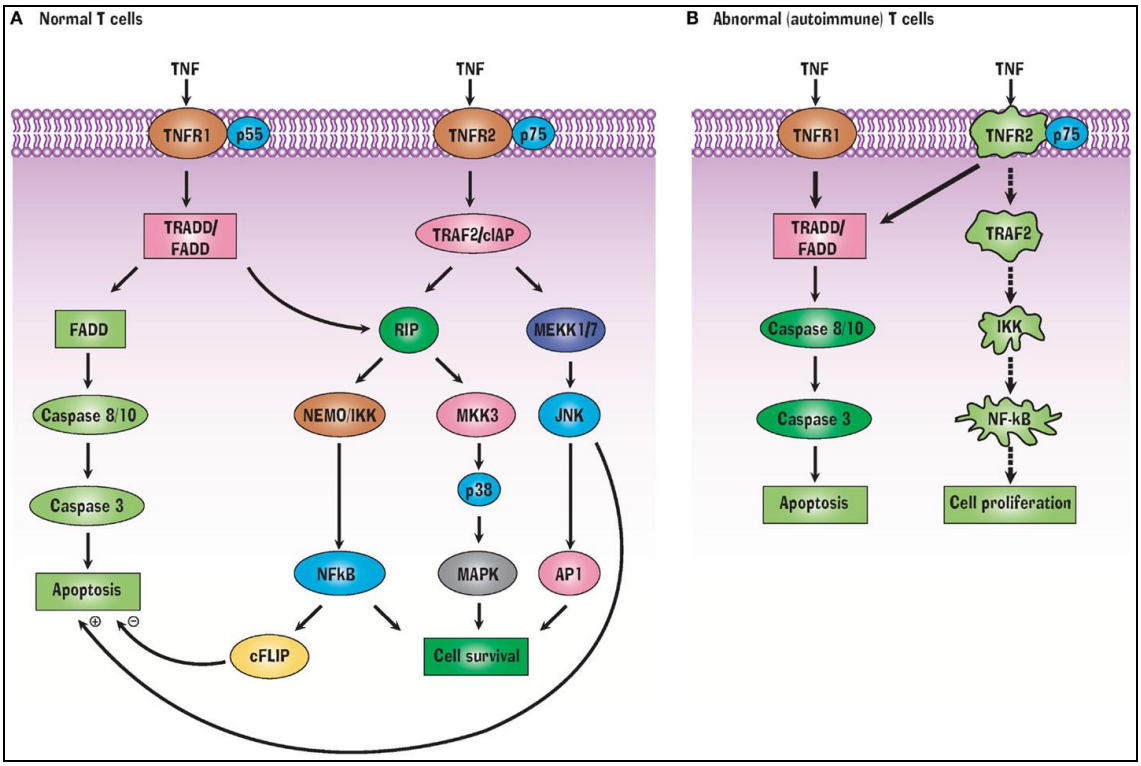

Figure 2: TNF signaling: TNF signals through TNFR1 and TNFR2 receptors (A) but abnormalities in this signaling pathway in autoimmunity (B) can favor a pathway of selective apoptosis of target cells due to excess of ligand or defects in downstream proteins involved in signaling pathway (Faustman & Davis,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

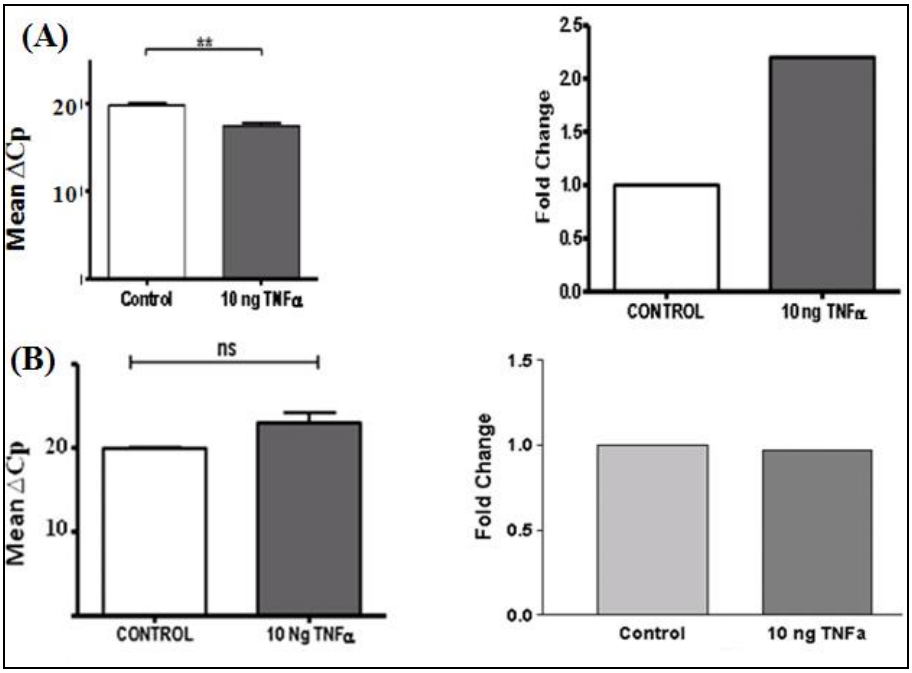

Figure 3: The NHM cells were treated with TNF-α (10ng/ml) and then *TNFR1* and *TNFR2* transcript levels were measured after 48 hrs of treatment.(A) Representation of mean ΔCp and fold change in expression of *TNFR1* at 10 ng/ml TNF-α at 48 hrs (n = 3; p = 0.0024). (B) Representation of Mean ΔCp and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

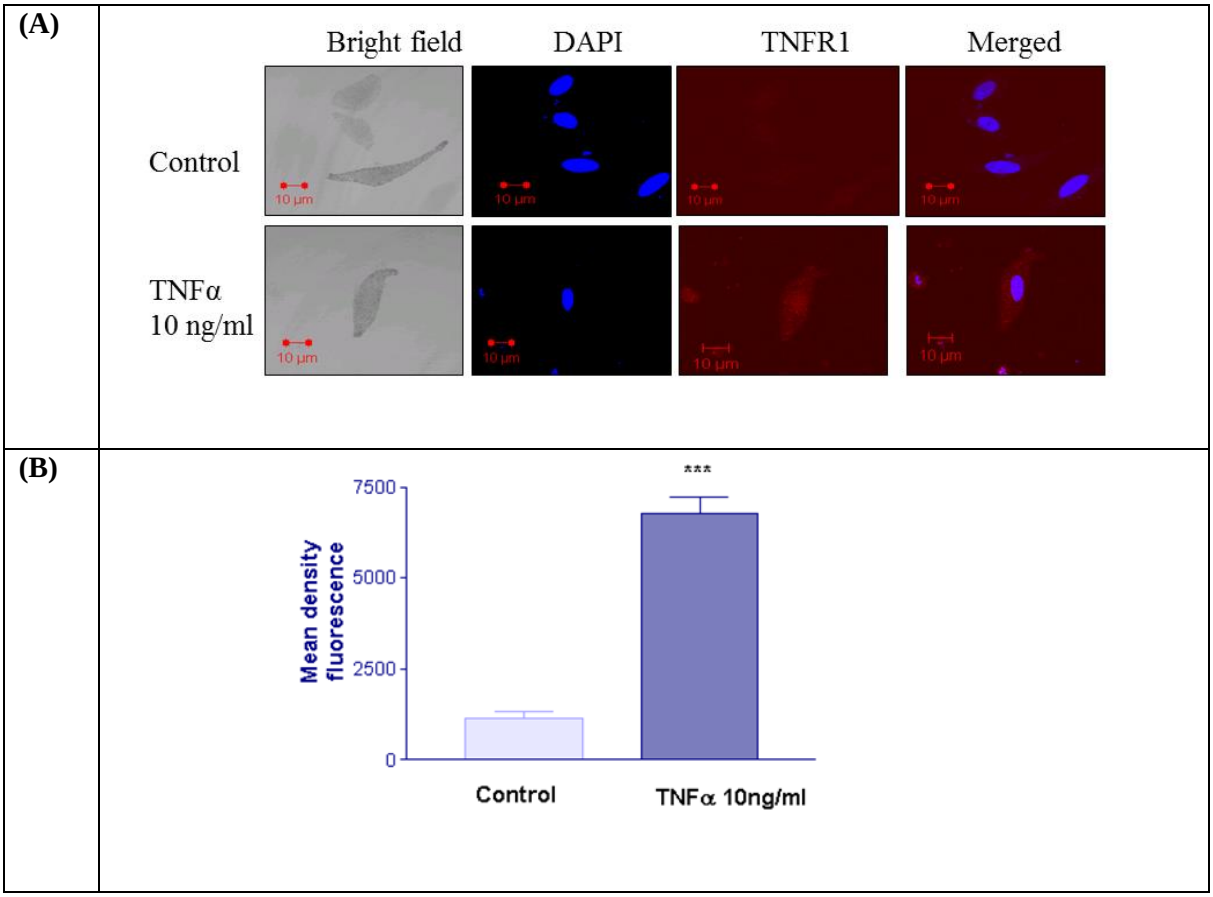

Figure 4: The NHM cells were treated with TNF-α (10ng/ml) and then TNFR1 expression levels were measured after 48 hrs of treatment. (A) Confocal microscopy analysis showed increased expression of TNFR1 on NHM. (B) Integrated mean density calculation showed significant increased expression of TNFR1 as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

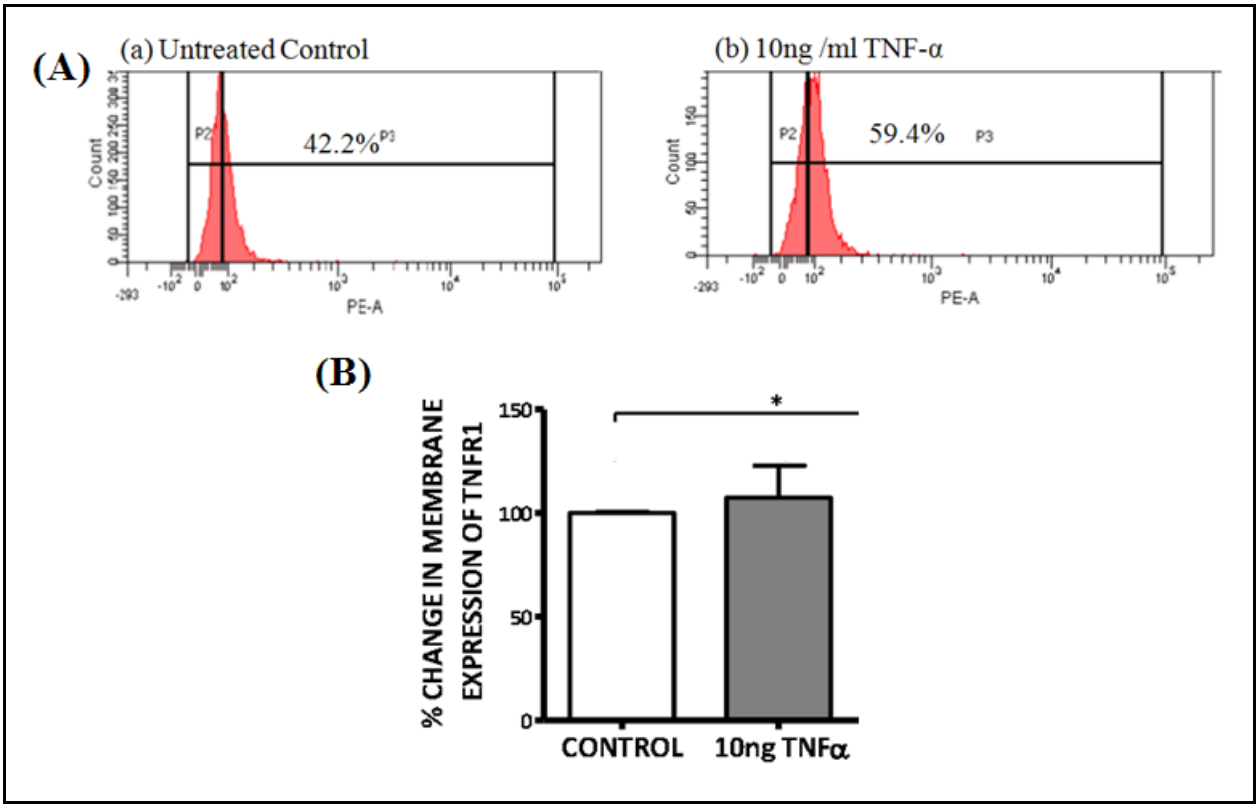

Figure 5: The NHM cells were treated with TNF-α (10ng/ml) and then TNFR1 expression levels were measured after 48 hrs of treatment. (A) Representative images showing the effect of TNF-α on expression of TNFR1 by Flow cytometry. (B) Mean % change in TNFR1 membrane expression at 10 ng/ml TNF-α at 48 hr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

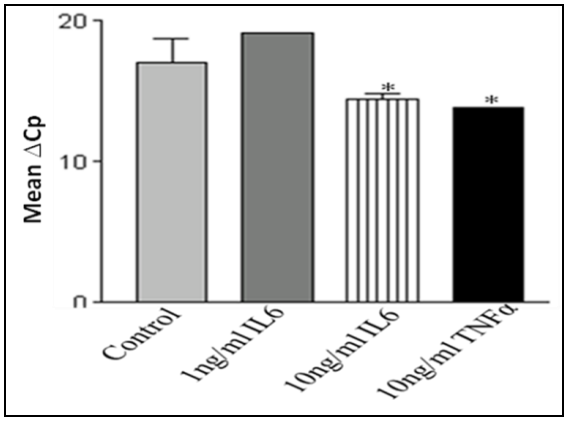

Figure 6: IL-6 and TNF-α induced *IL6R* gene expression in cultured melanocytes: The NHM cells were treated with IL-6 (1 and 10ng/ml; 48 hrs) and TNF-α (10ng/ml; 48 hrs) and analyzed for IL6R levels. Representation of Mean ΔCp showed significant increased expression of *IL6R* at 10 (ng/ml) treatment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

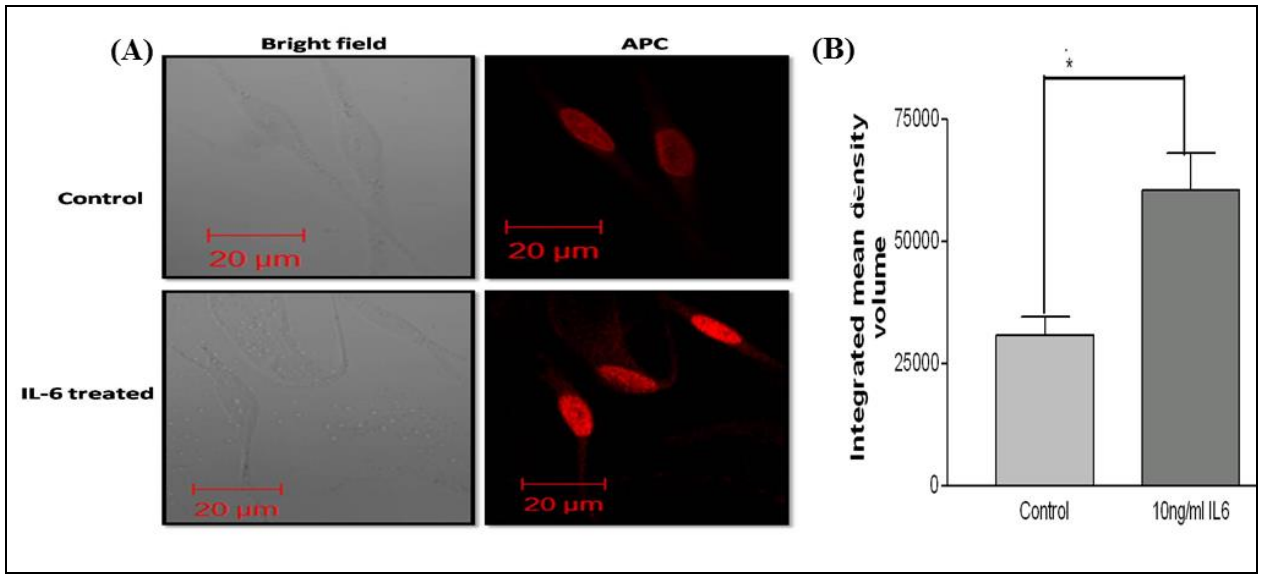

Figure 7 : IL-6 induced IL6 Receptor activation by confocal microscopy: The NHM cells were treated with IL-6 (10ng/ml) and then *IL6R* expression levels were measured after 48 hrs of treatment. (A) Representative images showing effect of IL-6 on IL6R expression by confocal microscopy. (B) Mean density 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.

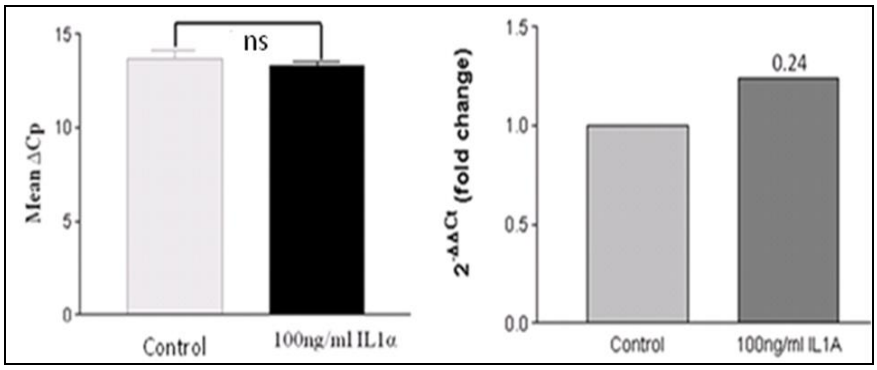

Figure 8 : Effect of IL-1αon IL1R1 expression in NHM: The NHM cells were treated with IL-1α (100ng/ml; 48 hrs). *IL1R1* transcript levels were measured after 48 hrs of treatment and the results showed no significant difference in the transcript levels of *IL1R1*. Representation of Mean ΔCp and Fold Change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

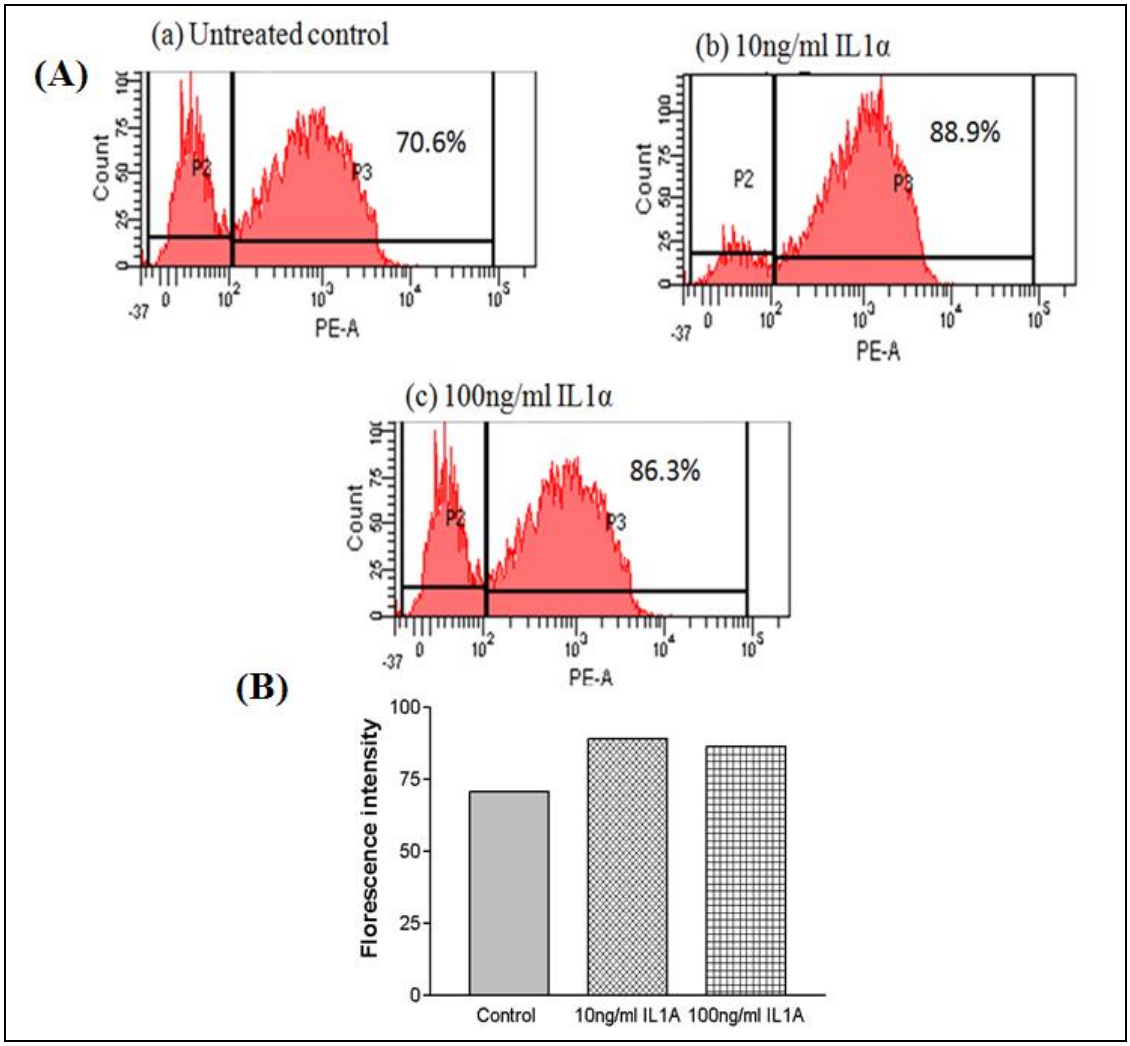

Figure 9: The NHM cells were treated with IL-1α (10 and 100ng/ml) and then IL1R1 expression levels were measured after 48 hrs of treatment. (A) Flow cytometry histogram shows increased expression of of IL1R1 upon IL-1α stimulation. (B) Graphical representation of fluorescence intensity of IL1R1 showed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

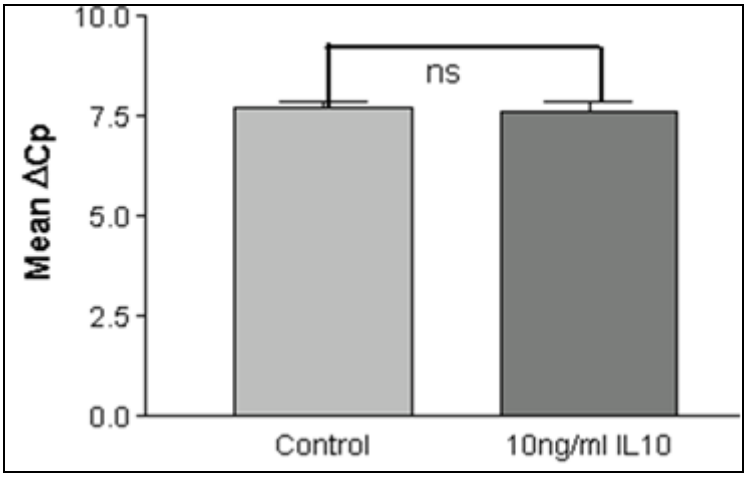

Figure 10: Effect of IL-10 on *IL10R* expression in NHM: The NHM cells were treated with IL-10 (10ng/ml) and *IL10R* transcript levels were measured after 48 hrs and the results showed no significant difference in the transcript levels of *IL10R*. Representation of Mean ΔCp of *IL10R* at 10 ng/ml IL-10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

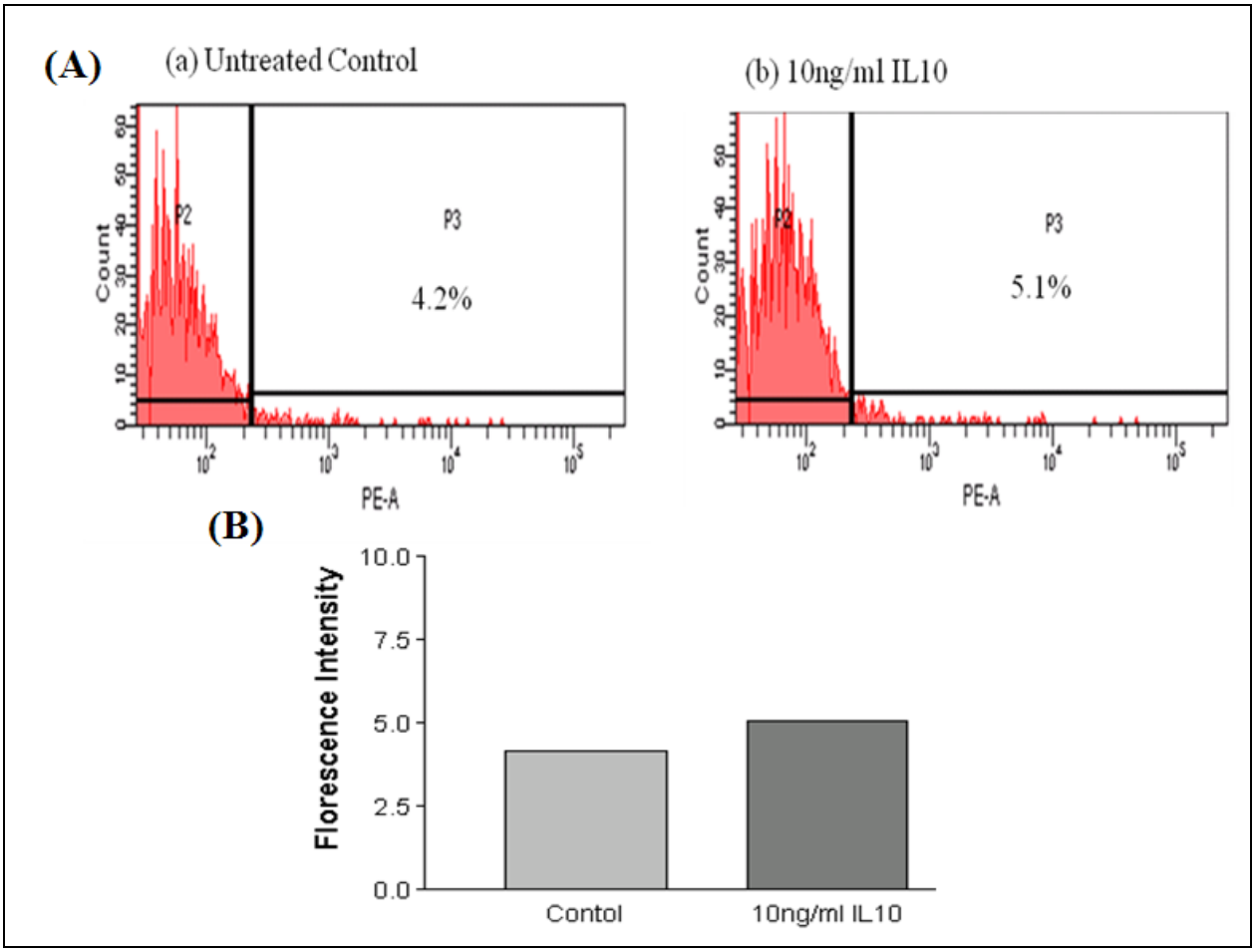

Figure 11: The NHM cells were treated with IL-10 (10ng/ml) and IL10R expression levels were measured after 48 hrs of treatment. (A) Effect of IL-10 on expression of IL10R by Flow cytometry. (B) Graphical representation of fluorescence intensity of IL10R upon exogenous stimulation of 10 ng/ml IL-10 for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

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6.1 INTRODUCTION

Oxidative stress has been implicated as the initial triggering event in vitiligo pathogenesis leading to melanocytes destruction (Laddha *et al*., 2013) and is marked by the accumulation of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al*., 2001). Our previous studies have suggested a significant increase in oxidative stress in vitiligo patients which is evident by high lipid peroxidation levels and an imbalance in the antioxidant enzymes (Laddha *et al*., 2013; Shajil, and Begum, 2006; Mansuri *et al*., 2016; Mansuri *et al*., 2017). Recent studies show that Poly (ADP-ribose) polymerase 1 (PARP-1), one of the major players of ROS-induced cell death, is involved in regulation of tissue inflammation, autoimmunity and apoptosis etc. (Kyeong *et al*., 2012). PARP-1 is a DNA repair enzyme that catalyses the poly (ADP-ribosyl)ation of proteins using NAD^+ as its substrate. PARP1 is activated 100- fold by DNA strand breaks and has a major role in base excision repair pathway (Caldecott, 2008). PARP-1 is known to have a role in the regulation of expression of various proteins that are implicated in the inflammation at the transcriptional level [(e.g., inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), [COX-2, and major histocompatibility complex class II (MHC Class II)]. The absence of functional PARP-1 (either genetic or pharmacological) decreased the expression of a host of pro-inflammatory mediators, including cytokines, chemokines, and adhesion molecules. The absence of PARP-1 also reduced tissue infiltration with activated phagocytes in experimental models of inflammation, circulatory shock, and ischemia reperfusion (Szabo, 2006). Studies have shown that PARP-1 is overexpressed in a variety of cancers and has been linked to the prognosis of cancers, most notably breast cancer. These seminal observations have been extended to show that PARP-1 further participates in the activation of other essential pro-inflammatory signaling cascades (Ha *et al*., 2002). A variety of endogenous and exogenous stress signals including those generated by oxidative, genotoxic, thermal, oncogenic, metabolic, and inflammatory stresses can trigger responses from PARP-1 leading to pathological conditions such as cancer, inflammation related diseases, autoimmune diseases, neurodegenerative diseases, and metabolic disorders.

Oxidative stress triggers extensive DNA damage, over activation of PARP and consequent depletion of the cellular NAD^+ levels impairing glycolysis, Krebs cycle, mitochondrial electron transport and eventually resulting in ATP depletion and consequent cell dysfunction and death by necrosis. During conditions of PARP over activation, pharmacological inhibition of PARP or genetic deletion of PARP-1 preserves cellular NAD^+ and ATP pools in oxidatively stressed cells, thereby allowing them to function normally, otherwise, if the apoptotic process has initiated it utilizes the apoptotic machinery and die by apoptosis instead of necrosis (Fiorillo *et al.*, 2006; Levrand *et al*. 2006). The inhibition of PARP by its inhibitors may offer therapeutic benefit in various disease conditions by preventing acute cell death. The ability of PARP inhibitors to suppress the expression of pro-inflammatory genes may be further exploited in various treatment modalities associated with acute and/or chronic inflammation (e.g., atherosclerosis, cardiovascular aging), inflammatory diseases, and various forms of cancer. 1,5-dihydroxyisoquinoline (DHQ) is a well characterized PARP inhibitor and was found to inhibit the PARP activity completely at non-cytotoxic concentrations (Shah *et al*., 1996). The recent clinical availability of the PARP inhibitors opens the door for their potential therapeutic use for various diseases. However, the role of PARP-1 activation and role of NFκB under oxidative stress in melanocytes has not been elucidated yet.

Therefore, in the present study, we aimed to monitor the effect of H_2O_2 on NHM and their rescue from H_2O_2 induced cell death using DHQ by assessing PARP1 activation and PARylation status. Moreover, we also monitored the effect of oxidative stress on NHM for MITM-M, *TYR*, and *ICAM1* expression.

6.2 MATERIALS AND METHODS

6.2.1 Ethics statement & Culture establishment of primary normal human melanocytes (NHM) These aspects are described in Chapter II.

6.2.2 Trypan blue exclusion assay

For cell viability assay, NHM were pretreated with 100 μM DHQ for 4 hrs, followed by 15 min exposure of H_2O_2 (0, 100, 250, 500 μM) along with media change**.** In this test, the cell suspension is mixed with trypan blue dye (1:10 dilution) and then visually examined

under light microscope to distinguish whether cells take up or exclude dye. (Strober *et al.*, 2001).

6.2.3 Western Blot analysis

To detect alterations in the protein levels, NHM were pre-treated with 100µM DHQ for 4 hrs, followed by a 15 min exposure of H_2O_2 (100 µM) and then cells were scraped on ice for cell harvesting. Total cell lysates were obtained by sonication (Using microprobe, 20% amplitude) using Laemmli Buffer (2% SDS, 0.0625 M Tris-HCl, pH 6.8, 10% glycerol, and 5% 2-β-mercaptoethanol) extraction. Thirty µg of total protein were subjected to electrophoresis in 10% and 6% SDS PAGE under reducing conditions for PARP-1 cleavage and PARylation respectively. Proteins were transferred to PVDF membrane and were blocked by blocking buffer (5% W/V BSA in PBS-T) for one hr. This was followed by probing Anti-PAR mouse mAb (10H) (Calbiochem, Germany) and Anti-PARP1rabbit antibody (cell signaling technology) overnight at 4ºC. Membrane was then probed with Peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) and Peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma) respectively, followed by six PBS-T and two PBS washes. Protein bands were detected by enhanced chemiluminescence system ECL (Amersham Pharmacia Biotech).

6.2.4 RNA isolation, cDNA synthesis and gene expression analysis

The transcript levels of different genes were estimated by semiQ PCR, using gene specific primers (Eurofins, Bangalore, India) as shown in Table 1. Expression of *GAPDH* gene was used as a reference. Details are described in Chapter IV.

Table 1. Primer sequences used for gene expression analysis.

Primer	**Sequence (5' to 3')**	**Amplicon size (bp)**	**Annealing Temperature (^{0}C)**
ICAM1FP	TCTGTTCCCAGGACCTGGCAATG	282	65

ICAM1RP	GGAGTCCAGTACACGGTGAGGAAG		
MITFM FP	CAAATGATCCAGACATGCGCTGG	180	61
MITFM RP	CTCGAGCCTGCATTTCAAGTTCC		
TYRFP	AGCACCCCACAAATCCTAACTTAC	92	63
TYRRP	ATGGCTGTTGTACTCCTCCAATC		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65
GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

'FP': forward primer; 'RP': reverse primer; 'bp': base pair.

6.2.5 Monitoring MITF-M levels upon H_2O_2 treatment by immunofluorescence

Details are described in Chapter IV.

6.2.6 Statistical analyses

All experiments were performed in triplicates using different batches of cells. Data are presented as the mean ± standard deviation (SD) and the statistical difference between two groups was analyzed by Student's t-test using GraphPad Prism version 3.02 software (GraphPad Software Inc. San Diego, CA, USA). *p*-values less than 0.05 were considered statistically significant.

6.3 RESULTS

6.3.1 Effect of PARP inhibitor (DHQ) on H_2O_2 induced NHM death:

Primary melanocytes were treated with different concentrations of H_2O_2 (0, 100, 250, 500 µM) for 15 min and incubated at 37ºC in humidified CO_2. NHM exhibited significant dose dependent decrease in the NHM viability upon H_2O_2 treatment (100, 250, 500 µM H_2O_2; p=0.0248, p= 0.0014 and p<0.0001 respectively Figure 1A). Further, we studied the dose-dependent effect of DHQ on NHM. NHM were treated with 0, 50, 100, 200 µM DHQ for 24 hours, followed by a cell count using trypan blue exclusion assay.

Concentration of DHQ up to 200 μM for 24 hours did not show any significant effect on cell morphology (Figure 1) and viability of NHM (Figure 2B). Therefore 100μM of DHQ was chosen for further experiments. In addition, we studied the effect of rescue of NHM death by PARP1 inhibitor in the presence and absence of H_2O_2. NHM were pre-treated with 100μM DHQ for 4 hours, followed by a 15 min exposure of H_2O_2 (0, 100, 250, 500 μM). Cell count was taken, using trypan blue exclusion assay. Pre-treatment with 100μM DHQ showed significant rescue from the cytotoxic effects of H_2O_2 both on cell morphology (Figure 1) and cell viability (Figure 2C). Significant rescue was observed at 100 μM, 250μM and 500μM of H_2O_2 (p=0.0471, p=0.0022 and p=0.0002 respectively).

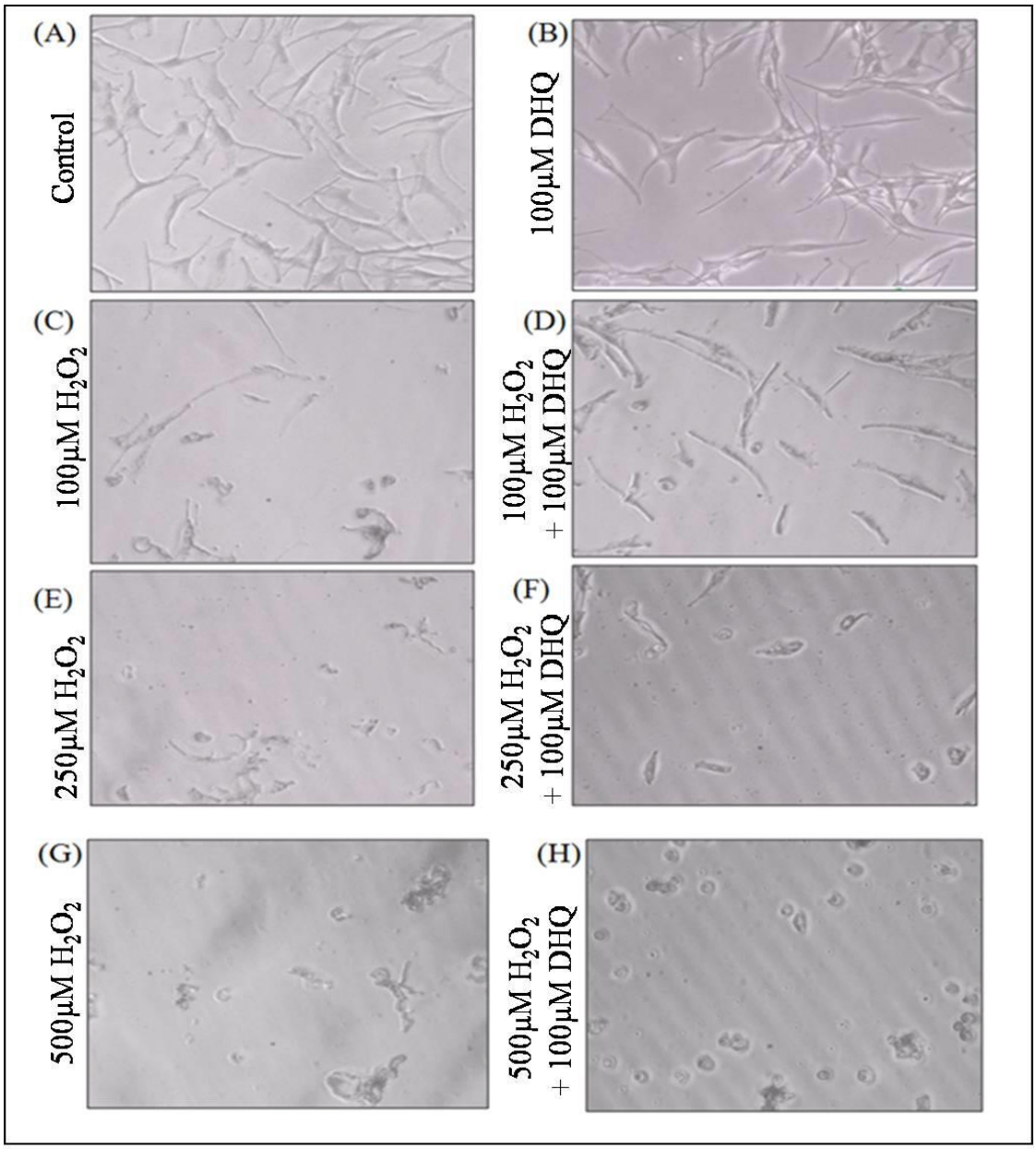

Figure 1: Morphological effect of DHQ on rescue of H_2O_2 induced NHM death: NHM were treated with respective doses as mentioned in figure (A) Control (B) 100μM DHQ (C) 100μM H_2O_2 (D) 100μM H_2O_2+ 100μM DHQ (E) 250μM H_2O_2 (F) 250μM H_2O_2+ 100μM DHQ (G) 500μM H_2O_2 (H) 500μM H_2O_2 + 100μM DHQ. Significant rescue was observed from dose dependent H_2O_2 mediated cell death upon pretreatment of DHQ.

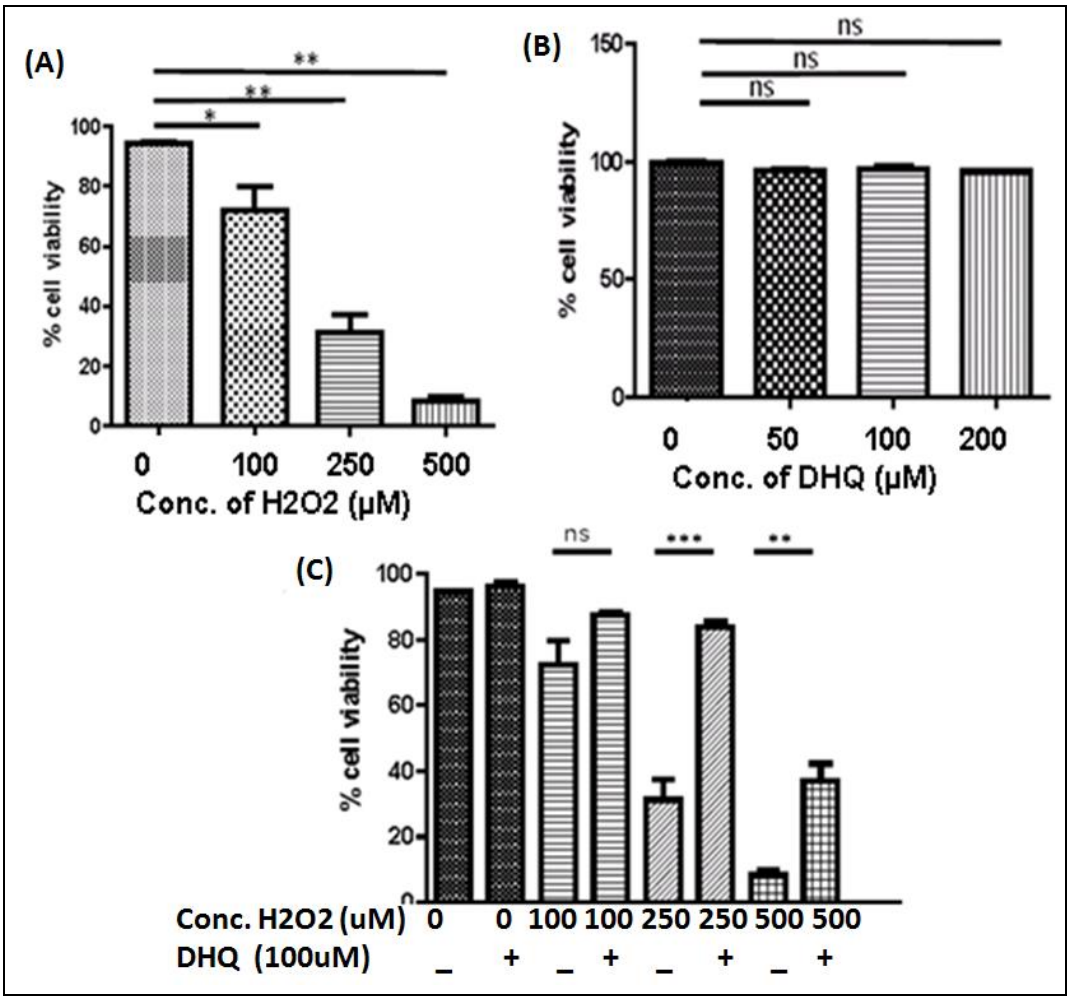

Figure 2: Effect of DHQ on rescue of H_2O_2 induced NHM death: (A) NHMs were treated with different doses of H_2O_2 (100μM, 250μM and 500μM) for 15 minutes followed by media change and cell viability was assayed by tyrpan blue exclusion assay. The results showed significant cell death (p=0.0248, p=0.0027 and p=0.0021 respectively; n=3).

(B) The cells were treated with different concentration of DHQ (50μM, 100 μM and 200μM) for 24 hours for cell viability analysis was performed by trypan blue assay. Dose dependent effect of DHQ on NHM viability by tyrpan blue exclusion showed no significant effect (n=3).

(C) Dose-dependent effect of H_2O_2 on NHM viability with and without pre-treatment (4 hours) of 100 μM DHQ. NHM were given 4 hours pretreatment with DHQ followed by

H_2O_2 exposure for 15 minutes. Media change was given after H_2O_2 treatment and cell count was taken by trypan blue exclusion assay. Significant rescue in cell death was observed at higher doses of H_2O_2 (p=0.0471; p=0.0002; p=0.0022); the values represent mean ±S.D. of three independent experiments.

6.3.2 Western blot analysis of PARylation and PARP1 activation upon DHQ mediated rescue from H_2O_2 induced NHM death:

NHM were treated with 100 µM H_2O_2 with and without DHQ for 15 min at 37ºC in humidified CO_2. DHQ exhibited significant restoration of a few NHM proteins which were degraded by H_2O_2 treatment. To confirm the same, we probed the samples (Control, 100µM H_2O_2, 100µM DHQ, 100µM DHQ +100µM H_2O_2) with anti-10H (PAR) antibody and PARP-1 antibodies. Our results demonstrated significant suppression of PARylation as well as PARP-1 activation upon DHQ treatment (Figures 3A and 3B). Also, NHM exhibited significant restoration of PARylation pattern of various proteins along with PARP-1 activation in DHQ + H_2O_2 group as compared to H_2O_2 group (Figures 3A and 3B). At higher concentration i.e., above 100µM of H_2O_2 there was complete degradation of proteins (data not shown).

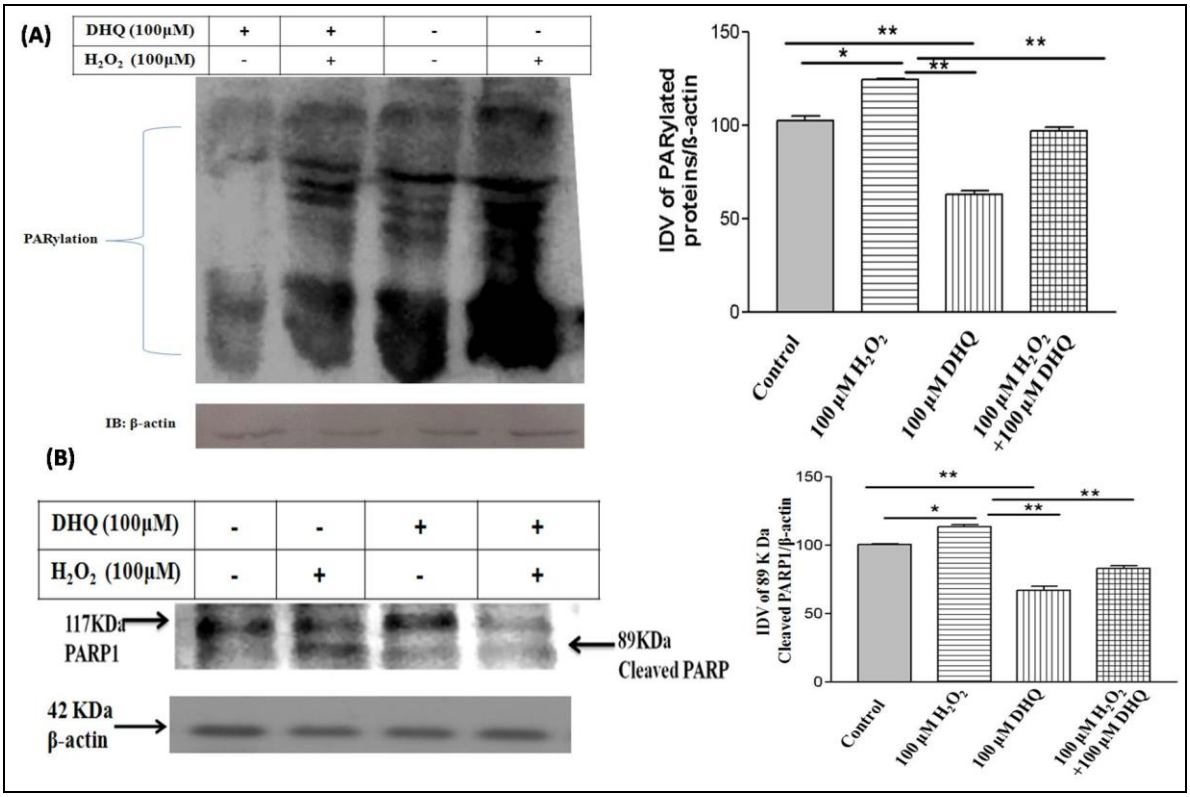

Figure 3: Analysis of PARylation and PARP1 activation upon DHQ mediated rescue from H_2O_2 induced NHM death: (A) Restoration of PARylation pattern of various proteins in DHQ+H_2O_2 group as compared to H_2O_2 group. DHQ treatment exhibited suppression of PARylation as compared to control group. Densitometric analysis for the DHQ and DHQ+H_2O_2 group reveled significant difference (p=0.0076) in terms of PARylation suppression.

(B) PARP-1 hyper activation was observed in H_2O_2 treated group (89 KDa cleaved fragment) which showed restoration in PARP1 inhibited conditions (DHQ + H_2O_2 group) as compared to H_2O_2 group. DHQ treatment suppressed PARP-1 activation. Densitometric analysis for the DHQ and DHQ+H_2O_2 group reveled significant difference (p=0.0062) in terms of PARP1 activation. β-actin was kept as a protein loading control for all western analysis (n=3).

6.3.3 Effect of oxidative stress on *MITF-M, TYR* and *ICAM-1* expression:

NHM were treated with 50 µM and 100 µM of H_2O_2 for 24 hrs. Significant decrease in *MITF-M* transcript levels was observed at both concentrations of H_2O_2 (p=0.0083 and p=0.0383 respectively) (Figure 4A). TYR is one of the target genes for MITF-M therefore we monitored the expression levels of *TYR*. Significant decrease in *TYR* transcript levels was observed with 50 µM and 100 µM of H_2O_2 (p=0.0439 and p=0.0109 respectively) (Figure 4B). However, there was no significant difference for *ICAM-1* transcript levels when treated with 50 µM and 100 µM of H_2O_2 (p=0.0772 and p=0.1325 respectively) (Figure 4C). We have also monitored effect of H_2O_2 on MITF-M protein expression in NHM**.** NHM upon treatment with 50 µM and 100 µM of H_2O_2 for 24 hrs significant decrease in MITF-M expression was observed in a dose dependent manner (p=0.0024 and p=0.0001 respectively) (Figures 5 A and B).

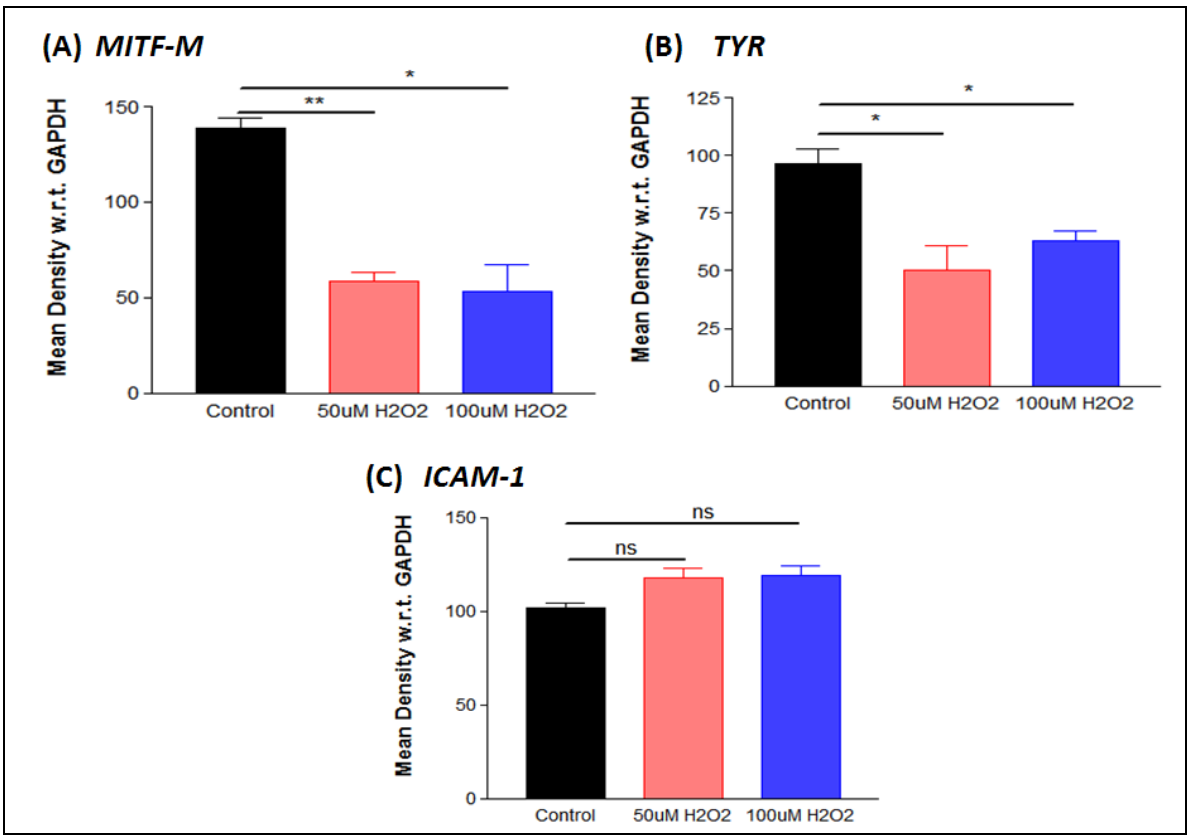

Figure 4: Effect of oxidative stress on *MITF-M, TYR* and *ICAM-1* mRNA expression: NHM were treated with 50 µM and 100 µM of H_2O_2 for 24 hrs (A) *MITF-M*

transcript levels were significantly reduced, p=0.0083 and p=0.0383 respectively. (B) *TYR* transcript levels were also significantly reduced, p=0.0439 and p=0.0109 respectively. (C) However, there was no significant difference in *ICAM-1* transcript levels, p=0.0772 and p=0.1325 respectively (n=3)

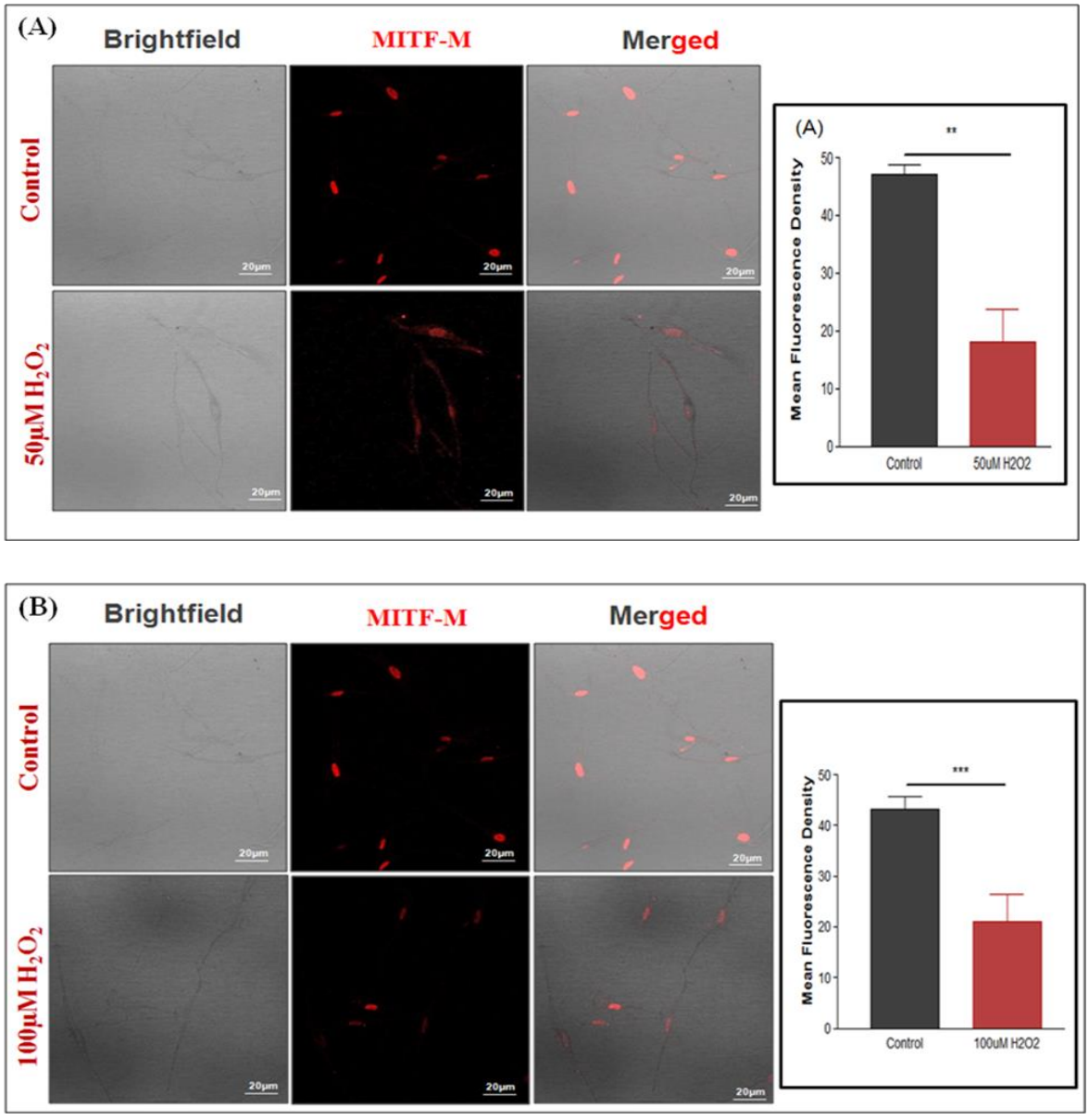

Figure 5: Dose dependent effect of H_2O_2 on MITF-M expression in NHM: Immunofluorescence analysis revealed significant decrease in MITF-M expression in NHM treated with (A) 50 μM and (B) 100 μM of H_2O_2 for 24 hrs (p=0.0024 and p=0.0001 respectively) (n=3; Magnification 630X: scale: 20μ).

6.4 DISCUSSION

Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis involving both genetic and non-genetic factors. The precise modus operandi for vitiligo pathogenesis has remained elusive (Laddha *et al*., 2013). PARP1 has been the most extensively studied member of PARP family. In response to DNA-strand breaks, it uses NAD^+ as a substrate to catalyze the covalent attachment of ADP-ribose units on the γ-carboxyl group of glutamate residues of acceptor proteins that are usually associated with DNA transactions, transmodification or on PARP1 itself (Hassa *et al*., 2008). PARP causes DNA damage-induced poly(ADPribosyl)ation of the N- and C-terminal tails of histone H1 and H2B and increases the access to breaks which in turn stimulates DNA repair signaling mechanism. Alteration of PARP1 activity may affect the development and functioning of cells through multiple processes. PARylation reaction is involved in several physiological processes such as differentiation, transcriptional regulation etc. However, the mechanism underlying the role of PARP1 in inflammatory response is still not clear. During inflammation, oxygen radicals are produced which induce massive DNA damage resulting in PARP1 over activation, leading to cell death by energy depletion. PARP1 is also found to modulate the transcription of inflammation linked genes. Thus, PARP1 regulates nuclear factor-κB (NF-κB)–dependent transcription and synthesis of inflammatory mediators, i.e., IL-1, IL-6, TNF-α, and inducible nitric oxide (NO) synthase (Oliver *et al*., 1999) which is a key element in the pathophysiology of inflammation and cell destruction. Various inflammatory cytokines, chemokines, necrotic cell products, bacteria and viruses stimulate NF-kB activation. Several investigators have proved the presence of oxidative stress in cultured melanocytes coupled with an increased susceptibility to pro-oxidants (Dell'Anna *et al*., 2007, Maresca *et al*., 1997; Boissy *et al*., 2004). *In vivo* oxidative stress has been attributed to accumulation of H_2O_2 in vitiligenous skin, which is associated with impaired catalase and glutathione peroxidase activities (Schallreuter *et al*., 1999). Also, millimolar levels of H_2O_2 lead to the inactivation of catalase due to substrate inhibition (Schallreuter *et al*., 1991); and glutathione peroxidase activities are found to be significantly decreased (Beazley *et al*., 1999, Agrawal *et al*., 2004). An alteration in melanocyte-specific proteins by the action of ROS results in the

generation of neoantigens, autoimmunity and melanocytorrhagy leading to defective apoptosis. In the present study, H_2O_2 exposure caused a significant dose-dependent death of NHM. Recent findings suggest PARPs as potential chemotherapeutic targets (Leung *et al*., 2011). The first PARP inhibitors were nicotinamide analogues like benzamide, 3-aminobenzamide (Durkacz *et al*., 1980). Other popular PARP inhibitors include 4-aminol & naphthalimide, 6 (5N)- and 2-nitro-6(5W) phenanthridinones, and DHQ etc (Banasik *et al*., 1992). We have used DHQ as it has a very low IC_{50} of 0.39 µM toward PARP and a very high IC_{50} of 890 µM toward mono(ADP-ribosyl) transferase. DHQ is also the only inhibitor to produce 100% inhibition of PARP activity in *in vitro* at a 1mM concentration (Banasik *et al*., 1992). PJ34 is another potent novel PARP inhibitor that dose-dependently inhibits purified PARP enzyme in a cell-free assay with half maximal effective concentration EC50 value of 20 nM. Unlike other PARP inhibitors (such as 3-AB), PJ34 does not possess any antioxidant properties but exhibits 10,000 times greater PARP inhibition than 3-AB (EC50 = 200 µM) (Huang *et al*., 2008). PJ34 has been found to have neuro-protective effects and enhance the chemotherapeutic effects in several tumor types. Study results have shown that PJ34 inhibits peroxynitrite-induced cell necrosis with EC50 value of 20 nM and dose-dependently suppresses the growth of HepG2 cells (Galaleldin *et al*., 2001).

Our results showed that DHQ, a potent PARP inhibitor did not show any significant effect on NHM viability and morphology up to 24 hrs of treatment. On the contrary PJ-34, another PARP inhibitor (10 µM) independent of DNA damage repair induces cell death in M14 melanoma cells (Chevanne *et al*., 2010). We have studied DNA damage-dependent role of PARP triggered by ROS in NHM. Pre-treatment with PARP inhibitor, DHQ showed a marked rescue from H_2O_2 induced melanocyte death, suggesting a significant role of PARP1 in the oxidative stress induced cell death. PARylation pattern was also studied in H_2O_2 mediated oxidative stress. We found significant decrease in H_2O_2 mediated PARylation and PARP activation upon pre-treatment with DHQ (100 µM). Previous reports have also suggested rescue of chondrocytes exposed to increased H_2O_2 concentrations (Shin *et al*., 2012). DHQ has also been shown to inhibit PARP activity and peroxynitrite-induced cell necrosis in mouse thymocytes (Garcia *et al*.,

2001). DHQ provided progenitors and mature oligodendrocytes with protection against H_2O_2 toxicity (Gabriela *et al*., 2004). Similar to observations by Liu *et al.*, (2009) and Dell'Anna *et al.*, (2006), our results demonstrated significant decrease in *MITF-M* expression at transcript and protein levels under oxidative stress conditions. Reduced expression of *TYR* was also observed by Jiménez-Cervantes *et al*., (2001). We did not find significant difference in *ICAM1* expression. However, increased *ICAM1* expression was reported in vitiligo patients (Dwivedi *et al.*¸ 2013; Al Badri 1993).

In conclusion, our results suggest that PARP-1 inhibitor, DHQ attenuates H_2O_2 induced NHM death and therefore, it could be used as a potential agent in vitiligo therapeutics.

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7A.1 INTRODUCTION

Vitiligo is an acquired hypomelanotic pigmentary disorder characterized by the presence of circumscribed depigmented macules in the skin. It is caused due to the loss of functional melanocytes of unknown etiology. Studies have revealed a worldwide incidence ranging from 0.04-2.16 % (Krüger *et al.,* 2012). As per our earlier studies 21.93% of vitiligo patients exhibit family history and 13.68% patients have first degree relatives affected, implicating involvement of genetic predisposition (Shajil & Begum, 2006). The etiology of vitiligo remains obscure despite being in focused debate for several years (Laddha *et al.,* 2013 ; Mansuri *et al.,* 2014).Various hypotheses have been proposed to understand vitiligo pathomechanisms for e.g. autoimmune, neural and oxidative stress theories which alone or in various combinations contribute towards vitiligo precipitation. Increasing evidences including our previous studies propose that genetic polymorphisms of *TNFA* (Laddha *et al.,* 2012) , *IL4* (Imran *et al.,* 2012), *IFNG* and *ICAM1* (Dwivedi *et al.,* 2013), *IL1B* (Laddha *et al.,* 2014), *TNFB* (Laddha *et al.,* 2013; Laddha *et al.,* 2014), different isoforms of *SOD* (Laddha *et al.,* 2013), *CAT* (Shajil *et al.,* 2007; Mansuri *et al.,* 2017), *NLRP1* (Dwivedi *et al.,* 2013), *CTLA4* (Dwivedi *et al*., 2011), *MYG* (Dwivedi *et al*., 2013), *GPX1* (Mansuri *et al*., 2016) might be playing a crucial role in vitiligo susceptibility. Genetic studies on vitiligo have revealed that the genetic variants, which are potentially involved in the autoimmunity could be responsible for conferring susceptibility or protection towards vitiligo. A key component in the inflammatory response is the increased production of pro-inflammatory cytokines such as *IL1B* (Laddha *et al*., 2014), *IFNG* (Dwivedi *et al.,* 2013; Natarajan *et al*., 2014) and TNF-α (Laddha *et al*., 2012). Moreover recently, we have further substantiated our previous findings by demonstrating miRNA mediated increased expression of *IL1B* and *IL1R1* in vitiligo patients (Mansuri *et al*., 2016).

The importance of IL-1 regulation is evident by the presence of natural antagonist, decoy receptor and other IL-1 family members for its fine regulation. The IL-1 family consists of the cytokines IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1RN), mapped on chromosome 2q14 (Smith *et al*., 2000; Patterson *et al.,* 1993). IL-1 has two

receptors IL-1RI is the functional receptor capable of mediating downstream signaling whereas IL-1RII acts as a decoy receptor (Arend and Guthridge, 2000). The IL-1RN is a unique naturally occurring cytokine that inhibits IL-1 activity by binding to the IL-1 receptors without signal transduction and acts as a negative regulator with anti-inflammatory effects (Granowitz *et al*., 1991). Apart from the presence of natural antagonist IL-1RN for IL1, various regulatory inhibitory molecules like IL-1RII, SIGIRR/TIR8, soluble IL-1RAcP, soluble IL-1RI or RII are present for the regulation of IL1 levels (Weber *et al*., 2010). Studies have shown that mice deficient in IL1RN exhibit reduced reproduction, stunted growth and develop spontaneous diseases in response to carcinogens (Zitvogel *et al*., 2012).

Aside from the nearness of normal rival IL-1RN for IL1, different administrative inhibitory particles like IL-1RII, SIGIRR/TIR8, solvent IL-1RAcP, dissolvable IL-1RI or RII are available for the direction of IL1 levels proposing significance of IL1 in keeping up resistant framework homeostasis

The *IL1RN* gene sequence shows 86-bp variable number tandem repeat (VNTR) in intron 2. This polymorphism has six alleles, comprising of 1-6 repeats of 86-bp sequence. The four-repeat (*IL1RN**1) and two-repeat (*IL1RN**2) alleles are most common, while the others occur at a combined frequency of lower than 5% (Table 3) (Tarlow *et al*., 1993; Vamvakopoulos *et al.,* 2002). The number of repeats may be of functional significance as these repeats contain putative binding sites for transcription factors which need further investigation (Tarlow *et al*., 1993). The role of *IL1RN* intron 2 VNTR (rs2234663) is well established with development of inflammatory disorders (Fischer *et al.,* 1992; McIntyre *et al.,* 1991; Xu *et al.,* 2011). The pro-inflammatory cytokine, IL1B and its antagonist, IL1RN are encoded by polymorphic genes (Wilkinson *et al*., 1991).

An association between polymorphism in the *IL1RN* intron 2 VNTR (rs2234663) and autoimmune disorders including vitiligo has been reported (Zaaber *et al*., 2014; Pehlivan *et al.,* 2009). Till date, there are no reports from India regarding rs2234663 and vitiligo. The aim of the present study was to investigate the association between *IL1RN* intron 2 VNTR polymorphism (rs2234663) with vitilgo; to assess *IL1RN*

transcript levels from PBMCs; and to perform possible genotype–phenotype correlation using case-control approach.

7A.2 MATERIALS AND METHODS

7A.2.1 Study Subjects

The study group included 307 vitiligo patients and 316 ethnically age and sex matched unaffected individuals who were referred to S.S.G. Hospital, Vadodara, Gujarat, India (Table 1). We used the clinical criteria to define the stability proposed by Falabella *et al*., (1995) discussed in the Vitiligo Global Issues Consensus Conference, 2012. (i) lack of progression of old lesions within the past 2 years; (ii) no new lesions developing within the same period. Active disease was defined as, appearance of new or spreading of existing lesions observed during past two years. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin and lack of association with other autoimmune disorders. The importance of the study was explained to all participants along with written consent form. The study plan was approved by the Institutional Ethics Committee for Human Research (IECHR).

Table 1: Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients	**Controls**
	(n = 307)	(n = 316)
Average age (mean age ± SD)	38.23 ± 19.15 yr	29.41 ± 12.16 yr
Sex: Male	156 (50.81%)	171(54.11%)
Female	151 (49.18%)	145 (45.88%)
Age of onset (mean age ± SD)	31.70±19.51 yr	NA
Duration of disease (mean ± SD)	7.723±9.154 yr	NA
Type of vitiligo		
Generalized	208(67.75%)	NA
Localized	99 (32.24%)	NA
Active vitiligo	242 (78.80%)	NA
Stable vitiligo	65 (21.19%)	NA

7A.2.2 Genotyping of *IL1RN* intron 2 VNTR (rs2234663) polymorphism

Polymerase chain reaction (PCR) was used to genotype rs2234663 polymorphism of *IL1RN*. The primers used for genotyping are mentioned in Table 2 and the product size for different alleles along with number of VNTR repeats has been described in Table 3.

The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mMdNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a Mastercycler gradient (Eppendorf, Germany) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table S2) for 30 seconds, and 72°C for 30 seconds. The amplified products along with 50 bp DNA ladder (Invitrogen, USA) were resolved on 3.5% agarose stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

7A.2.3 Genomic DNA Preparation

Five ml venous blood was collected from the patients and healthy subjects in K_3EDTA coated vacutainers (BD, Franklin Lakes, USA). Genomic DNA was extracted from whole blood using QIAamp DNA Blood Kit (QIAGEN Inc., Valencia, USA) according to the manufacturer's instructions. After extraction, concentration and purity of DNA was estimated spectrophotometrically. Quality of DNA was also determined on 0.8% agarose gel electrophoresis and DNA was stored at -20^{o}C until further analyses.

7A.2.4 RNA extraction and cDNA synthesis

Total RNA from whole blood was isolated and purified using Ribopure^{TM}- blood Kit (Ambioninc. Texas, USA) following the manufacturer's protocol. RNA integrity was verified by 1.5% agarose gel electrophoresis while the RNA yield and purity was determined spectrophotometrically at 260/280 nm. RNA was treated with DNase I (Ambioninc. Texas, USA) before cDNA synthesis to avoid DNA contamination. One microgram of total RNA was used to prepare cDNA. cDNA synthesis was performed

using the Verso cDNA Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions in the CG Palm Cycler (Genetix, India).

7A.2.5 Real-time PCR

The expression of *IL1RN* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table S2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10s, 66°C and 65°C for 20 s for *IL1RN* and *GAPDH* respectively, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out to check the specificity of the products formed. The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

Table 2: Primers used for genotyping and gene expression analysis.

Gene/Polymorphism Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing Temperature
IL1RN intron 2 VNTR F	CTCAGCAACACTCCTATTG	160+ n*86 (n=2-6)	62°C
IL1RN intron 2 VNTR R	TCATCTTCCTGGTCTGCA		
IL1RN expression F	GCTGGAGGCAGTTAACATC	165	66°C
IL1RN expression R	CTACTCGTCCTCCTGGAAG		

GAPDH expression F	CATCACCATCTTCCAGGAGCGAG	122	65°C
GAPDH expression R	CCTGCAAATGAGCCCCAGCCT		

Table 3: Different alleles of *IL1RN* intron 2 VNTR (rs2234663) polymorphism.

Allele	Size (bp)	No. of repeats
A1	418	4
A2	246	2
A3	332	3
A4	504	5
A5	590	6
A6	160	1

7A.2.6 Statistical analyses

The distribution of the genotypes and allele frequencies of rs2234663 for patients and control subjects were compared using chi-squared test with 2×2 contingency tables using Prism 3 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Relative expression of *IL1RN* and genotype-phenotype correlation analysis in patient and control groups was plotted and analyzed by nonparametric unpaired t-test using Prism 3 software. The A1/1 is considered as ancestral genotype, A2/2 is considered as mutant genotype while all the heterozygous genotypes grouped together with fewer repetitions genotypes. Finally, to compare the expected genotype frequencies against the observed frequencies, we used a chi-square test applied through the Hardy–Weinberg equilibrium.

7A.3 RESULTS

7A.3.1. Analysis of association between *IL1RN* intron 2 VNTR polymorphism (rs2234663) and susceptibility to vitiligo:

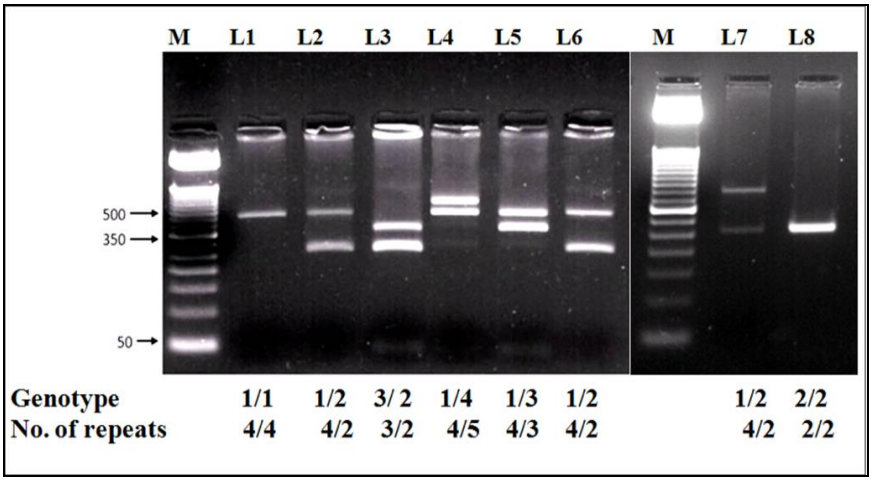

Figure 1: PCR amplification analysis of *IL1RN* VNTR polymorphism on 3.5% Agarose gel: M: DNA Marker; Lane 1: *ILRN**1/1; Lanes 2, 6 and 8: *ILRN**1/2; Lane 3: *ILRN**3/2; Lane 4: *ILRN**1/4; Lane 5: *ILRN**1/3; Lane 7: *ILRN**1/2.

Eight genotypes were identified in Gujarat population as shown in Figure1. Both patients and controls were under Hardy Weinberg equilibrium p = 0.6835 and p = 0.6003, respectively. Our studies suggested that there was no significant association of genotype or allele frequency among vitiligo patients and controls. When genotypes were compared between patients and controls we found: *IL1RN**1/2 (p=0.8946), *IL1RN**2/2 (p=0.8390), *IL1RN**3/2 (p=0.3144), *IL1RN**3/1 (p=0.9909), *IL1RN**4/2 (p=0.7250), *IL1RN**1/4 (p=0.5066), *IL1RN**5/2 (p=0.1623). Similarly when alleles were compared between patients and controls we found non-significant difference: *IL1RN**A2 (p =0.7177), *IL1RN**A3 (p=0.5554), *IL1RN**A4 (p =0.6805) and *IL1RN**A5 (p =0.1611) (Table 4). However, we observed *IL1RN**3/2 genotype to be present only in the vitiligo patients, whereas *IL1RN**5/2 genotype was observed only in the control group. Further stratification of results based on type, activity and gender of vitligo revealed non-significant difference both in genotype and allele frequencies among different groups. Intrestingly, we observed significant difference in *IL1RN**A1/2 genotype distribution between active vitiligo (AV)

(47.10%) and stable vitiligo (SV) pateints (33.84%) (p=0.0172). Significant difference was observed in genotype frequencies between SV (6.15%) and controls (14.87%) of *IL1RN**A2/2 (p=0.0246). Additionally, we found significant difference in allele frequency of *IL1RN**A2 between AV (35.95%) and SV (23.07%) (p=0.0060); and when SV (23.07%) were compared to controls (36.07%) (p=0.0046).

Further, when genotypes were compared between AV and SV we found: *IL1RN**1/2 (p=0.0172), *IL1RN**2/2 (p=0.0639), *IL1RN**3/2 (p=0.3740), *IL1RN**4/2 (p=0.3740), *IL1RN**1/4 (p=0.7219). Similarly upon allele comparison between AV and SV we found: *IL1RN**A2 (p =0.0060), *IL1RN**A3 (p =0.4225), *IL1RN**A4 (p =0.7192). When genotypes were compared between controls and AV we found: *IL1RN**1/2 (p=0.2146), *IL1RN**2/2 (p=0.5455), *IL1RN**3/2 (p=0.0940), *IL1RN**3/1 (p=0.4022), *IL1RN**4/2 (p=0.6952), *IL1RN**1/4 (p=0.6439), *IL1RN**5/2 (p=0.2369). Similarly when alleles were compared between controls and AV we found: *IL1RN**A2 (p =0.9599), *IL1RN**A3 (p =0.4182), *IL1RN**A4 (p =0.9971), *IL1RN**A5 (p =0.2157).

When genotypes were compared between controls and SV we found: *IL1RN**1/2 (p=0.1016), *IL1RN**2/2 (p=0.0246), *IL1RN**3/1 (p=0.5970), *IL1RN**4/2 (p=0.2916), *IL1RN**1/4 (p=0.4605), *IL1RN**5/2 (p=0.4550). Similarly when alleles were compared between controls and SV we found: *IL1RN**A2 to be significantely (p =0.0046), *IL1RN**A3 (p =0.6190), *IL1RN**A4 (p =0.7080), *IL1RN**A5 (p =0.4820) (Table 5).

Genotype and allele frequencies when compared among generalized vitiligo, localised vitilgo and controls; male and female; duration of disease and genotypes no association was found within different sub groups analysis (Tables 6 and 7; Figure 3).

7A.3.2 Relative gene expression analysis of *IL1RN* using case-control approach

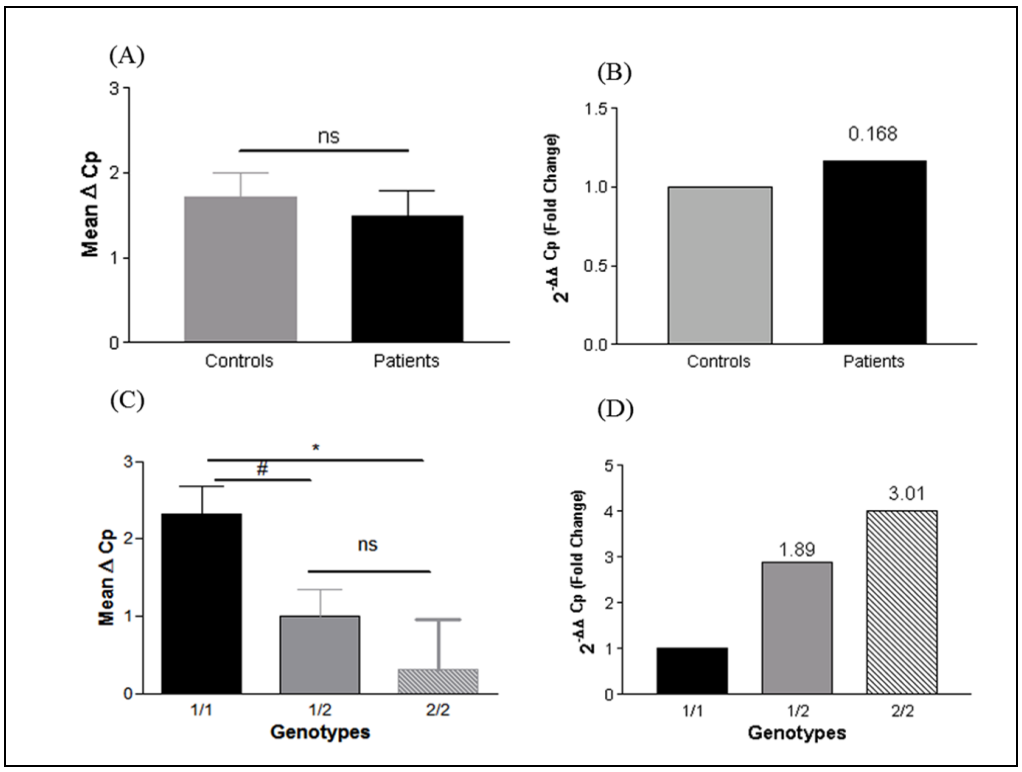

Figure 2: Relative gene expression analysis of *IL1RN* in vitiligo patients and controls. **(A)** Expression of *IL1RN* transcripts in controls (n=36) and vitiligo patients (n=36), as suggested by Mean ΔCp. Vitiligo patients showed non-significant difference in mRNA levels of *IL1RN* as compared to controls ($p = 0.5962$). **(B)**Vitiligo patients showed 1.163 fold higher *IL1RN* expressions as compared to controls, as determined by $2^{-\Delta\,\Delta Cp}$ method. **(C)** Genotype-phenotype correlation of *IL1RN* transcript levels: When mean ΔCp for frequent genotypes *IL1RN**2/2 and *IL1RN**1/1 were compared we got significant increase in *IL1RN* expression in *ILRN* A2 carriers (*p value= 0.03); additionally when mean ΔCp for frequent genotypes *IL1RN**1/1 and *IL1RN**2/1 were compared significant increase in *IL1RN* expression was observed ($^{\#}p$ value=0.01). However, when *IL1RN**2/2 and *IL1RN**1/2 mean ΔCp for genotypes were compared we obtained non-significant difference (^{ns}p value=0.45). **(D)** 1.89 increase in the fold change of *IL1RN* expression was observed when *IL1RN**1/1 and *IL1RN**2/2 genotype were compared; and 3.01 increase in

the fold change of *IL1RN* expression was observed when *IL1RN**1/1 and *IL1RN**1/2 genotype were compared.

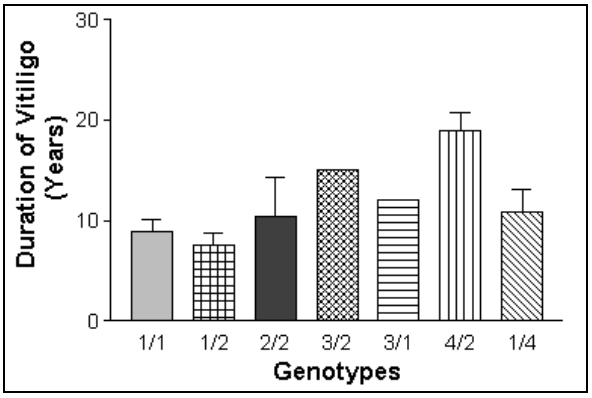

Figure 3: Correlation analysis for duration of disease and different genotypes of vitiligo patients. No significant difference was observed among different genotypes and duration of disease.

Relative gene expression analysis in 36 patients and 36 controls revealed a non-significant difference in expression of *IL1RN* transcripts in patients as compared to controls (Mean ΔCp ± SEM: 1.784±0.61659 vs 1.940±0.3340; p=0.5962), after normalization with *GAPDH*. The $2^{-\Delta\Delta Cp}$ analysis showed non-significant difference (0.168 fold increase) in the expression of *IL1RN* in patients, compared to controls (Figure 2 A, B). However, further stratification of data based on type, activity and gender of vitligo revealed no significant difference in expression level (data not shown).

7A.3.3 Genotype-phenotype correlation analysis of *IL1RN* levels:

Genotype-phenotype correlation of *IL1RN* levels showed significant increase in *IL1RN* expression in *IL1RN**A2 genotypes. Significant increase in transcript level was observed when mean ΔCp for frequent genotypes *IL1RN**2/2 and *IL1RN**1/1 were compared (p =0.03). Also, when mean ΔCp was compared for *IL1RN**1/1 and *IL1RN**2/1 genotypes, *IL1RN**A2 containing genotypes showed higher expression (p=0.01). However, non-significant difference in expression level of *IL1RN* was found for genotypes *IL1RN**2/2 and *IL1RN**2/1 (p=0.45) (Figure 2 C, D).

Table 4: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in vitiligo patients and controls from Gujarat population.

Genotype or allele	Vitiligo Patients (Freq. %)	Controls (Freq. %)	*p* value	Odds ratio	CI (95%)
Genotype	**n= 307**	**n=316**			
*IL1RN** (A 1/1)	123 (40.06)	125(39.55)	R	1	-
*IL1RN** (A 1/2)	123 (40.06)	128(40.50)	0.8946	0.9766	0.6874-1.387
*IL1RN** (A 2/2)	44 (14.33)	47(14.87)	0.8390	0.9514	0.5883-1.539
*IL1RN** (A 3/2)	1 (0.32)	0(0)	0.3144	3.049	0.1229-75.62
*IL1RN** (A 3/1)	1 (0.32)	1(0.31)	0.9909	1.016	0.06282-16.44
*IL1RN** (A 4/2)	3 (0.97)	4(1.26)	0.7250	0.7622	0.1671-3.478
*IL1RN** (A 1/4)	12 (3.90)	9(2.84)	0.5066	1.355	0.5511-3.332
*IL1RN** (A 5/2)	0(0)	2(0.63)	0.1623	0.2032	0.009652-4.280
Allele					
*IL1RN**A1	382 (62.21)	388(61.39)	R	1	-
*IL1RN**A2	215 (35.01)	228(36.07)	0.7177	0.9578	0.7581-1.210
*IL1RN**A3	2 (0.32)	1 (0.15)	0.5554	2.031	0.1833-22.51
*IL1RN**A4	15 (2.44)	13 (2.05)	0.6805	1.172	0.5502-2.496
*IL1RN**A5	0 (0)	2 (0.31)	0.1611	0.2031	0.0097-4.248

'n' represents number of Patients/ Controls,
'R' represents reference group
CI refers to Confidence Interval,
(P) refers to Patients and (C) refers to Controls,
[a]Vitiligo Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,
[b] Vitiligo Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table, A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

Table 5: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in active and stable vitiligo patients & controls from Gujarat population.

Genotype or allele	Active Patients (Freq.%)	Stable Patients (Freq.%)	Controls (Freq.%)	*p* value	Odds ratio	CI (95%)
Genotype	**n= 242 (78.80)**	**n=65 (21.19)**	**n=316**			
*IL1RN**(A 1/1)	88 (36.36)	35 (53.84)	125 (39.55)	R	1	-
*IL1RN**(A 1/2)	114 (47.10)	22 (33.84)	128 (40.50)	0.0172[a]	2.061 [a]	1.129 - 3.761 [a]
				0.2146[b]	1.265[b]	0.8724 – 1.835[b]
				0.1016[c]	0.6138[c]	0.3411- 1.105[c]
*IL1RN**(A 2/2)	28 (11.57)	4 (6.15)	47 (14.87)	0.0639 [a]	2.787 [a]	0.9095- 8.522 [a]
				0.5455[b]	0.8462[b]	0.4923- 1.455[b]
				0.0246[c]	0.3040[c]	0.1024- 0.9020[c]
*IL1RN**(A 3/2)	2 (0.82)	0 (0.00)	0 (0)	0.3740 [a]	2.006 [a]	0.0938 - 42.86 [a]
				0.0940[b]	7.090[b]	0.3360- 149.6[b]
				-	-	-
*IL1RN**(A 3/1)	0 (0.00)	0 (0.00)	1 (0.31)	-	-	-
				0.4022[b]	0.4727[b]	0.0190- 11.75[b]
				0.5970[c]	1.178[c]	0.0469 – 29.58[c]

*IL1RN**(A 4/2)	2 (0.82)	0 (0.00)	4 (1.26)	0.3740 [a]	2.006 [a]	0.0938-42.86 [a]
				0.6952[b]	0.7102[b]	0.1272-3.965[b]
				0.2916[c]	0.3928[c]	0.0206-7.476[c]
*IL1RN**(A 1/4)	8 (3.305)	4 (6.15)	9 (2.84)	0.7219 [a]	0.7955 [a]	0.2250-2.812 [a]
				0.6439[b]	1.263[b]	0.4687-3.401[b]
				0.4605[c]	1.587[c]	0.4610-5.465[c]
*IL1RN**(A 5/2)	0 (0.00)	0 (0.00)	2 (0.63)	0.2369[b]	0.2836[b]	0.0134-5.984[b]
				0.4550[c]	0.7070[c]	0.0331-15.08[c]

Allele						
*IL1RN**A1	298 (61.57)	96 (73.84)	388 (61.39)	R	1	-
*IL1RN** A2	174 (35.95)	30 (23.07)	228 (36.07)	0.0060 [a]	1.868 [a]	1.191 – 2.932 [a]
				0.9599[b]	0.9936[b]	0.7750-1.274[b]
				0.0046[c]	0.5318[c]	0.3420-0.8270[c]
*IL1RN** A3	2 (0.41)	0 (0.00)	1 (0.15)	0.4225 [a]	1.6160 [a]	0.07688-33.99 [a]
				0.4182[b]	2.604[b]	0.2349- 28.87[b]
				0.6190[c]	1.342[c]	0.0542-33.22[c]
*IL1RN**	10 (2.06)	4 (3.07)	13 (2.05)	0.7192	0.8054	0.2469-2.627 [a]

A4				[a]	[a]	
				0.9971[b]	1.002[b]	0.4331-2.316[b]
				0.7080[c]	1.244[c]	0.3965-3.900[c]
*IL1RN** A5	0 (0.00)	0 (0.00)	2 (0.31)	-	-	-
				0.2157[b]	0.2603[b]	0.0124 –5.446 [b]
				0.4820[c]	0.8052[c]	0.0383 –16.92[c]

'n' represents number of Patients/ Controls,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Active Vitiligo vs. Stable Vitiligo,
[b]Active Vitiligo vs. Controls,
[c]Stable Vitiligo vs. Controls.
A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

Table 6: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in generalised and localized vitiligo patients & controls from Gujarat population.

Genotype or allele	Generali-sed Patients (Freq.%)	Localised Patients (Freq.%)	Control	*p* value	Odds ratio	CI (95%)
Genotype	**n= 208**	**n=99**	**n=316**			
*IL1RN** (A 1/1)	92 (44.23)	37 (37.37)	125 (39.55)	R	1	-
*IL1RN** (A 1/2)	87 (41.82)	40 (40.40)	128 (40.50)	0.6235[a]	0.8747 [a]	0.5125-1.493 [a]
				0.6837[b]	0.9235 [b]	0.6296-1.355 [b]
				0.8350[c]	1.056 [c]	0.6336-1.759 [c]
*IL1RN**	21	17	47	0.0629[a]	0.4968[a]	0.2359-1.046[a]

(A 2/2)	(10.09)	(17.17)	(14.87)	0.0903[b]	0.6071 [b]	0.3396-1.085 [b]
				0.5543 [c]	1.222 [c]	0.6283-2.377[c]
*IL1RN** (A 3/2)	1 (0.48)	0 (0)	0 (0)	0.5266[a]	1.216[a]	0.0484-30.56[a]
				0.2452 [b]	4.070 [b]	0.1638-101.1 [b]
				-	-	-
*IL1RN** (A 3/1)	1 (0.48)	0 (0)	1 (0.31)	0.5266[a]	1.216[a]	0.0484-30.56[a]
				0.8286 [b]	1.359 [b]	0.0838-22.02 [b]
				0.5867 [c]	1.116 [c]	0.0444-27.98 [c]
*IL1RN** (A 4/2)	1 (0.48)	0 (0)	4 (1.26)	0.5266[a]	1.216[a]	0.0484-30.56[a]
				0.3156 [b]	0.3397 [b]	0.0373 - 3.091 [b]
				0.2783 [c]	0.3719 [c]	0.0195 – 7.070 [c]
*IL1RN** (A 1/4)	5 (2.40)	5 (5.05)	9 (2.84)	0.1573[a]	0.4022[a]	0.1099-1.472[a]
				0.6234 [b]	0.7548 [b]	0.2448-2.328 [b]
				0.2782 [c]	1.877 [c]	0.5923-5.948 [c]
*IL1RN** (A 5/2)	0 (0)	0 (0)	2 (0.63)	-	-	-
				0.2266 [b]	0.2714 [b]	0.0129-5.724 [b]
				0.4425 [c]	0.6693 [c]	0.0314-14.26 [c]

Allele						
*IL1RN**A1	277 (66.58)	119 (60.10)	388 (61.39)	R	1	-
*IL1RN**A2	131 (31.49)	74 (37.37)	228 (36.07)	0.1323[a]	0.7605[a]	0.5322-1.087[a]
				0.1073[b]	0.8048[b]	0.6177-1.049[b]
				0.7391[c]	1.058[c]	0.7584-1.477[c]
*IL1RN**A3	2 (0.48)	0 (0)	1 (0.15)	0.3545[a]	2.153[a]	0.1025-45.22[a]
				0.3808[b]	2.801[b]	0.2526-31.07[b]
				0.5798[c]	1.084[c]	0.0438 - 26.80[c]
*IL1RN**A4	6 (1.44)	5 (2.52)	13 (2.05)	0.2736[a]	0.5155[a]	0.1543 - 1.723[a]
				0.3793[b]	0.6465[b]	0.2427-1.722[b]
				0.6725[c]	1.254[c]	0.4380 – 3.590[c]
*IL1RN**A5	0 (0)	0 (0)	2 (0.31)	-	-	-
				0.2326[b]	0.2800[b]	0.0134-5.859[b]
				0.4338[c]	0.6502[c]	0.0310-13.65[c]

'n' represents number of Patients/ Controls,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Generalised vitiligo vs. Localised vitiligo,
[b]Generalised vitiligo vs. Controls
[c]Localised vitiligo vs. Controls.

A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

Table 7: Distribution of genotypes and alleles for *IL1RN* intron 2 VNTR (rs2234663) in male and female vitiligo patients from Gujarat population.

Genotype or allele	Male Patients (Freq.%)	Female Patients (Freq.%)	*p* value	Odds ratio	CI (95%)
Genotype	**n= 156**	**n=151**			
*IL1RN**(A1/1)	52 (33.33)	64 (42.39)	R	1	-
*IL1RN**(A1/2)	76 (48.71)	61 (40.39)	0.0915	0.6521	0.3965 – 1.073
*IL1RN**(A2/2)	20 (12.82)	18 (11.92)	0.4027	0.7313	0.3508 – 1.524
*IL1RN**(A3/2)	2 (1.28)	0 (0)	0.1205	1.628	0.0076- 3.468
*IL1RN**(A 3/1)	-	-	-	-	-
*IL1RN**(A4/2)	3 (1.92)	0 (0)	0.0584	0.1163	0.0059-2.304
*IL1RN**(A1/4)	3 (1.92)	8 (5.29)	0.2614	2.167	0.5468-8.585
*IL1RN**(A5/2)	-	-	-	-	-
Allele					
*IL1RN**A1	183 (58.65)	197 (65.23)	R	1	-
*IL1RN**A2	121 (38.78)	97 (32.11)	0.0837	0.7447	0.5330 – 1.041
*IL1RN**A3	2 (0.64)	0 (0)	0.1434	0.1858	0.0088-3.899
*IL1RN**A4	6 (1.92)	8 (2.64)	0.6966	1.239	0.4216-3.696
*IL1RN**A5	-	-	-	-	-

'n' represents number of Male/Female Patients,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Male vs. Female Patients (genotype) using chi-squared test with 2 × 2 contingency table,
[b] Male vs. Female Patients (allele) using chi-squared test with 2 × 2 contingency table.
A are different alleles of *IL1RN* intron 2 VNTR (rs2234663).

7A.4 DISCUSSION

Cytokine imbalance in the skin and systemic circulation in vitiligo is well reported. The balance between IL-1 and IL-1RN in local tissues plays an important role in the susceptibility to and severity of many diseases (Fischer *et al*., 1992; Arend, 2000). Polymorphisms in regulatory gene regions of cytokines that affect transcription may account for differences at individual levels for cytokine production (Danis *et al*., 1995). The rs2234663 polymorphism has been associated with multiple diseases primarily of epithelial and endothelial origin along with various autoimmune disorders. The *IL1RN* intron 2 86 bp VNTR involvement may be resulting in an imbalance in the IL-1 system with increased IL-1β and reduced production of the major intracellular isoform of IL-1RN (Arend, 2000). Altogether vitiligo is considered to be an autoimmune disorder due to cytokine imbalance (between pro- and anti- inflammatory) with increased levels of IL-1α and *IL1B* in skin and PBMCs in vitiligo patients respectively (Laddha *et al*., 2014; Birol *et al*., 2006).

The *IL1RN* intron 2 VNTR of 86 bp repeat may be of functional significance as these repeats contain putative binding sites for transcription factors. Number of repeats may affect the strength of enhancer binding sequence which in turn influences the transcription of *IL1RN* (Tarlow *et al*., 1993). Interestingly, the differences in circulating levels of IL-1RN have been reported with different alleles of *IL1RN* VNTR (Danis *et al*., 1995; Mandrup-Poulsen *et al*., 1994). The rs2234663 has been reported to be associated with Hashimoto thyroiditis (Zaaber *et al*., 2014), juvenile idiopathic inflammatory myopathies (Rider *et al*., 2000), systemic lupus erythematosus (SLE) (Blakemore *et al*., 1994), and ulcerative colitis (UC) (Mansfield JC, Holden *et al*., 1994) including vitiligo (Pehlivan *et al.*, 2009). Conversely, no association was found for rs2234663 with SLE in Italians (D'Alfonso *et al*., 2000) suggesting the importance of ethnicity in disease predisposition. We found lack of *IL1RN**A6 (single repeat of 86 bp) in the present study. Our results showed that genotype and allele frequencies for rs2234663 did not differ between vitiligo patients and controls. Exceptionally, we found significant difference in *IL1RN**A1/2 genotype distribution between AV and SV

(p=0.0172). Significant difference was observed in genotype frequencies between SV and controls for *IL1RN**A2/2 (p=0.0246). Additionally, we found significant difference in allele frequency of *IL1RN**A2 between AV and SV (p=0.0060); and when SV were compared to controls (p=0.0046). Also, we observed *IL1RN**3/2 genotype only in vitiligo patients confering suspectibility towards vitiligo while *IL1RN**5/2 genotype was observed only in controls. Also, the pro-inflammatory immune response of individuals homozygous for the *IL1RN**A2 allele was found to be more prominent compared to other genotypes (Witkin *et al.,* 2002). The influence of the *IL1RN**A2 allele has been widely studied in multiple diseases such as inflammatory bowel disease, SLE (Blakemore *et al*., 1994), UC (Mansfield *et al*., 1994), Graves' disease, nephropathy in diabetes mellitus, alopecia areata, chronic inflammatory diseases and psoriasis. *IL1RN**A2 was associated with increased production of IL-IRN, and also reduced production of IL-lα by monocytes (Danis *et al*., 1995). On the contrary, *IL1RN**A2 is associated with reduced levels of IL-1RN and *IL1RN* mRNA in the colonic mucosa (Carter *et al*., 2004). The association of low IL-lα production with *IL1RN**A2 polymorphism may be a consequence of higher IL-1RN production in individuals with *IL1RN**A2 genotype (Danis *et al*., 1995). It has also been demonstrated that *IL1RN**A2 is associated with significantly reduced levels of IL-1RN in human umbilical vein endothelial cells (Dewberry *et al*., 2000). It has been speculated that the impact of this polymorphism is different in cells synthesizing different mRNA splice variants; those synthesizing the intracellular form produce less IL1RN when carrying *IL1RN**A2, in contrast to monocytes that synthesize predominantly sIL-1RN and produce more protein when of genotype *IL1RN*A2* (Danis *et al*., 1995). Whereas it did not alter steady state levels of *IL1RN* mRNA in cultured keratinocytes (Clay *et al*., 1996).

Similarly, there are reports on vitiligo from Turkish population (n=48) and from Korean population (n=31), where they have reported the absence of *IL1RN**1/5 genotypes and *IL1RN**A5 in vitiligo patients and lack of association of rs2234663 polymorphism respectively (Pehlivan *et al*., 2009; Lee *et al*., 1995). In accordance with this we did not observe *IL1RN**5 genotype and allele in pateints, which was present

only in conrols implicating its possible protective role in vitiligo predisposition. Interestingly, genotype-phenotype correlation showed that *IL1RN**A2 of *IL1RN* VNTR was found to be associated with increased *IL1RN* transcript levels, suggesting important role of *IL1RN**A2 of *IL1RN* VNTR in *IL1RN* regulation.The IL1RN family of molecules include one secreted isoform (sIL1RN) and three intracellular isoforms (icIL1RN1, 2 and 3). Numerous studies indicate that the sole biological function of sIL1RN seems to competitively inhibit IL-1 binding to cell-surface receptors. Overall, suggesting the importance of gene regulation of various *IL1RN* isoforms which may exhibit tissue specific expression.

In the present study, non-significant difference was observed for *IL1RN* transcript levels and this can be attributed to the presence of various genotypes and fewer *IL1RN* VNTR 2/2 samples in expression analysis. TNF-α induces the production of IL-1α, IL-13, IL-lRN and IL-6 by monocytes (Santtila *et al*., 1998). Recently, we have reported up-regulation of *IL1RN* in normal human melanocytes upon IL-1α stimulation (Singh *et al*., 2016). There are reports suggesting involvement of various cytokines in vitiligo pathogenesis (Singh *et al*., 2014) and increased levels of IL-1α (Birol *et al*., 2006), TNF-α (Laddha *et al*., 2012; Birol *et al*., 2006), IFN-γ (Dwivedi *et al*., 2013; Natarajan *et al*., 2014) in vitiligo patients. IFN-γ down-regulates the production of IL-1RN while up regulating the production of IL-1α, IL-1β, IL-6 and TNF-α (Danis *et al*., 1995). Due to the importance of IL-1RN in regulation of IL-1 family, it has been used in human clinical trials for various autoimmune and inflammatory disorders. Variations in *IL1RN* can modulate the effectiveness of IL-1 signaling and thereby predispose to disease condition. The present study will be the second report of *IL1RN* VNTR polymorphism in vitiligo worldwide with sufficient sample size. Therefore, our study demonstrates that pateints with vitiligo carrying a less common allele (allele 2, containing two repeats) was associated with increased *ILIRN* transcript levels, and are associated with progressive vitiligo. Moreover, the allele 2 carrier *IL1RN**3/2 was observed only in vitiligo patients while *IL1RN**5/2 was seen exclusively in controls.

The present study demonstrates the association of allele and genotype of *IL1RN* intron 2 VNTR (A2) polymorphism with active vitiligo patients and

increased *IL1RN* expression (allele 2 carriers), suggesting *IL1RN**A2 as a risk factor for progressive vitiligo in Gujarat population. Larger studies with different ethnicities are required to find out the impact of *IL1RN* VNTR polymorphism as a risk factor for developing vitiligo.

7A.5 REFERENCES

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7B.1 INTRODUCTION

Generalized vitiligo is an acquired, non-contagious disorder in which progressive, patchy loss of pigmentation from skin, overlying hair, and oral mucosa results from autoimmune loss of melanocytes from the involved areas (Boissy *et al.,* 2006). The clinical hallmark of vitiligo is loss of melanin pigment due to decreased number of functioning melanocytes or their complete absence in the lesional skin (Passeron and Ortonne, 2005). The inheritance of vitiligo does not follow the simple Mendelian pattern of an autosomal dominant or autosomal recessive or X- linked inheritance. Therefore, it has been proposed that vitiligo is a polygenic disease influenced by a set of recessive alleles occurring at several unlinked autosomal loci that collectively confer the vitiligo phenotype (Nath*et al.* 1994). IL-10 is a cytokine, which is expressed by cells of the innate and the adaptive immune system, including dendritic cells (DCs), macrophages, mast cells, natural killer (NK) cells, eosinophils, neutrophils, CD4 & CD8 T cells and B cells. IL-10 has potent anti-inflammatory properties, repressing the expression of cytokines such as TNF-α, IL-6 and IL-1. The balance between TNF- α and IL-10 is important for the maintenance of immune homeostasis (Shmarina *et al.,* 2001). It has been reported that low levels of IL-10 are governed by its gene promoter polymorphisms (Abanmi *et al.,* 2008). The *IL10* gene is located on the chromosome 1 and IL-10 production appears to be genetically encoded, which has been reported to cause 75% of the variation in IL-10 levels. The highly polymorphic promoter region of human IL-10 gene has been found to be associated with numerous autoimmune diseases (Asadullah, 2003). Hence, it becomes pertinent to investigate *IL10* promoter polymorphisms and its levels in vitiligo.

In view of the above, we aimed to examine the association of *IL10* promoter polymorphisms: -819 (C/T; rs1800871),-592 (C/A; rs1800872) and -1082 (G/A; rs1800896) in Gujarat vitiligo patients and controls using a case control approach. Furthermore, IL-10 transcript and plasma levels were monitored in PBMCs and plasma samples respectively. Also, genotype-phenotype correlation, haplotype analysis and linkage disequilibrium were performed that may provide a better understanding of the role of *IL10* polymorphisms in vitiligo pathogenesis.

7B.2 MATERIALS AND METHODS

7B.2.1 Study Subjects

The study group included 404 vitiligo patients where 294 patients were having generalized vitiligo (including acrofacial vitiligo and vitiligo universalis) and 110 were with localized vitiligo; 195 males and 209 females; 313 patients were with active vitiligo and 91 with stable vitiligo who were referred to S.S.G. Hospital, Vadodara, Gujarat, India (Table 1). The mean age (years ± SD) of patients and healthy controls were 29.41 ± 12.09 and 32.22 ± 11.75 respectively (Table 1).

7B.2.2 Genomic DNA Preparation For details refer Chapter VIIA.

Table 1: Demographic characteristics of Gujarat vitiligo patients and controls.

	Vitiligo Patients	**Controls**
	(n = 404)	(n = 481)
Average age (mean age ± SD)	32.22 ± 11.75 yr	29.41 ± 12.09 yr
Sex: Male	195 (48.26%)	237 (49.27%)
Female	209 (51.73%)	244 (50.72%)
Age of onset (mean age ± SD)	22.07 ± 12.12 yr	NA
Duration of disease (mean ± SD)	9.11 ± 6.64 yr	NA
Type of vitiligo		
Active vitiligo	313 (77.47%)	NA
Stable vitiligo	91 (22.52%)	NA
Localized vitiligo	110 (27.22%)	NA
Generalized vitiligo	294 (72.77%)	NA

Table 2:Primers and restriction enzymes used for expression and genotyping of *IL10* promoter -819 (C/T; rs1800871); -592 (C/A; rs1800872) and -1082 (G/A; rs1800896) SNPs.

Primer	Sequence (5' to 3')	Amplicon size (bp)	Annealing Temperature (°C) & Restriction Enzyme (Digested Products) nn
IL10 -819 C Allele specific FP:	ACCCTTGTACAGGTGATGAAC	270	57°C
IL10 -819 T Allele specific FP:	ACCCTTGTACAGGTGATGTAAT		
IL10 -819 RP :	CTGTCGACTTCTCCACCTT		
HGH FP:	CCTTCCCAACCATTCCCTTA	428	62°C
HGH RP:	TCACGGATTTCTGGTGTTTC		
IL10 -592 FP:	TGGTGAGCACTACCTGACTAGC	413	58 °C & *RsaI* (235 & 178 bp)
IL10 -592 RP:	CCTAGGTCACAGTGACGTGGAC		
IL10-1082 FP:	CTCGCCGCAA CCCAACTGGC	180	64°C & *MnlI* (150 & 30bp)
*IL*10 -1082 RP:	GGTCCCTTACTTTCGTCTTACCTATCC		
IL10 FP	ACCTGGGTTGCCAAGCCTT	189	62^0C
IL10 RP	CCACGGCCTTGCTCTTGTT		
GAPDH FP	CATCACCATCTTCCAGGAGCGAG	122	65^0C

GAPDH RP	CCTGCAAATGAGCCCCAGCCT		

7B.2.3 Genotyping of *IL10* promoter -819 (C/T; rs1800871) polymorphism

IL10 promoter C/T genotyping was done using amplification refractory mutation system-polymerase chain reaction (ARMS-PCR) method. DNA was amplified in two different PCR reactions with a generic antisense primer and one of the two allele specific sense primers (Table 2). To assess the success of PCR amplification in both the reactions, an internal control of 428 bp was amplified using a pair of primers designed from the nucleotide sequence of the human growth hormone (*HGH*) (Table 2). The reaction mixture of the total volume of 15 µL included 3 µL (100 ng) of genomic DNA, 4.9 µL nuclease-free H_2O, 1.5 µL 10x PCR buffer, 1.5 µL 10 mM dNTPs , 1 µL of 10 µM allele-specific and common primers (MWG Biotech, India), 1 µL of 10 µM control primers (*HGH*) and 0.1 µL (10U/µL) Taq Polymerase. Amplification was performed using a Mastercycler Gradient PCR according to the protocol: 95°C for 10 minute followed by 15 cycles of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, then 24 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The amplified products were separated by electrophoresis on 3.5% agarose gel and stained with ethidium bromide. The gel was visualized under a UV transilluminator with a 100 base pair DNA ladder and photographed. Two amplicons were available for each sample (one each specific for T or C allele of the *IL10* polymorphism).

7B.2.4 Genotyping of *IL10* promoter -592 (C/A; rs1800872) polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype C/A (rs1800872) polymorphism of *IL10* gene (Figure 1). The primers used for genotyping are mentioned in Table 2.

7B.2.5 Genotyping of *IL10* promoter -1082 (G/A; rs1800896) polymorphism

IL10 promoter -1082 (G/A; rs1800896) genotyping was done using PCR-RFLP method. Similar to above details regarding primers, annealing temperature, amplicon size, restriction enzyme etc. used for genotyping are mentioned in Table 2.

7B.2.6 Estimation of plasma IL-10 levels by enzyme-linked immunosorbent assay

Plasma levels of IL-10 in patients with vitiligo and controls were measured by enzyme-linked immunosorbent assay (ELISA) using the eBioscience Human IL-10 ELISA kit (Human IL-10 ELISA Ready-SET-Go!®, eBioscience; Catalog Number: 88-7106) as per the manufacturer's protocol.

7B.2.7 Determination of *IL10* mRNA expression

The transcripts levels of *IL10* in whole blood of vitiligo patients and controls were estimated using gene specific primers (Eurofins, Bangalore, India) by SYBR green method, real-time PCR as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Details are described in Chapter III.

7B.2.8 Data analysis

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for *IL10* promoter polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-square analysis. The distribution of the genotypes and allele frequencies of polymorphisms in different groups, considering the major genotype / allele as a reference group and were compared using chi-square test with 2×2 contingency tables using Prism 4 software (Graph Pad Software, USA, 2003). Odds ratio (OR) with 95% confidence interval (CI) for disease susceptibility was also calculated. Bonferroni's correction was applied for multiple testing of polymorphisms. Haplotype analysis was carried out using http://analysis.bio-x.cn/myAnalysis.php (Shi and He, 2005). The LD coefficients D' and r^2-values for the pair of most common alleles at each site were estimated using the Haploviewprograme version 4.1(Barrett *et al*., 2005). IL-10 mRNA and plasma level expression were compared between different groups and genotype/ haplotype-phenotype correlation was analyzed and plotted by non-parametric unpaired t-test using Prism 4 software to determine the statistical significance of data. *p*-values less than 0.05 were considered as statistically significant. Fold change in mRNA was calculated according to $2^{-\Delta\Delta Ct}$ method. Statistical power of detection of association with the disease at 0.05 level of significance was determined by using G* Power software.

7B.3 RESULTS

7B.3.1 Analysis of association between *IL10* promoter -819 (C/T; rs1800871) polymorphism and susceptibility to vitiligo:

Genotyping of *IL10* promoter -819 (C/T; rs1800871) SNP was performed by ARMS PCR method in 481 controls and 402 vitiligo patients. HGH was used as a PCR positive control. Three genotypes were identified in both patients and controls: CC, CT and TT for *IL10* -819 C/T promoter polymorphism (Figure.1). The genotype frequencies differed significantly between controls and patients population (p<0.0001). Also, significant difference in allele frequencies of T allele was observed in controls and vitiligo patients (p<0.0001) (Table 3). Hence, there is significant association of the *IL10* promoter -819 (C/T; rs1800871) with vitiligo in Gujarat population. The distribution of genotype frequencies for *IL10*-819 C/T SNP was not consistent with Hardy-Weinberg expectations in both patient and control groups (p=0.0468 and p<0.0001).

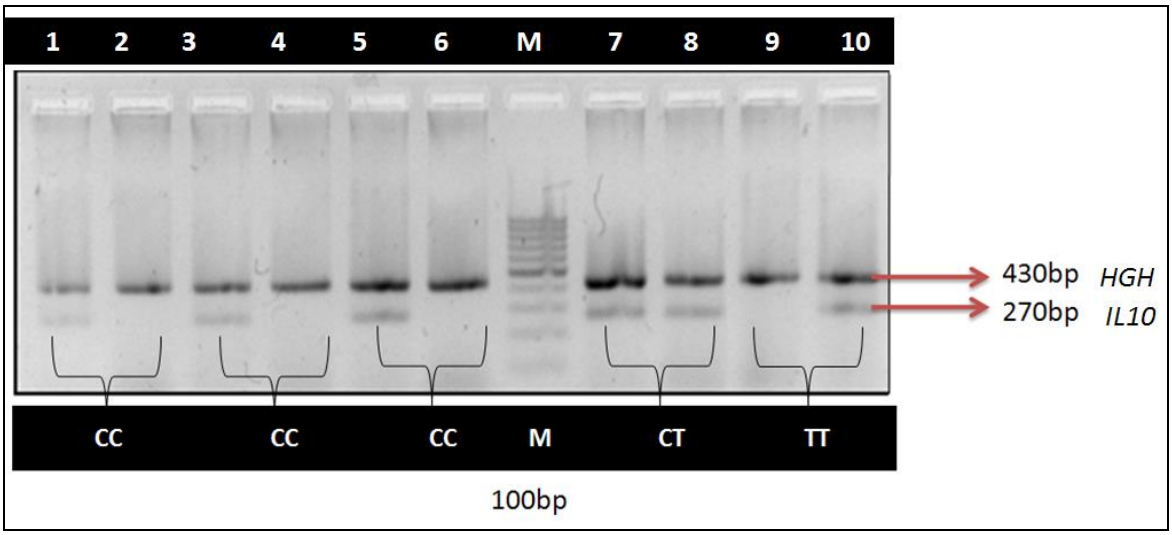


Figure 1: Representative gel image for the genotyping of *IL10*-819 C/T SNP using ARMS-PCR. Lanes 1-6 represent CC genotype, lanes 7, 8 represent CT genotype and lanes 9, 10 represent TT genotype. HGH: PCR positive control.

Table 3: Distribution of *IL10* -819 C/T (rs1800871) polymorphism in Gujarat vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 402)	(n = 481)			
IL10	CC	171 (42.54)	143 (29.73)	<0.0001[a]	0.0468	0.4880
-819 C/T (rs	CT	168 (41.79)	155 (32.22)		(P)	(0.4029 to
1800871)	TT	63 (15.67)	183 (38.05)		<0.0001	0.5910)
	Allele				(C)	
	C	510 (0.63)	441 (0.46)	<0.0001[b]		
	T	294 (0.37)	521 (0.54)			

[a]Vitiligo Patients vs. Controls using chi-square test with 3 × 2 contingency table,

[b]Vitiligo Patients vs. Controls using chi-square test with 2 × 2 contingency table.

7B.3.2 Analysis of association between *IL10* promoter -592 (C/A; rs1800872) polymorphism and susceptibility to vitiligo:

We estimated the genotype frequency at this locus in 406 controls and 391 vitiligo patients. Three genotypes were identified in both patients and controls: CC, CA and AA for *IL10* -592 C/A promoter polymorphism (Figure. 2). *RsaI* was used for amplicon digestion and it cleaves if A allele is present in the amplicon and undigested product remains if C allele is present. Non-significant difference in genotype as well as allele frequencies of C/A allele was observed in controls and vitiligo patients (p=0.5796 and p=0.7096) (Table 4). Hence, there is no significant association of the *IL10* promoter -592 (C/A; rs1800872) with vitiligo. The distribution of genotype frequencies for *IL10* promoter -592 (C/T; rs1800872) were consistent with Hardy-Weinberg expectations in both patient and control groups (p=0.1861 and p=0.9594).

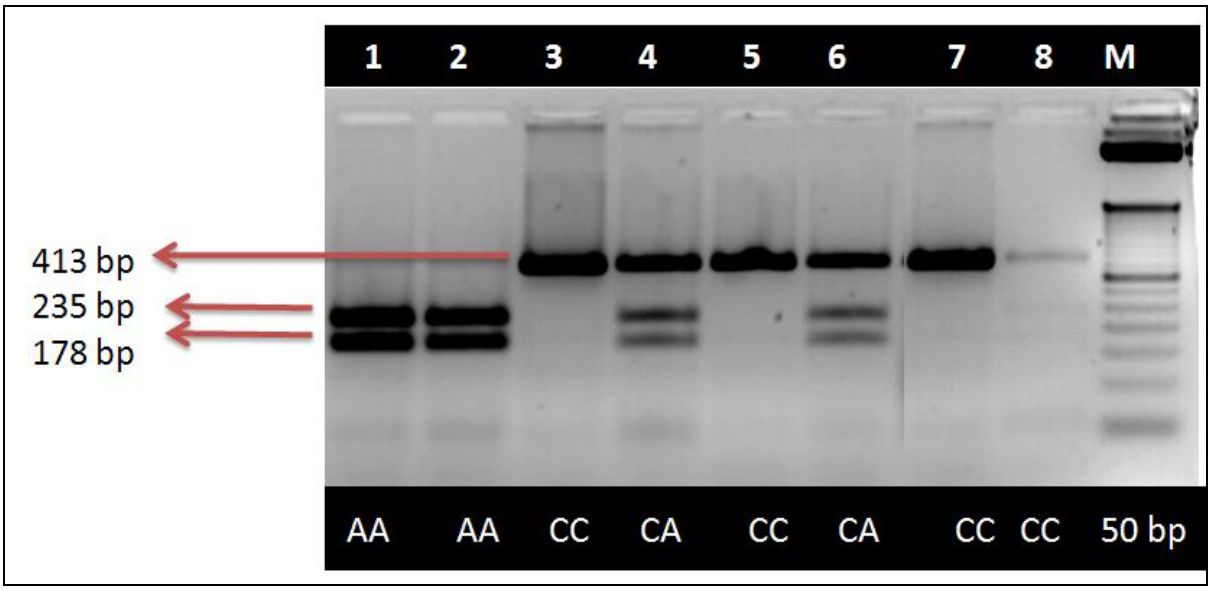


Figure 2: Representative gel image for the genotyping of *IL10* -592 C/A SNP using PCR-RFLP. Lanes 1and 2 represent AA genotype, Lanes 4 and 6 represent CA genotype and lanes 3,5,7 and 8 represent CC genotype.

Table 4: Distribution of *IL10* -592 C/A (rs1800872) polymorphism in Gujarat vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 391)	(n = 406)			
IL10	GG	182 (46.55)	178 (43.84)	0.5796[a]	0.1861	0.9612
-592 C/A (rs	GA	161 (41.18)	182 (44.83)		(P)	0.7803 to
1800872)	AA	48 (12.28)	46 (11.33)		0.9594	1.184
	Allele				(C)	
	G	525 (0.67)	538(0.66)	0.7096[b]		
	A	257 (0.33)	274 (0.34)			

[a]Vitiligo Patients vs. Controls using chi-square test with 3 × 2 contingency table,

[b] Vitiligo Patients vs. Controls using chi-square test with 2 × 2 contingency table.

7B.3.3 Analysis of association between *IL10* promoter -1082 (G/A; rs1800896) polymorphism and susceptibility to vitiligo:

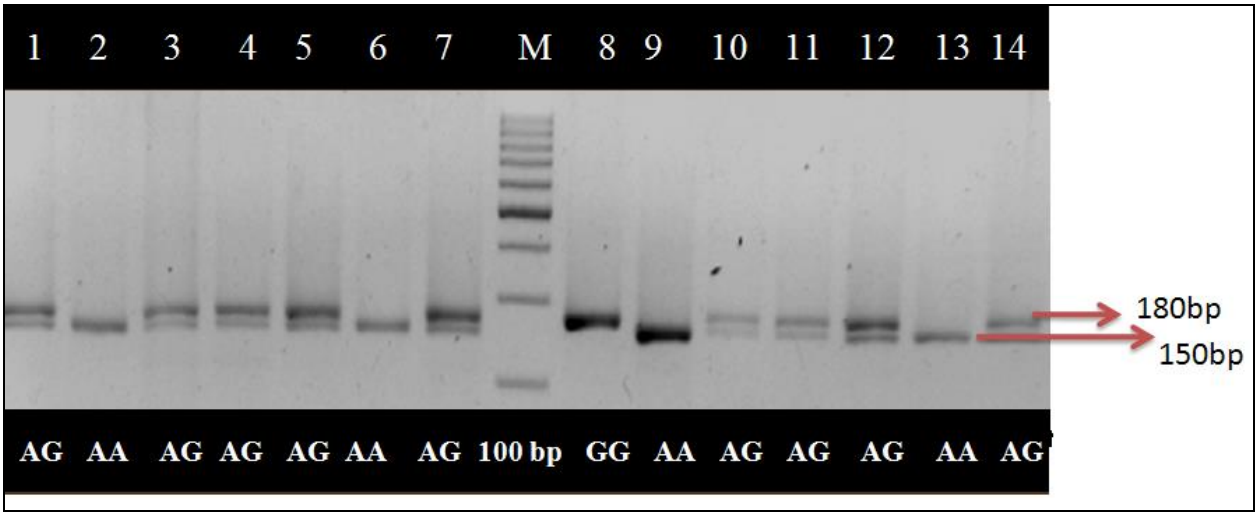


Figure 3: Representative gel image for the genotyping of *IL10* -1082 G/A using PCR-RFLP. Lanes 2, 6, 9 and 13 represent AA genotype, lanes 1, 3, 4, 5, 7, 10, 11, 12 & 14 represent AG genotype and lane 8 represents GG genotype.

We have estimated the genotype frequency at this locus in 446 controls and 404 vitiligo patients. Three genotypes were identified in both patients and controls: GG, GA and AA for *IL10* -1082 G/A promoter polymorphism as illustrated in representative figure (Figure. 3). *MnlI* restriction enzyme was used for amplicon digestion. *MnlI* cleaves if G allele is present in the amplicon and undigested product remains if 'A'allele is present. Significant difference in allele frequency of 'A' allele was observed in controls and vitiligo patients (p=0.0311) (Table 5). However, the genotype frequency did not differ significantly between patients and controls (p=0.1445). Also, the distribution of genotype frequencies for *IL10* promoter -1082 G/A SNP was not consistent with Hardy-Weinberg expectations in both patient and control population (p<0.0001 and p<0.0001 respectively).

Table 5: Distribution of *IL10* -1082 G/A (rs1800896) polymorphism in Gujarat vitiligo patients and controls.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for Association	*p* for HWE	Odds ratio (95% CI)
	Genotype	(n = 404)	(n = 446)			
IL10	GG	147 (36.39)	134 (30.04)	0.1445[a]	<0.0001	0.8107
-1082 G/A (rs	GA	106 (26.24)	127 (28.48)		(P)	(0.6697 to
1800896)	AA	151 (37.38)	185 (41.48)		<0.0001	0.9813)
	Allele				(C)	
	G	400 (0.50)	395 (0.44)	0.0311[b]		
	A	408 (0.50)	497 (0.56)			

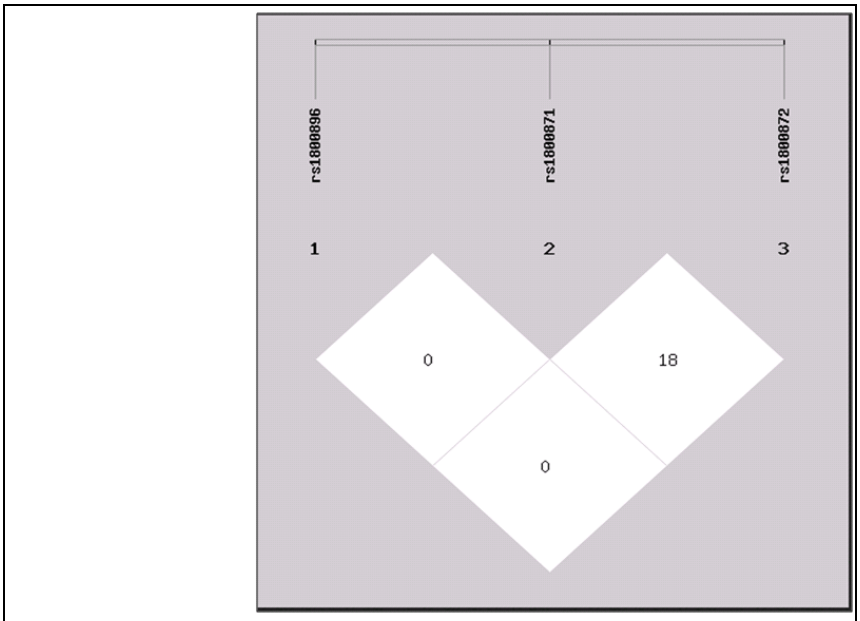
[a]Vitiligo Patients vs. Controls using chi-square test with 3 × 2 contingency table,

[b]Vitiligo Patients vs. Controls using chi-square test with 2 × 2 contingency table.

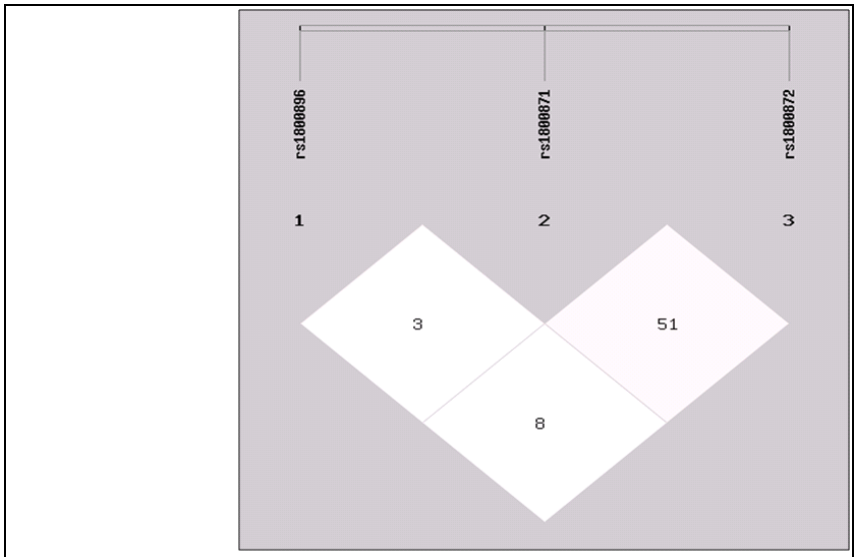
Table 6: Haplotype analysis of *IL10* promoter -592 (C/A; rs 1800872), -819 (C/T; rs 1800871) and -1082 (G/A; rs 1800896) SNPs in Gujarat vitiligo patients and controls.

Haplotypes (*IL 10*)	Patients Freq in% N=534	Control Freq in % N=592	P for association	P global	Odds ratio 95% CI
A C A*	21.34(0.040)	30.73(0.052)	0.340182		0.760 [0.432~1.338]
A C C*	168.98(0.316)	129.25(0.218)	0.000196		1.657 [1.269~2.165]
A T A*	49.95(0.094)	82.63(0.140)	0.016703		0.636 [0.438~0.923]
A T C*	28.73(0.054)	94.39(0.159)	$1.45e^{-008}$	$5.15e^{-011}$	0.300 [0.194~0.463]
G C A*	28.09(0.053)	26.56(0.045)	0.546307		1.182 [0.686~2.036]
G C C*	133.59(0.250)	114.46(0.193)	0.021619		1.392 [1.049~1.847]
G T A*	79.62(0.149)	61.08(0.103)	0.019947		1.523 [1.067~2.175]
G T C*	23.70(0.044)	52.90(0.089)	0.002765		0.473 [0.287~0.780]

CI represents Confidence Interval, (Frequency <0.03 in both control & case has been dropped and was ignored in analysis).



r2: rs1800871 rs1800872
rs1800896 0.001 0.005
rs1800871 - 0.186



r2: rs1800871 rs1800872
rs1800896 0.035 0.089
rs1800871 - 0.516

Figure 4: Haplotype analysis of *IL10* promoter -592 (C/A; rs 1800872), -819 (C/T; rs 1800871) and -1082 (G/A; rs 1800896) SNPs

The two polymorphisms investigated were in moderate LD association (*IL -819 C/T: IL 10 – 592 C/A;* D'= 0.516, r^2 = 0.186). The haplotype ATC (p=1.45 e^{-008}) was found to be less frequent in vitiligo patients, suggesting its crucial role in disease protection (Table 6) whereas, ACC (p=0.000196), GCC (p=0.021619), GTA (p= 0.019947) haplotypes were found to be significantly associated with patients, suggesting their importance in vitiligo susceptibility. However, ACA (p=0.3401) and GCA (p=0.546307) haplotypes were not found to be associated with vitiligo.

7B.3.4 Relative gene expression of *IL10* in PBMCs of vitiligo patients:

The *IL10* transcript levels were compared in 80 PBMCs of vitiligo patients and 88 age matched unaffected controls after normalization with *GAPDH* transcript levels. The *IL10* transcript levels in vitiligo patients were significantly higher when compared to controls (p = 0.0150) as suggested by mean ΔCp values (Figure 5A). The $2^{\Delta\Delta Cp}$ analysis showed approximately 2.42 fold higher expression of *IL10* transcript in patients as compared to controls (Figure 5B).

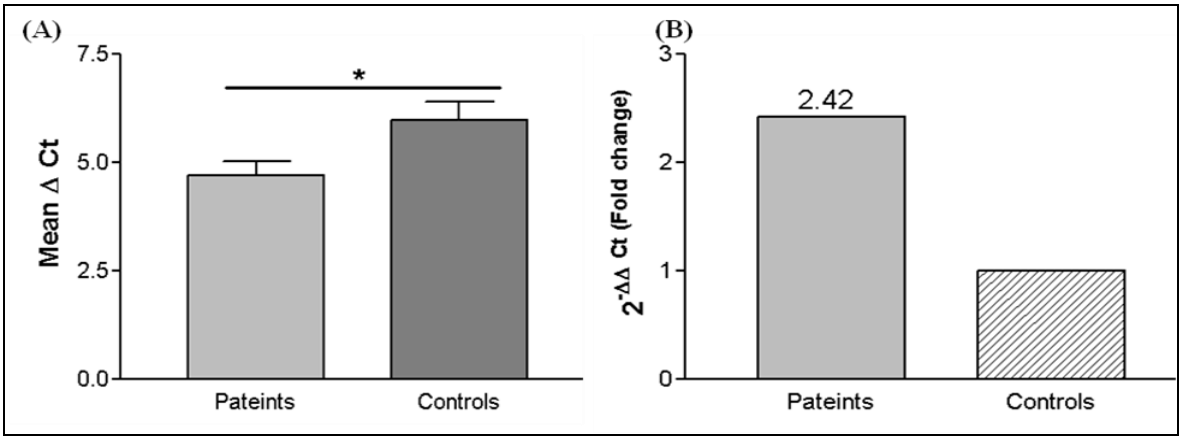


Figure 5: Relative gene expression of *IL10* in PBMCs of controls and vitiligo patients: (A) Expression of *IL10* transcripts in 88 controls and 80 vitiligo patients as suggested by mean ΔCp. Patients showed significantly increased *IL10* expression as compared to controls (p=0.0150). (B) Expression fold change of *IL10* transcripts in 80 vitiligo patients against 88 controls showed 2.42-fold change as determined by $2^{-\Delta\Delta Cp}$ method.

7B.3.5 Estimation of plasma levels of IL10 vitiligo patients and controls:

Plasma IL10 levels in 92 vitiligo patients and 72 controls were estimated by ELISA. IL10 levels were found to be significantly increased in patients with vitiligo when compared with controls [p=0.0041 Figure. 6; Mean ± SEM of patients 4.150 ± 0.2485 (N=92) and controls 3.214 ± 0.1783 (N=72)]. These results are in accordance with transcript analysis where we observed 2.42 fold increased expression of *IL10* in patients as compared to controls.

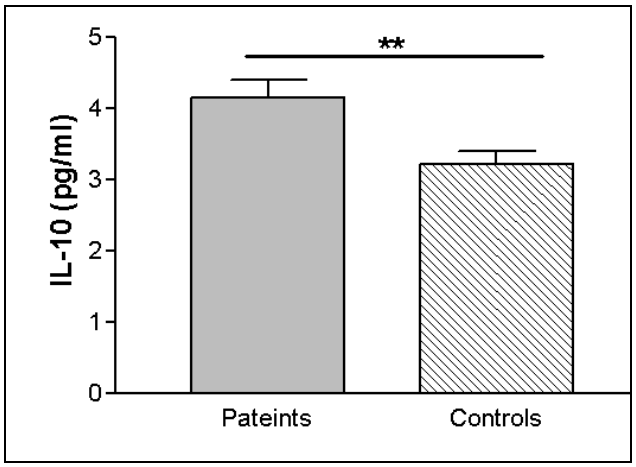


Figure 6: Plasma IL10 levels in controls and vitiligo patients: Comparison of plasma IL10 levels (pg/ml) in 72 controls and 92 vitiligo patients showed significantly increased IL10 levels in vitiligo patients (p=0.0041), as determined by ELISA.

7B.3.6 Genotype - phenotype correlation analysis:

In order to observe how SNPs alone and in combination are affecting the transcript and protein levels of IL10 in studied subjects, we carried out possible genotype - phenotype correlation with respect to different genotypes and haplotypes observed in the present study. Interestingly, among nine genotypes of three promoter polymorphisms of *IL10*, we observed *IL10*-819 C/C genotype was exhibiting increased expression of *IL10* as compared to TT genotype (p=0.0121). However, the ELISA results for the same showed no difference in the IL10 levels (p=0.9858). Moreover, there was no difference in the transcript as well as protein levels of IL10 for different observed genotypes (Figure. 7).

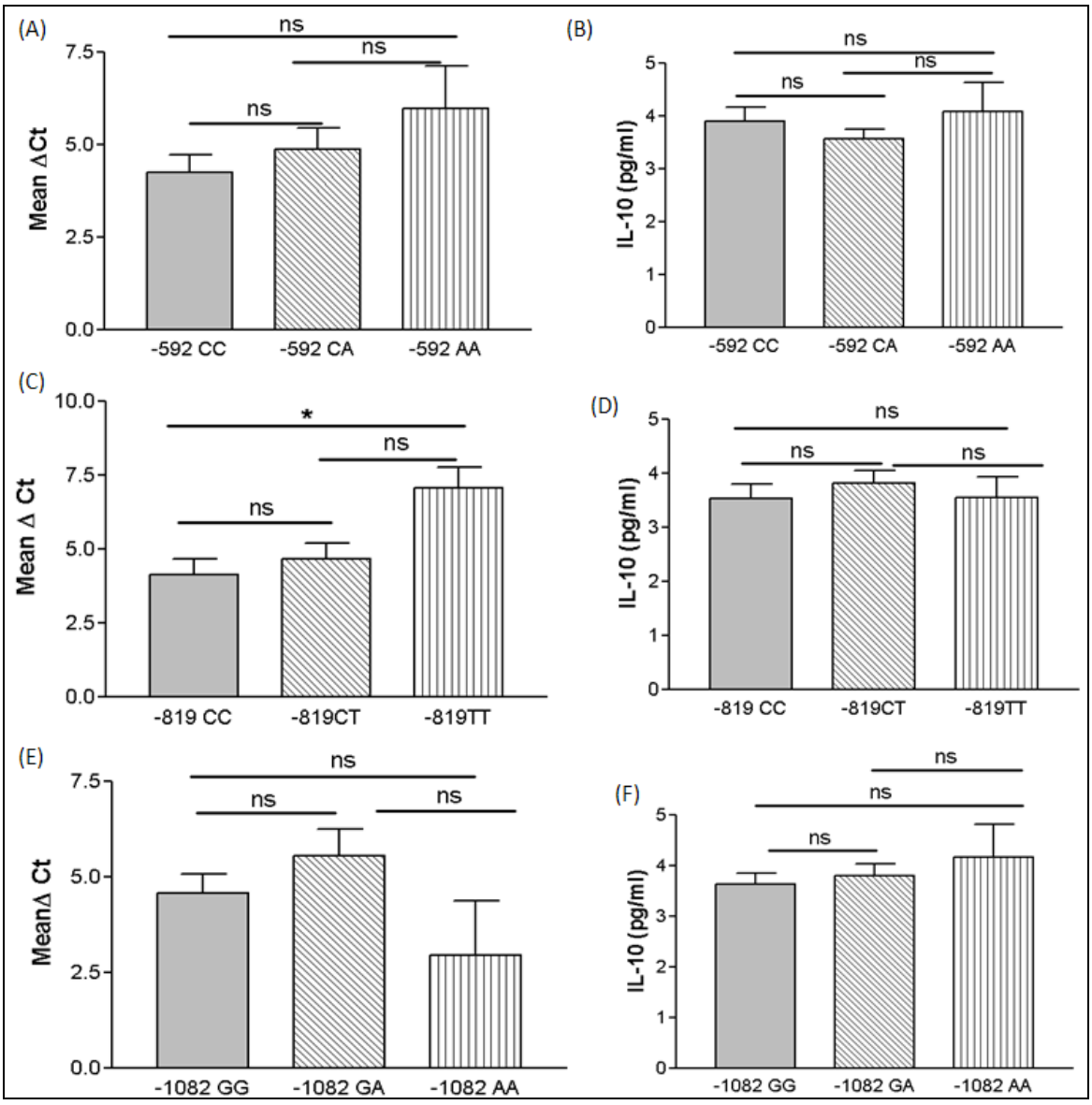


Figure 7: Genotype-phenotype correlation of -592 C/A, -819 C/T and -1082 G/A promoter polymorphisms of *IL10* transcript and plasma levels in controls and vitiligo patients: (A) Relative mRNA expression of *IL10* with respect to -592 C/A genotypes in vitiligo patients. None of the three genotypes CC vs CA (p=0.4142); CC vs AA (p=0.1535) and CA vs AA (p=0.4184) showed significant effect on *IL10* transcript levels as suggested by Mean ΔCp. (B) Similarly, the plasma levels among different genotypes CC vs CA (p=0.3013); CC vs AA (p=0.7514) and CA vs AA (p=0.2456) of -592 C/A also showed no significant difference (C) Relative mRNA expression of *IL10* with respect to -819 C/T genotypes in vitiligo patients. *IL10*-819 C/C genotype was exhibiting increased expression of *IL10* as compared to TT genotype (p=0.0121).

However, other genotypes CC vs CT (p=0.5045) and CT vs TT (p=0.0922) showed no difference for *IL10* transcript levels as suggested by Mean ΔCp. (D) Similarly, the plasma levels among different genotypes CC vs CT (p=0.4277); CC vs TT (p=0.9858) and CT vs TT (p=0.7016) of -819 C/T showed no significant effect on IL10 plasma levels. (E) Relative mRNA expression of *IL10* with respect to -1082 G/A genotypes in vitiligo patients. None of the three genotypes GG vs GA (p=0.2501); GG vs AA (p=0.2296) and GA vs AA (p=0.1012) showed significant effect on *IL10* transcript levels as suggested by Mean ΔCp. (F) Similarly, the plasma levels among different genotypes GG vs GA (p=0.5926); GG vs AA (p=0.3577) and GA vs AA (p=0.7951) of -1082 G/A showed no significant effect on IL10 plasma levels.

7B.3.7 Effect of IL10 expression on disease progression:

The levels of IL10 were analyzed with respect to progression of disease in 58 patients with active vitiligo (AV) and 22 patients with stable vitiligo (SV). As suggested by mean ΔCp and ELISA results analysis, there was no difference in transcript as well as plasma levels of IL10 p=0.8560 and p=0.0665 respectively between AV and SV (Figure 8).

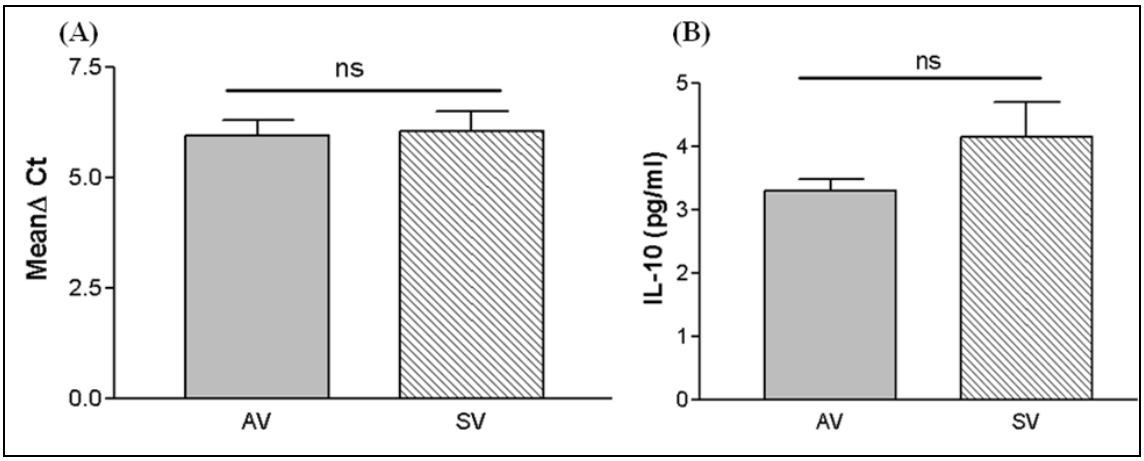


Figure 8: Effect of IL10 expression on disease progression showed no difference in IL10 transcript as well as protein levels between AV and SV (p=0.8560 and p=0.0665 respectively).

7B.3.8 Gender based analysis of IL10 expression:

Expression of IL10 was analyzed with respect to gender differences in 43 male patients and 37 female patients with vitiligo. The female patients showed significant increase in *IL10* mRNA levels as compared to male vitiligo patients (p = 0.0216, Figure 9A). Also,

the plasma levels of IL10 were significantly increased in female vitiligo patients compared to male vitiligo patients (p=0.0454; Figure 9B).

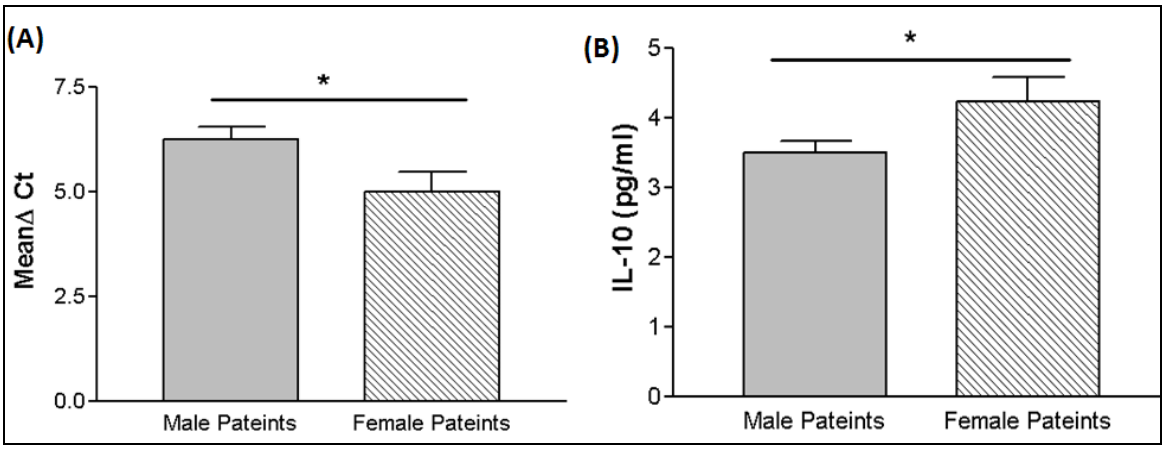


Figure 9: **Effect of IL10 expression on gender biasness showed significant difference in IL10 (A) transcript as well as (B) protein levels between male and female vitiligo patients p=0.0216 and p=0.0454 respectively**

7B.3.9 Effect of IL10 expression in different clinical types of vitiligo:

The levels of *IL10* were analyzed with respect to progression of disease in 50 patients with generalized vitiligo (GV) and 30 patients with localized vitiligo (LV). As suggested by mean ΔCp and ELISA, there was no difference in transcript as well as plasma levels of IL10 between GV and LV (p=0.8680 and p=0.6747) respectively (Figure 10).

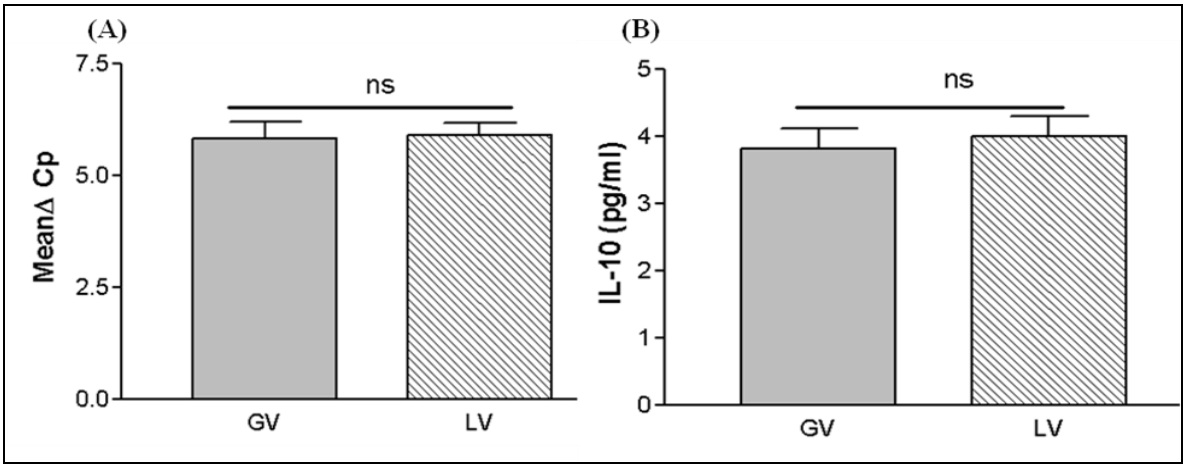


Figure 10: **Effect of IL10 expression on GV and LV showed no difference in IL10 (A) transcript as well as (B) protein levels between GV and LV (p=0.8680 and p=0.6747) respectively.**

7B.3.10 Haplotype based analysis:

Further, the transcript as well as plasma levels of IL10 were analyzed with respect to haplotypes generated from investigation of three promoter polymorphisms of *IL10*. However, there was no significant difference in transcript as well as plasma levels of IL10 among various haplotypes was observed (p>0.05; Figure 11).

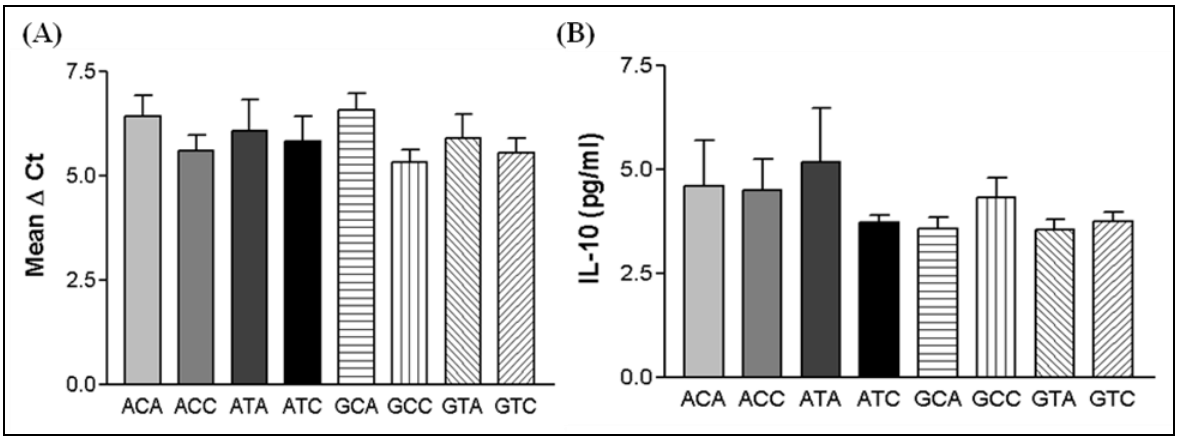


Figure 11: **Correlation of *IL10* transcript levels with -819 (C/T; rs1800871), -592 (C/A; rs1800872) and -1082 (G/A; rs1800896) promoter polymorphisms with different haplotypes showed no difference in the (A) transcript as well as (B) plasma levels of IL10 (p>0.05 for all comparisons).**

7B.4 DISCUSSION

Major hypotheses which could explain pathogenesis of vitiligo are oxidative stress, autoimmune and genetic hypotheses. IL10 acts as an anti-inflammatory cytokine wherein Zhao *et al.,* (year) and Ala *et al.,* (2015) showed significant decrease in the expression of IL10 in vitiligo. In addition to this, Taher *et al.,* (2009) have found increased levels of IL10 after application of topical tacrolimus treatment to patients with vitiligo. On the contrary, Grimes *et al.,* (2004) and Aydingoz *et al.,* (2014) have reported increased levels of IL10 in skin and serum samples of vitiligo patients respectively; and after topical application of tacrolimus, Grimes *et al.,* (2004) didn't find any significant difference in the IL10 levels. Polymorphism studies of *IL10* -1082, -592 and -819 promoter SNPs revealed association of *IL10* polymorphisms with vitiligo susceptibility (Aydingoz *et al.,* 2014, Abanmi *et al.,* 2008). Previous studies as mentioned in Table 7 have shown

significant difference in the expression of IL10 in vitiligo patients as compared to controls.

Table 7: **Reports on IL10 expression in vitiligo patients:**

Cytokine	Reference	No. of vitiligo patients & Sample type	Levels of IL 10 (Technique used)
IL10	Grimes *et al.*, 2004	19, tissue	significantly increased (real time -PCR)
	Basak*et al.*, 2009	40, serum	non-significant (ELISA)
	Taher *et al.*, 2009	20, tissue	significant increase post treatment (ELISA)
	Ala *et al.*, 2010	280, serum	significantly decreased (ELISA)
	Zhao *et al.*, 2010	20, PBMC	significantly decreased (real time -PCR)
	Tembhre*et al.*, 2012	80 (untreated), 25 (narrow band ultraviolet B treated vitiligo), serum	significantly increased (ELISA)

In the present study, we have investigated three polymorphisms in promoter region of *IL10* gene for their role in susceptibility to vitiligo, by using an approach of case-control study. Our results showed that genotype and allele frequencies of *IL10* -819 C/T (p<0.0001; p<0.0001 respectively) were significantly different between vitiligo patients and unaffected controls, suggesting the significant association of -819T allele with vitiligo susceptibility. Additionally, the allele frequency of -1082 G/A differed significantly between vitiligo patients and controls (p=0.0311). However, the genotype

and allele frequencies for *IL10* -592C/A (p= 0.5796; p= 0.7096 respectively) and genotype frequencies of *IL10* -1082G/A (p = 0.1445) did not differ significantly between vitiligo patients and controls, suggesting the non-association of these SNPs with vitiligo susceptibility. The two polymorphisms investigated were in moderate LD association (*IL -819 C/T: IL 10 – 592 C/A;* D'= 0.516, r^2 = 0.186). The haplotype ATC (p=1.45 e^{-008}) was found to be less frequent in vitiligo patients, suggesting its crucial role in disease protection. Whereas, ACC (p=0.000196), GCC (p=0.021619), GTA (p= 0.019947) haplotypes were more found to be significantly associated with patients, suggesting their importance in vitiligo susceptibility. However, ACA (p=0.3401) and GCA (p=0.546307) haplotypes were not found to be associated with vitiligo.

Additionally, as mentioned in chapter III, we have estimated the transcript levels of *IL10* in skin samples from vitiligo patients and controls (n=12) and we observed siginificant decrease in *IL10* levels (p=0.0357) in lesional skin compared to control skin. However, this study further needs to be carried out in different populations with higher sample size. Moreover, we have estimated the transcript levels of *IL10* in PBMCs of vitiligo patients and controls and found significant increase in *IL10* transcript levels in vitiligo patients and gender biasness of females as compared to male vitiligo patients is also observed. However, there was no significant difference in *IL10* transcript levels with respect to disease progression (Av vs SV) and type of vitiligo (GV vs LV) in our studies. Also, to monitor the effect of polymorphism on the circulatory levels of IL10, we analyzed plasma levels of IL10 in vitiligo patients and controls and found significant increase in IL10 levels in vitiligo patients with gender biasness of females as compared to male vitiligo patients. However, there was no significant difference in IL10 levels with respect to disease progression (AVvs SV) and type of vitiligo (GV vs LV) in our studies.

Furthermore, in our genotype-phenotype corelation studies among nine genotypes of three promoter polymorphisms of *IL10*, we observed *IL10*-819 C/C genotype was exhibiting increased expression of IL10 as compared to TT genotype (p=0.0121). However, the ELISA results for the same showed no significant difference in the IL10 levels (p=0.9858). Moreover, there was no significant difference in the transcript as well as protein levels of IL10 when analyzed for different observed genotypes and haplotypes.

The present study supports the autoimmune hypothesis of vitiligo pathogenesis with significant association of *IL10* -819 C/T polymorphism in vitiligo patients thus, suggesting the crucial role of *IL10* in vitiligo pathogenesis. Also, the increased levels of transcript as well as plasma levels of lL10 in vitiligo patients indicate its potential role in vitiligo pathogenesis. The present study might pave the pathway for understanding the higher prevalence of vitiligo in Gujarat population.

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7C.1 INTRODUCTION

Vitiligo is a progressive depigmenting disorder affecting 0.45–2.16% of the population worldwide (Kruger *et al.*, 2012). There is an imperative need for investigating the genetic predisposition of candidate genes in vitiligo as it cannot be explained by simple Mendelian genetics; however, it is characterized by incomplete penetrance, multiple susceptibility loci, and genetic heterogeneity (Zhang *et al.*, 2005).

We have earlier reported that 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have at least one first-degree relative affected (Shajil *et al.*, 2006 a). The etiology of vitiligo remains obscure despite being in focused debate for more than six decades (Le Poole *et al.*, 1993; Taieb, 2000; Shajil *et al.*, 2006 b), hence, it is important to unravel the underlying pathomechanisms of vitiligo.

In addition to the cosmetic and psychosocial implications, there are evidences of association of vitiligo with other autoimmune diseases,, such as systemic lupus erythematosus, hypothyroidism, diabetes etc. (Laddha *et al.*, 2014, Deretzi *et al.*, 2010; Kocer *et al.*, 2009; Nikiforidis *et al.*, 1993; Ramagopalan *et al.*, 2007; Rashtak and Pittelkow, 2008; Sabate *et al.*, 1999; Varoglu *et al.*, 2010; Yacubian *et al.*, 2001).

One of the emerging concepts favoured by autoimmune hypothesis is the role of epidermal cytokine imbalance in vitiligo pathogenesis. Cytokine imbalance between pro and anti-inflammatory cytokines is well reported in vitiligo. Complex cytokine network exists in skin cells which regulates melanocyte activity by paracrine mechanisms (Imokawa *et al.*, 2004). Keratinocytes surround melanocytes and form a structural and functional unit called the epidermal melanin unit (Jimbow *et al.*, 1976). Keratinocytes also secretes cytokines, such as Interleukin (IL)-6 and tumor necrosis factor (TNF)-α, which act as paracrine inhibitors of melanocyte growth and proliferation (Swope *et al.*, 1991). Altered levels of keratinocyte-derived mediators have been recently described in vitiligo epidermis (Moretti *et al.*, 2002 a,b ; Kitamura *et al.*, 2004; Lee *et al.*, 2005), suggesting a role of epidermal cytokines in vitiligo pathogenesis.

IL6 is a pleiotropic Th2 cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation etc. IL-6 induces and enhances Intercellular

Adhesion Molecule (ICAM)-1 expression on melanocytes, which promotes melanaocyte-leukocyte attachment (Yohn *et al*., 1990). IL6 stimulates the inflammatory and auto-immune processes in many diseases such as diabetes, atherosclerosis, depression, alzheimer's disease, systemic lupus erythematosus, multiple myeloma, prostate cancer, behçet's disease, and rheumatoid arthritis (RA) (Dubiński and Zdrojewicz 2007). Blockade of IL-6 activity with a soluble anti-IL-6 molecule 'tocilizumab' has been found to be promising for treatment of various autoimmune disorders like RA (Tanaka *et al*., 2014). Increased levels of *IL6* have been reported in vitiligo patients (Tu *et al*., 2003; Singh *et al*., 2012; Pichler *et al*., 2009). Human IL6 gene has been mapped to chromosome 7p21. There are two biallelic polymorphisms in the promoter region of *IL6* that may be associated with differences in IL-6 production: −174G/C (rs1800795) and −572G/C (rs1800796). These single nucleotide polymorphisms consist of a change from guanine (G) to cytosine (C) at positions −174 and −572 in the promoter region. Also, several allelic variants have been identified in the promoter region of *IL6*, responsible for its gene regulation at transcriptional level (Fishman *et al*., 1998; Terry *et al*., 2000). Polymorphisms solely do not exist in isolation, and it may be the combination of base changes at different loci, i.e. the haplotype, that may be influencing the function. Therefore, the present study aims to analyze the association of two promoter polymorphisms -174 G/C (rs1800795), -572 G/C (rs1800796) of *IL6* as well as its transcript levels in peripheral blood mononuclear cells (PBMCs) using a case-control approach.

7C.1 MATERIALS AND METHODS

7C.2.1 Study Subjects

The study group included 322 vitiligo patients (181 males and 141 females) wherein 239 patients were having generalized vitiligo (including acrofacial vitiligo and vitiligo universalis) and 83 were with localized vitiligo, who were referred to S.S.G. Hospital, Vadodara, Gujarat, India (Table 1). The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. A total of 343, ethnically age and sex

matched unaffected individuals (186 males and 157 females) were included in the study (Table 1).

Table 1: Demographic characteristics of vitiligo patients and unaffected controls.

	Vitiligo Patients		**Controls**
	(n = 322)		(n = 343)
Average age (mean age ± SD)	38.23 ± 19.15 yr		29.41 ± 12.16 yr
Sex: Male	181 (56.21%)		186 (54.2%)
Female	141 (43.7%)		157 (45.77%)
Age of onset (mean age ± SD)	31.70±19.51 yr		NA
Duration of disease (mean ± SD)	7.723±9.154 yr		NA
Type of vitiligo			
Generalized	239 (74.22%)		NA
Localized	83 (25.77%)		NA
Active vitiligo	235 (72.98%)		NA
Stable vitiligo	87 (27.01%)		NA

7C.2.2 Genomic DNA Preparation

As discussed in Chapter VII A.

Table 2: Primers used for gene expression and polymorphism analysis.

Gene Primer	Sequence	Annealing Temperature	Restriction Enzyme (Digested Products)
IL6 -174 F	5'-TCAAGACATGCCAAAGTGCTGAG-3'	62°C	Nsp1 (129&59 bp=188bp)
IL6 -174 R	5'- AGCGGGTGGGGCTGATTGG-3'		
IL6 -572 F	5'-GGAGACGCCTTGAAGTAACTGC -3'	58°C	Aci1 (366 & 76 bp=442bp)
IL6 -572 R	5'-GTTTCCTCTGACTCCATCGCAG-3'		
IL6 F	5'-AAATTCGGTACATCCTCGACGGCA-3'	64°C	
IL6 R	5'-AGTGCCTCTTTGCTGCTTTCACAC-3'		-
GAPDH F	5'- CATCACCATCTTCCAGGAGCGAG - 3'	65°C	-
GAPDH R	5'- CCTGCAAATGAGCCCCAGCCT- 3'		

7C.2.3 Genotyping of *IL6* promoter -174 G/C (rs1800795) polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype G/C (rs1800795) polymorphism of *IL6* gene (Figure 1 A). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic- DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a Mastercycler gradient (Eppendorf, Germany) according to the

protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2% agarose gel stained with ethidium bromide. Restriction enzyme NspI (Fermentas, Lithuania) was used for digestion of PCR amplicons of *IL6* for genotyping of G/C (rs1800795) SNP (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 bp DNA ladder (Invitrogen) were resolved on 3.5% agarose stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

7C.2.4 Genotyping of *IL6* promoter -572 G/C (rs1800796) polymorphism

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to genotype G/C (rs1800796) polymorphism of *IL6* gene (Figure 1B). The primers used for genotyping are mentioned in Table 2. The reaction mixture of the total volume of 20 µL included 5 µL (100 ng) of genomic DNA, 10 µL nuclease-free H_2O, 2.0 µL 10x PCR buffer, 2 µL 2 mM dNTPs (SIGMA Chemical Co, St. Louis, Missouri, USA), 0.3 µL of 10 µM corresponding forward and reverse primers (Eurofins, Bangalore, India), and 0.3 µL (5U/µL) Taq Polymerase (Bangalore Genei, Bangalore, India). Amplification was performed using a PTC-100 thermal cycler (MJ Research, Inc., Watertown, Massachusetts, USA) according to the protocol: 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, primer dependent annealing (Table 2) for 30 seconds, and 72°C for 30 seconds. The amplified products were checked by electrophoresis on a 2% agarose gel stained with ethidium bromide. Restriction enzyme AciI (Fermentas, Lithuania) was used for digestion of PCR amplicons of *IL6* for genotyping of G/C (rs1800796) SNP (Table 2). 5 µL of the amplified products were digested with 5 U of the restriction enzyme in a total reaction volume of 25 µL as per the manufacturer's instruction. The digested products with 50 bp DNA ladder (Invitrogen) were resolved on 3.5% agarose stained with ethidium bromide and visualized under UV transilluminator. More than 10% of the samples were randomly selected for confirmation and the results

were 100% concordant (analysis of the chosen samples was repeated by two researchers independently).

7C.2.5 Determination of *IL6* and GAPDH mRNA expression As discussed in Chapter VII A.

7C.2.6 Real-time PCR

The expression of *IL6* and *GAPDH* transcripts were measured by real-time PCR using gene specific primers (Eurofins, Bangalore, India) as shown in Table 2. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed in duplicates in 20 µl volume using LightCycler®480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) following the manufacturer's instructions and carried out in the Light Cycler 480 Real-Time PCR (Roche Diagnostics GmbH, Mannheim, Germany). The thermal cycling conditions included an initial activation step at 95°C for 10 min, followed by 45 cycles of denaturation, annealing and amplification (95°C for 10s, 62°C and 65°C for 20 s for *IL6* and *GAPDH* respectively, 72°C for 20 s). The fluorescence data collection was performed during the extension step. At the end of the amplification phase a melt curve analysis was carried out to check the specificity of the products formed. The value of Cp was determined by the first cycle number at which fluorescence was greater than the set threshold value.

7C.2.7 Statistical analyses

Evaluation of the Hardy-Weinberg equilibrium (HWE) was performed for all four polymorphisms in patients and controls by comparing the observed and expected frequencies of the genotypes using chi-squared analysis. The distribution of the genotypes and allele frequencies of *IL6* polymorphisms for patients and control subjects were compared using chi-squared test with 3×2 and 2×2 contingency tables respectively using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003). Odds ratio (OR) with respective confidence interval (95% CI) for disease susceptibility was also calculated. Relative expression of *IL6* and genotype-phenotype correlation analysis of genotypes of *IL6* SNPs and *IL6* expression in patient and control groups was plotted and

analyzed by nonparametric unpaired t-test using Prism 4 software (Graphpad software Inc; San Diego CA, USA, 2003).

7C.3 RESULTS

7C.3.1 Analysis of association between *IL6* -174 G/C promoter polymorphism and susceptibility to vitiligo:

IL6 -174 G/C promoter polymorphism was found to be monogenic for 'G' allele and only 'GG' genotype was detected in 100 vitiligo patients and healthy age and gender matched controls in Gujarat population (Table 3; Figure 1A).

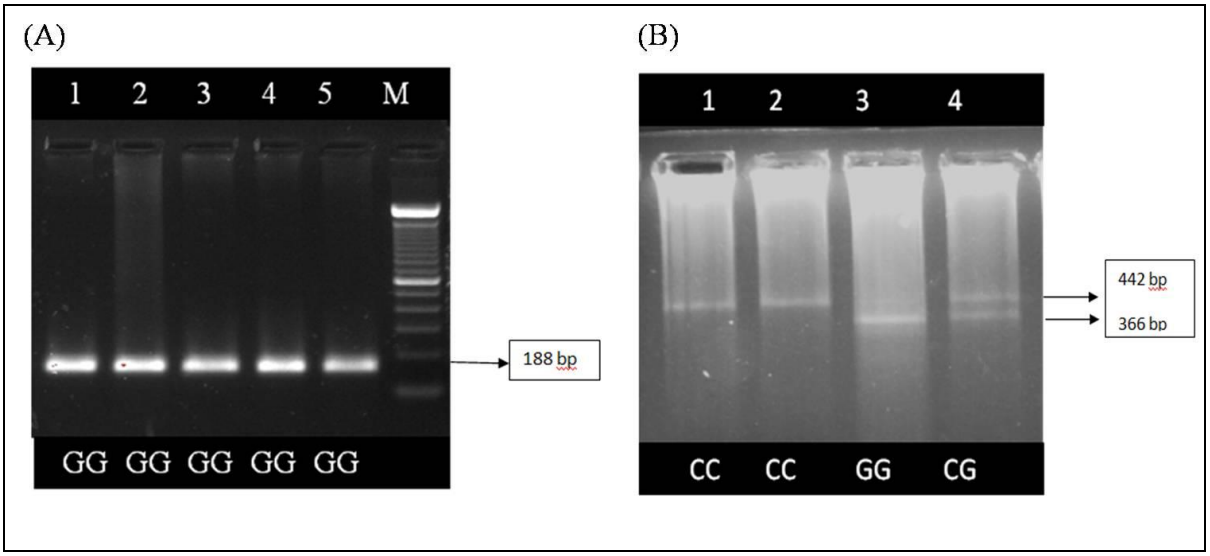

Figure 1: Genotyping of *IL6* gene polymorphisms: (A) PCR-RFLP analysis of *IL6* (-174 G/C) promoter polymorphism on 3.5 % agarose gel electrophoresis: lanes: All lanes exhibit homozygous (GG) genotypes. M shows 50 bp DNA ladder. (B) PCR-RFLP analysis of *IL6* (-572 G/C) promoter polymorphism on 3.5 % agarose gel electrophoresis: lanes 1 & 2 show homozygous (CC) genotypes; lane 3 shows homozygous (GG) genotypes and lane 4 shows heterozygous (CG) genotype.

Table 3: Distribution of Genotypes and Alleles for *IL6* -572 G/C (rs1800796) and -174 G/C (rs1800795) in vitiligo patients and controls from Gujarat.

SNP	Genotype or allele	Vitiligo Patients (Freq.)	Controls (Freq.)	*p* for HWE	*p* for Association	Odds ratio	CI (95%)
IL6 -572 G/C (rs1800796)	Genotype	n= 322	n = 343				
	GG	67 (0.21)	52 (0.15)		R	1	-
	GC	186 (0.58)	225 (0.66)	<0. 0001 (C)	**0.0336**[a]	0.6416[a]	0.42 -0.9678[a]
	CC	69 (0.21)	66 (0.19)		0.4078[a]	0.8114[a]	0.49 – 1.331[a]
	Allele			0.0053 (P)			
	G	320 (0.50)	329 (0.48)		R	1	-
	C	324 (0.50)	357 (0.52)		0.5281[b]	0.9331[b]	0.75- 1.157[b]
IL6 -174 G/C (rs1800795)	Genotype	n= 100	n = 100				
	GG	100 (1.00)	100 (1.00)	-	-	-	-
	GC	00 (0.00)	00 (0.00)		-	-	-
	CC	00 (0.00)	00 (0.00)	-	-	-	-
	Allele						
	G	100 (1.00)	100 (1.00)		-	-	-
	C	00 (0.00)	00 (0.00)		-	-	-

'n' represents number of Patients/ Controls,
'R' represents reference group,
HWE refers to Hardy-Weinberg Equilibrium,
CI refers to Confidence Interval,
(P) refers to Patients and (C) refers to Controls,
[a] Vitiligo Patients vs. Controls (genotype) using chi-squared test with 2 × 2 contingency table,
[b] Vitiligo Patients vs. Controls (allele) using chi-squared test with 2 × 2 contingency table

7C.3.2 Analysis of association between *IL6* -572 G/C promoter polymorphism and susceptibility to vitiligo:

Three genotypes GG, GC and CC were identified in both patients and controls for *IL6* -572 G/C promoter polymorphism (Figure 1B). Ancestral 'GG' genotype was considered as reference genotype for the analyses. The heterozygous 'GC' genotype was found to be significantly higher in control group as compared to vitiligo patients (p=0.0336, OR=0.6416, CI=0.42-0.9678; Table 3). In addition, frequency of 'CC' genotype did not differ significantly between patients and controls for *IL6* -572 G/C SNP (p=0.4078, OR=0.8114, CI=0.49 – 1.331; Table 3). Similarly, the allele frequency also did not differ significantly between patients and controls (p=0.5281, OR=0.9331, CI=0.75 – 1.157; Table 3). Further, we analysed the vitiligo patient's data in subgroups based on gender, disease progression and type of vitiligo. Analysis based on gender revealed increased frequency of the 'CC' genotype in males as compared to females (22.0% versus 9.0%, p=0.0282). However, there was no significant difference between 'GC' genotype and minor allele frequency of 'C' (p=0.3322, OR=0.7781, CI=0.46-1.293 and p=0.1172, OR=1.286, CI=0.93-1.761 respectively; Table 4).

Analysis based on the disease progression revealed significantly decreased frequency of the minor allele 'C' in patients with active vitiligo (AV) compared to controls (43.0% versus 52.0%, p=0.0025; Table 5), suggesting the important role of 'C' allele in disease progression. However, there was no significant difference in the frequency of 'C' allele between stable vitiligo (SV) patients and controls (p=0.0892; Table 5). Interestingly, the 'GC' genotype was predominant in controls as compared to AV and SV patients (65.0% versus 57.0% and 50.0% respectively; p=0.0003 and 0.0020 respectively; Table 5). Further, analysis based on the type of vitiligo revealed significantly decreased frequency of the minor allele 'C' in patients with generalised vitiligo (GV) (43.0% versus 52.0%, p=0.0024) as compared to controls (Table 6). However, there was no significant difference between localised vitiligo (LV) and controls (p=0.329; Table 6). Interestingly, the 'GC' genotype was predominant in controls compared to GV (66.0% versus 54.0%, p<0.0001) The 'CC' genotype was predominant in controls as compared to GV (19.0% versus 16.0%, p=0.0013) suggesting the important protective role of 'C' allele in GV.

However, there was no significant difference in genotype and allele frequencies based onage of onset, family history of patients (data not shown).

Table 4: Association of *IL6* SNP in male and female vitiligo patients of Gujarat population.

SNP	Genotype or allele	Male (Freq.)	Female (Freq.)	*p* for Association	Odds ratio	CI (95%)
	Genotype	n= 181	n = 141			
IL6 -572 G/C (rs1800796)	GG	52(0.28)	40 (0.28)	R	1	-
	GC	88(0.48)	87 (0.61)	0.3322[a]	0.7781[a]	0.46 – 1.293[a]
	CC	41(0.22)	14 (0.09)	**0.0283[a]**	2.253[a]	1.08 – 4.69[a]
	Allele G	192 (0.53)	167(0.59)	R	1	-
	C	170(0.47)	115(0.41)	0.1172[b]	1.286[b]	0.93 – 1.761[b]

'n' represents number of Male/Female Patients,

'R' represents reference group,

CI refers to Confidence Interval,

[a] Male vs. Female Patients (genotype) using chi-squared test with 2 × 2 contingency table,

[b] Male vs. Female Patients (allele) using chi-squared test with 2 × 2 contingency table

Table 5: Distribution of Genotypes and Alleles for *IL6* -572 G/C (rs1800796) in patients with active and stable vitiligo from Gujarat.

SNP	Genotype or allele	Active Vitiligo (Freq.)	Stable Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	Odds ratio	CI (95%)
	Genotype	n = 235	n= 87	n = 343			
IL6 -572 G/C (rs1800796)	GG	67 (0.28)	26 (0.29)	52 (0.15)	R	1	-
					0.5632[a]	1.182[a]	0.67-2.08[a]
	GC	134 (0.57)	44 (0.50)	225 (0.65)	**0.0003[b]**	0.462[b]	0.30-0.70[b]
					0.0020[c]	0.391[c]	0.22-0.69[c]
	CC	34 (0.14)	17 (0.19)	66 (0.19)	0.5691[a]	0.776[a]	0.37 – 1.62[a]
					0.0010[b]	0.399[b]	0.23 – 0.69[a]
					0.0655[c]	0.515[c]	0.25 – 1.04[c]
	Allele						
	G	268 (0.57)	96 (0.55)	329(0.48)	R	1	-
					0.6743 [a]	0.927[a]	0.65 – 1.31[a]
	C	202 (0.43)	78 (0.45)	357(0.52)	0.0025[b]	0.694[b]	0.62 – 0.87[b]
					0.0892[c]	0.748[c]	0.76 -1.04[c]

'n' represents number of Patients/ Controls,

'R' represents reference group,

CI refers to Confidence Interval,

[a]Active Vitiligo vs. Stable Vitiligo,

[b]Active Vitiligo vs. Controls,

[c]Stable Vitiligo vs. Controls,

Table 6: Association of *IL6* SNPs in patients, with generalized and localized vitiligo from Gujarat.

SNP	Genotype or allele	Generalized Vitiligo (Freq.)	Localized Vitiligo (Freq.)	Controls (Freq.)	*p* for Association	Odds ratio	CI (95%)
	Genotype	n = 299	n= 44	n = 343			
IL6 -572 G/C (rs1800796)	GG	82(0.29)	9 (0.21)	52 (0.15)	R	1	-
	GC	154(0.54)	26 (0.63)	225 (0.66)	0.2911[a] **<0.0001[b]** 0.3292[c]	0.650[a] 0.434[b] 0.667[c]	0.29 – 1.45[a] 0.28 – 0.64[b] 0.29 – 1.51[c]
	CC	45 (0.16)	6 (0.14)	66 (0.19)	0.7273[a] **0.0013[b]** 0.2434[c]	0.823[a] 0.432[b] 0.525[c]	0.27 – 2.46[a] 0.25 – 0.72[b] 0.17 – 1.57[c]
	Allele						
	G	318 (0.57)	44(0.54)	329(0.48)	R	1	-
	C	244 (0.43)	38 (0.46)	357(0.52)	0.6180[a] **0.0024[b]** 0.32910[c]	0.888[a] 0.707[b] 0.795[c]	0.55 - 1.41[a] 0.56 – 0.88[b] 0.50 - 1.26[c]

'n' represents number of Patients/ Controls,
'R' represents reference group,
CI refers to Confidence Interval,
[a]Generalized vitiligo vs. Localized vitiligo,
[b]Generalized vitiligo vs. Controls
[c]Localized vitiligo vs. Controls,

7.C.3.3 Relative gene expression analysis of *IL6* in PBMCs of vitiligo patients and controls:

Relative gene expression analysis in PBMCs of 70 patients and 74 controls revealed no significant difference in transcript levels of *IL6* in patients as compared to controls (Mean ΔCp ± SEM: 11.89 ± 0.7297 versus 12.09 ± 0.6649; *p* =0.8354) after normalization with

GAPDH. The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.147-fold increase in the expression of *IL6* transcript levels in patients as compared to controls (Figure 2). Moreover, analysis based on type of the disease revealed non-significant difference in *IL6* transcript levels between GV and LV (p=0.8744); furthermore, GV as well as LV also showed no significant difference in *IL6* transcript levels compared to controls (p=0.1660 and p=0.6135 respectively; Figure 3A). Similarly, analysis based on activity of the disease revealed no significant difference in *IL6* transcript levels between AV and SV (p=0.6041); furthermore, AV as well as SV also exhibited no significant difference in *IL6* transcript levels compared to controls (p=0.1343 and p=0.7148 respectively; Figure 3B). Gender based analysis showed no significant difference in *IL6* transcripts in both the groups (p=0.8995; Figure 3C).

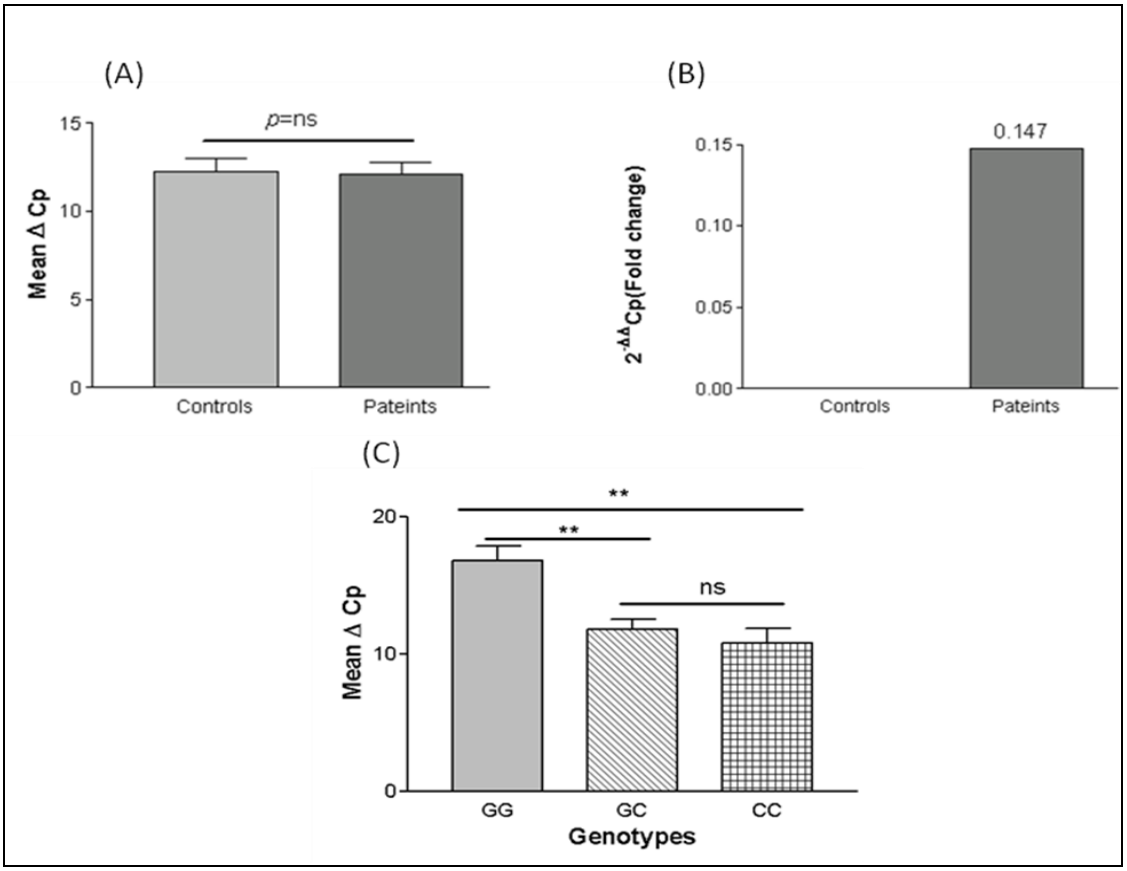

Figure 2: Analysis of *IL-6* transcript levels in controls and vitiligo patients:

(A) Expression of *IL6* transcripts in 74 controls and 70 vitiligo patients as suggested by mean ΔCp. Patients showed no significant difference in the mRNA levels of *IL6* as compared to controls (Mean ΔCp ± SEM: 12.29 ± 0.7436 versus 12.09 ± 0.6649 ; p =0.8423).

(B) Expression fold change of *IL6* transcripts in 70 vitiligo patients against 74 controls showed 0.147-fold change as determined by $2^{-\Delta\Delta Cp}$ method.

(C) Genotype-phenotype correlation analysis showed increased *IL6* expression in carriers of GC genotype as well as CC genotype as compared to carriers of GG genotype (p=0.0060 and p=0.0014, respectively) as suggested by mean ΔCp.

There was non-significant difference in the *IL6* transcript between GC genotype carriers and CC genotype carriers (p=0.4635).

7.C.3.4 Genotype-phenotype correlation analysis:

The expression of *IL6* was analyzed with respect to G/C (-572) genotypes (Figure 2C). Individuals with GC genotype showed significantly increased *IL6* expression levels (p=0.0060) as compared to individuals with GG genotype. Similarly, increased *IL6* expression levels were found in CC genotype as compared to GG genotype (p=0.0014). There was non-significant difference in the *IL6* transcript levels between GC and CC genotype (p=0.4635) (Figure 2 C). The results indicate increased *IL-6* expression is associated with individuals carrying 'C' allele as compared to 'G' allele.

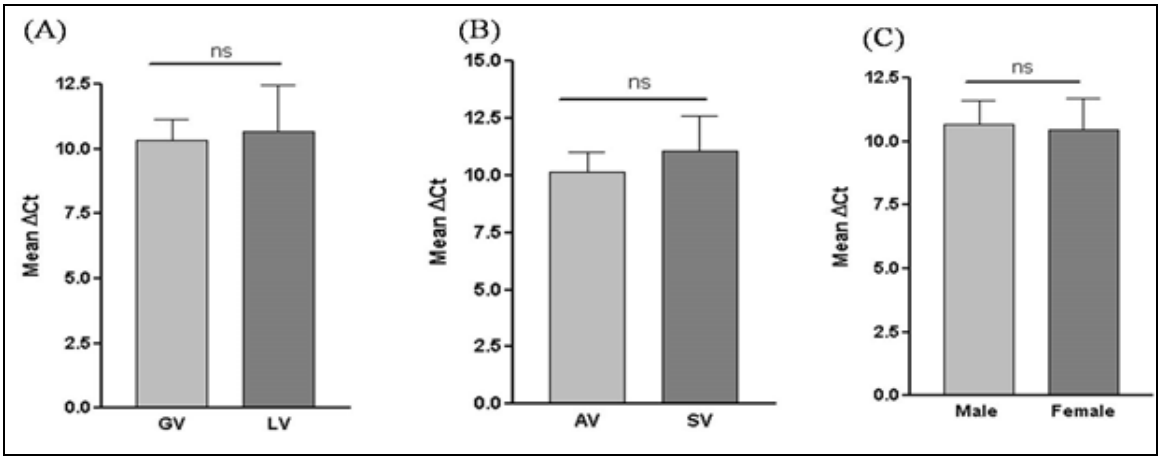

Figure 3 Analysis based on type of the disease revealed no significant difference in *IL6* transcript levels (A) between GV and LV patients (p=0.8744); furthermore GV as well as LV patients showed non-significant difference in *IL6* transcript levels compared to controls (p=0.1660 and p=0.6135 respectively.

(B) analysis based on activity of the disease revealed non-significant difference in *IL6* transcript levels between AV and SV patients (p=0.6041); furthermore AV as well as SV patients showed non-significant difference in *IL6* transcript levels compared to controls (p=0.1343 and p=0.7148 respectively).

(C) Gender based analysis showed no significant difference in *IL6* transcripts in both the groups (p=0.8995).

7C.4 DISCUSSION

Genetic predisposition of key molecules involving cytokines, their receptors and other immune regulatory molecules in addition to epidermal cytokine imbalance advocates autoimmune component for vitiligo precipitation IL-6 plays an important role during the transition from innate to acquired immunity. Also, IL-6 is capable of creating an immunological imbalance between Th-17 cells and Treg cells, resulting in an autoimmune pathology (Kimura *et al*., 2010). Increased levels of IL-6 in skin (Moretti *et al*., 2009) as well as in serum (Farhan *et al*., 2014; Singh *et al*., 2012; Yu *et al*., 1997; Tu *et al*., 2003) of vitiligo patients are well documented. However, a few studies concerning IL6 expression in vitiligo patients are summarized in Table 7.

Table 7: **Reports on IL6 levels in vitiligo:**

Population	IL6 Expression Levels (No. of patients and controls)	Reference
Austrian	Increased levels in Serum (40 patients and 40 controls)	Pichler *et al*., 2009
Chinese	Increased levels in Serum	Tu *et al*., 2003

	(50 patients and 20 controls)	
Indian	Increased levels in Serum (80 patients and 50 controls)	Singh *et al*., 2012
Turkish	No difference in serum levels (40 patients and 40 controls)	Basak *et al*., 2009
Turkish *IL6* promoter -174 C/G (rs1800795) Turkish	No association of *IL6* SNP and vitiligo (105 patients and 211 controls)	Aydıngoz *et al*., 2015
	No difference in serum levels (105 patients and 211 controls)	Aydıngoz *et al*., 2015
Caucasian population	No difference in skin samples as well as serum (15 patients and 14 controls)	Miniati *et al*., 2014
Caucasian	Increased expression levels in skin biopsies (15 patients and 5 controls)	Moretti *et al*., 2009
Taiwanese	MNCs increased (12 patients and 12 controls)	Yu *et al*., 1997
	Increased levels in Serum (39 patients and 42 controls)	Farhan *et al*., 2014
Egypt	Increased levels in Serum (74 patients and 75 controls)	Rashed *et al*., 2015

Saudi Arabia	Increased expression in PBMCs, (32 patients and 32 controls)	Zailaie, 2005

IL6 -174 G/C and -572 G/C promoter polymorphisms are located adjacent to cis-acting regulatory elements involved in controlling *IL6* transcript levels, suggesting that they may influence the interaction of proteins with DNA at these sites (Tanabe *et al*., 1988). Additionally, various findings suggest that promoter polymorphism of *IL6* have regulatory effect on its transcript as well as protein levels, implicating the importance of investigation of *IL6* genetic variants in an autoimmune disorders like vitiligo (Fishman *et al*., 1998). There is a paucity of data on the study of *IL6* genetic variants and vitiligo susceptibility. However, Aydingoz *et al.,* (2015) observed lack of association between *IL6* polymorphism (-174 G/C) and vitiligo with a sample size of 105 vitiligo patients and 211 controls. In the present study, for the first time -572 G/C promoter polymorphism has been addressed in the context of vitiligo. *IL6* -174 G/C and -572 G/C promoter polymorphisms have already been reported in various autoimmune diseases like Systemic Lupus Erythematosus, Multiple Sclerosis, Rheumatoid arthritis. However, Psoriasis vulgaris, Primary Sjogren Syndrome, Dermatomyositis etc. exhibited no association with *IL6* polymorphisms (Table 8).

Table 8: *IL6* promoter polymorphisms and correlation with other diseases.

Disease	**SNP position**	**Result**	**Reference**
Rheumatoid arthritis	-572G/C -174G/C	Associated	Pawlik *et al.*,2005
Kaposi sarcoma	-174G/C	Associated	Foster *et al*.,2000
Type 2 Diabetes	-174G/C	Associated	Huth *et al.*, 2006
Celiac Disease	-174G/C	Associated	Dema *et al*.,2009
Coronary Heart	-174G/C	Associated	Humphries *et al*.,

Disease			2001
Psoriasis Vulgaris	-174G/C	Not Associated	Baran *et al.*, 2008
Primary Sjogren Syndrome	-174G/C	Not Associated	Hulkkonen *et al.*, 2001

Our results suggest that *IL6* -174 G/C is monogenic in Gujarat population which is in accordance with SNPedia report (https://www.snpedia.com/index.php/Rs1800795), stating monomorphic G allele in the promoter region of the *IL6* at position -174 for Asian population. Interestingly, our analysis for -572G/C SNP indicated that the 'GC' genotype was predominant in controls compared to vitiligo patients. The 'CC' genotype was predominant in controls compared to GV patients. Also, our results demonstrated no significant difference in *IL6* transcript levels between lesional and non-lesional skin of patients, or between patient and control skin which are in accordance with the results obtained by Miniati *et al*., (2014). Contrary to this, Moretti *et al*., (2009) have demonstrated increased levels of IL6 in vitiligo skin. Similar to skin *IL6* levels, our PBMC expression analysis also revealed no difference in the expression levels of *IL6* among vitiligo patients and controls as well as upon further analysis based on type of disease, activity and gender. Aydıngoz *et al*., (2014) and Basak *et al*., (2009) reports are in accordance with our results where no-significant difference was found in IL6 levels of vitiligo patients. However, as mentioned in Table 7, Yu *et al*., (1997), Farhan *et al*., (2014), Singh *et al*., (2012); Zailaie, (2005); Tu *et al*., (2003) have reported increased levels of IL6 in vitiligo.

Overall, present study suggests that the -572 G/C promoter polymorphism of *IL6* maybe a genetic risk factor for vitiligo susceptibility in Gujarat population.

7C.5 REFERENCES

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Vitiligo is an acquired hypomelanotic, multifactorial and polygenic skin disorder characterized by circumscribed depigmented macules resulting from the loss of functional melanocytes. Despite extensive research in molecular and genetic aspects of vitiligo, no universally accepted hypothesis can explain the entire spectrum of vitiligo pathomechanism. Loss of melanocytes in vitiligo occurs through a combination of several mechanisms that act in concert. However, it is hypothesized to be of autoimmune origin due to its frequent association with various autoimmune diseases, the presence of anti-melanocyte antibodies and auto-reactive CTLs. Cytokines are important mediators of immunity and play a vital role in the pathogenesis of autoimmune disorders. In the present study we have explored the role of cytokines in vitiligo pathogenesis using population based studies with case-control approach and *in-vitro* studies on cultured melanocytes.

We have carried out the histopathological examination of skin biopsies from vitiligo patients and healthy controls. A significant increase in the epidermal thickness of the lesional skin as compared to the non-lesional skin was observed, suggesting the absence of melanocytes from epidermis deprives the skin of its protective effect. A few other studies also have reported the development of epidermal hyperplasia in vitiligo skin, including hyperkeratosis and acanthosis, as a means of compensation for the absence of pigmentation. 'Rete-ridges' pattern is important for melanocyte proliferation and function. Interestingly, we have found the loss of rete-ridges in vitiligo skin as compared to control. Moreover, scattered melanocytes are observed in the lesional and peri-lesional skin biopsies. Overall, our *in situ* results provide a further clue for in-depth analysis of parameters that might affect melanocyte biology.

We investigated the expression of *TNFA, IL1A, IL1B, IL4, IL6, IL10,* (*IL1R1*), (*IL-1RN*) and *IFNG* in control and vitiliginous human skin samples. Gene expression analysis in the skin samples revealed that the levels of pro-inflammatory cytokines: *TNFA, IFNG* were significantly higher in lesional skin of vitiligo patients as compared to controls. Further, non lesional skin of vitiligo patients exhibited significantly increased expression of *TNFA, IFNG,IL1B* as compared to controls. An interesting finding was that there is

significant increase in the expression of *IL1B* in non- lesional skin of vitiligo patients as compared to lesional skin, suggesting its important role in the progression of lesions. Conversely, the expression of anti-inflammatory cytokine *IL10* was significantly decreased in the lesional skin of vitiligo patients as compared to control skin (Figure 1). Therefore this epidermal cytokine imbalance between pro and anti-inflammatory cytokines and correlation with vitiligo suggests the need for further in depth analysis of the role of these cytokines in melanocyte biology.

We have monitored the effect of pro-inflammatory cytokines (TNF-α, IL1-α, IL-6) and anti-inflammatory cytokine IL-10 on primary cultured normal human melanocytes (NHM). Our results showed a significant decrease in NHM viability upon treatment with TNF-α, IL1-α, IL-6; however, IL-10 didn't show any significant effect on NHM viability even at higher concentration (100ng/ml). TNF-α was exerting most potent effect on NHM viability therefore, we investigated the combinatorial effect of TNF-α with IL1-α, IL-6 and IL-10 as well as with H_2O_2. Interestingly, we found that TNF-α was synergistically acting with IL1-α, IL-6 and H_2O_2 , whereas IL-10 was ameliorating TNF-α mediated NHM cytotoxicity. Few other interesting findings on the effect of cytokines on NHM are summarized below:

TNF-α: Exogenous stimulation of melanocytes with TNF-α caused significantly decreased viability with significant alteration in following parameters: increased cellular and mitochondrial ROS levels; ~20% decrease in mitochondrial complex 1 activity; decrease in melanin content via shedding of dendrites and down regulation of *MITF-M*, *TYR* andincreased *TNFR1, IL6, ICAM1* expression, while *TNFR2* levels remained unaltered. Upon TNF-α stimulation, LC3 I-LC3 II conversion at 12 hrs and caspase-8 activation at 48 hrs were observed, which disappeared at could not be seen after 48 hrs (LC3 I-LC3 II conversion) and 12 and 24 hrs (caspase-8 activation) respectively. Overall, the above studies advocate the crucial role of TNF-α in melanocyte homeostasis and autoimmune pathogenesis of vitiligo.

IL1-α: The dose dependent effect of IL1-α on melanocytes showed ~12% melanocyte death and ~22% increase in IL1R1 membrane expression upon IL1-α (100 ng/ml) treatment for 48 hrs. Further, *IL1RN, IL1A, IL1B, IL6, TNFA, ICAM1* showed significantly increased expression and *MITF-M* showed significantly decreased expression uponIL1-α (10 and 100 ng/ml, 48 hrs) stimulation on NHM; while *TYR, TYRP1, IL8 and IL1R1* showed non-significant difference. Overall, the present study suggests the crucial role of IL1-α in melanocyte destruction in vitiligo by regulating MITF and other immunomodulatory molecules.

IL-6: Effect of IL6 treatment on NHMshowed significant decrease in melanocyte viability, significant increase in IL6R protein expression,*ICAM1*expression and significant decrease in *MITF-M* as well as *TYR* expression suggesting crucial role of IL6 in melanocyte homeostasis.

IL-10: IL10 treatment on NHMshowed non-significant effect on cell viability, *TYR, MITF-M* and *ICAM-1* expressionbut significant increase in the MITF-M protein expression. However, IL10 treatmentameliorates TNF-α induced cytotoxicity.

MITF-M is a master transcriptional regulator of melanogenesis, melanocyte survival and plays a key role in melanocyte biology. Cytokines and oxidative stress also affect key molecules like TYR and ICAM-1 expression. We monitored MITF-M levels *in situ* in skin biopsies derived from patients and controls to obtain a better insight and our results suggest decreased levels of MITF-M in vitiligo patients as compared to controls. MITF-M transcript and protein levels were also monitored in primary normal human melanocytes upon exogenous stimulation with TNF-α, IL-1α, IL-6, IL-10 along with H_2O_2.

Cytokines mediate their action by interacting with their respective receptors. The regulation of receptor expression plays an important role in immune homeostasis. However, there is paucity of data with regard to the regulation of cytokine receptors in melanocytes. Hence, for better understating of the role of cytokine mediated melanocyte destruction in vitiligo or other pigmentary disorders, we monitored transcript as well as protein expression levels of the cytokine receptors TNFR1, IL1R1, IL6R and IL10R on NHM upon stimulation with the

respective cytokines. We found significant up regulation of TNFR1 transcript and protein levels while there was non- significant difference for TNFR2 transcript levels upon TNF-α treatment on NHM. IL-1α, IL-6 also mediated their action *via* receptor up regulation on melanocytes. Contrary to this there was non- significant difference in the expression of IL10R at transcript as well as protein levels upon treatment of NHM with IL-10. Our results showed that cytokines stimulated the membrane expression of their respective receptors, indicating auto-regulation of cytokines via their receptors.

Our results showed significant decrease in NHM viability upon H_2O_2 treatment in a dose dependent manner. Inhibition of PARP1 by pretreatment of NHM with DPQ (PARP1 inhibitor) showed significant rescue in H_2O_2 induced cell death. Amelioration of H_2O_2 mediated cytotoxicity by PARP-1 inhibitor was also evident by PARP-1 cleavage and PARylation pattern. The present study indicates that PARP1 might be playing a crucial role in melanocyte biology under oxidative stress, which could be the initial triggering factor in vitiligo pathogenesis. As DPQ attenuates H_2O_2 induced NHM death, we suggest that PARP1 inhibitors could be used in vitiligo therapeutics.

Individuals with genetic predisposition develop autoimmunity when exposed to endogenous or exogenous stresses that affect melanocytes. This could be the initial event, leading to the second step of a local inflammatory reaction with the activation of innate immune responses and subsequent generation of melanocyte /cell-specific cytotoxic immune responses. The incidence of vitiligo is higher in Gujarat population, hence we have investigated polymorphisms of the cytokine genes: *IL10* -819 C/T (rs1800871), -592C/A (rs1800872) and -1082G/A (rs 1800896); *IL6* -174 G/C (rs1800795) -572 G/C (rs1800796) and *IL1RN* intron 2 VNTR (rs2234663). Also, transcript levels were analyzed for the respective genes for possible genotype-phenotype correlation analysis. We have found that *IL10* -819 C/T (rs1800871), *IL6*-572 G/C (rs1800796) and *IL1RN* intron 2 VNTR (rs2234663) polymorphisms were significantly associated with vitiligo susceptibility in Gujarat population.

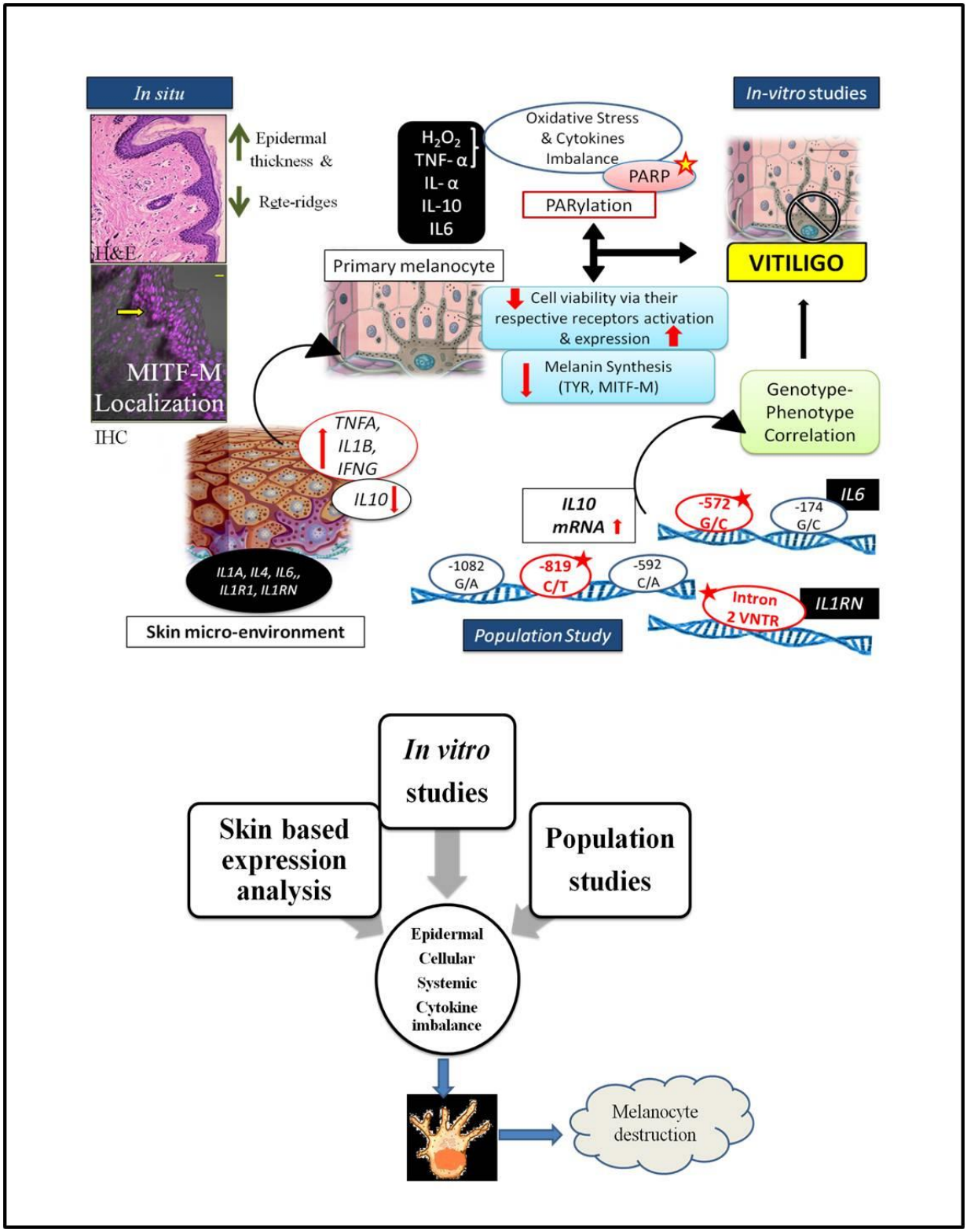

Figure 1. Possible cellular, molecular events and genetic factors responsible for cytokine imbalance mediated melanocyte destruction in vitiligo: Increased levels of pro inflammatory cytokines *TNFA, IFNG, IL1B* and decreased levels of anti-inflammatory cytokine *IL10* were observed in the skin microenvironment of vitiligo patients as compared to controls. Our histopathological examination studies of the ***skin biopsies revealed***increased viable epidermal thickness, loss of rete-ridges as well as decreased levels of MITF-M in the vitiliginous skin. Pro inflammatory cytokines TNF-α, IL-1α, IL-6 as well as H_2O_2 decreased melanocyte viability alone as well as in synergism. Additionally, the pro inflammatory cytokines altered expression of melanin synthesis genes (*TYR, MITF-M*), cell adhesion molecule (*ICAM-1*) as well as other immunoregulatory genes. On the contrary, IL-10 rescued TNF-α induced cytotoxicity in melanocytes. TNF-α induces cellular as well as mitochondrial ROS in melanocytes and increases epidermal oxidative stress in vitiligo. Furthermore, oxidative stress causes PARP-1 activation in NHM, which was ameliorated by the use of PARP inhibitor DPQ. Analysis of polymorphisms of *IL6, IL10* and *IL1RN* revealed an association with vitiligo susceptibility in Gujarat population. The genotype-phenotype analysis revealed a significant association of *IL6, IL10*and *IL1RN* with vitiligo susceptibility. Overall, cytokine imbalance at tissue and cellular levels coupled with genetic susceptibility aggravates the compromised state of melanocytes, advocating autoimmune mediated disease progression in vitiligo pathogenesis.

Overall, our studies suggest compromised melanocyte microenvironment may be attributed to genetic predisposition of susceptible individuals coupled with environmental factors such as oxidative stress. This results in systemic as well as epidermal cytokine imbalance that could trigger melanocyte loss in vitiligo pathogenesis (Figure 1). Our findings are in accordance with our previous lab studies and thus substantiate the role of cytokine imbalance in vitiligo pathogenesis. Thus, our results might lead to a better understanding of personalized treatment modalities with respect to polymorphism association and cytokine expression profile in vitiligo patients.

CONTROL CLINICAL PROFORMA

Department of Biochemistry
Faculty of Science
The Maharaja Sayajirao University of Baroda,
Vadodara, Gujarat- 390 002

Date:

Name: _____ Age: _____ Sex: _____

Address_____

Marital status: Married/Single Religion: _____

Blood Group:

Occupation: _____ Income: _____

Education: _____ Native place: _____

1. Any Disease (including Vitiligo):
   _____

---

2. Personal history
   Diet: Veg/ nonveg/ ovoveg/ mixed

   Routine food: _____

3. Habits: Smoking/ tobacco chewing/ alcoholism

4. Treatment:
   _____
   _____

I, _____ have understood the aim of this study and willing to donate 5 ml blood sample for this purpose.

Signature

Date:

APPENDIX -A

Vitiligo Clinical Proforma

Department of Biochemistry, The Maharaja Sayajirao University of Baroda, Faculty of Science, Vadodara, Gujarat - 390 002

Dr. _____ Date: _____

Name: _____ Age: _____ Sex: _____

Address_____

_____

Marital status: Married/Single Religion: _____

Occupation: _____ Income: _____

Education: _____ Native Place: _____

History of illness

1. Age of onset: _____
2. Site of onset: _____
3. Duration: _____
4. Lesions: Number_____ Size: _____ Shape_____
5. Condition of hair: no/ black/ gray
6. Any associated symptoms: Itching/ burning/ pain
7. Mode of spread: Static/ growing/ receding
8. Use of any drugs before onset of illness

   _____

   _____

   _____

9. Aggravating factors: occupational/ hobbies/ trauma/ drug/ work/sunlight/ emotional factors/ menstruation/ pregnancy/ food/ cosmetics/ chemicals/ any other:_____
10. What does the patient associate it with as cause_____

    _____ _____

11. Treatment: yes/ no Regular/ Irregular
12. Recovery: Some/ good/ poor/ no response
13. Sudden repigmentation: yes/ no
14. Local sensitivity (photo):

    _____

APPENDIX - B

15. Associated diseases:

_____

---

16. Family history
    A. Ist degree relatives: father/mother/sister/brother/daughter/son
    B. 2nd degree relatives: paternal grandmother/paternal grandfather/ maternal grandmother/maternal grandfather/maternal or paternal uncles or aunts
    C. 3rd degree relatives: cousins/nephews/nieces

17. Personal history
    Diet: veg/ nonveg/ ovoveg/ mixed

    Routine food: _____

18. Habits: smoking/ tobacco chewing/ alcoholism

19. Types of vitiligo
    a. Generalized/ Localized
    b. Unilateral/ Bilateral
    c. Symmetrical/ Symmetrical
    d. Universal
    e. Acrofacial
    f. Segmental
    g. Focal
    h. Liptip vitiligo
    i. Trichrome
    j. Quadrichrome

20. Treatment:

_____

_____

21. Koebners phenomenon: Yes/ No

The purpose of the study has been explained to me. I..... hereby agree to donate 5 ml of blood sample for the research purpose.

Signature of Patient

Date:

APPENDIX - B

Publications

1. Jadeja SD, Mansuri MS, **Singh M**, Laddha NC, Dwivedi M, Begum R.(2017) A case-control study on association of proteasome subunit beta 8 (PSMB8) and transporter associated with antigen processing 1 (TAP1) polymorphisms and their transcript levels in Vitiligo from Gujarat. *PLoS ONE* 12(7):e0180958.

2. Mansuri MS, Jadeja SD, **Singh M**, Laddha NC, Dwivedi M, Begum R.(2017) Catalase (CAT) promoter and 5'-UTR genetic variants lead to its altered expression and activity in vitiligo. *Br J Dermatol.* May 25. doi: 10.1111/bjd.15681. [Epub ahead of print]

3. Sahay II, Ghalsasi PS, **Singh M,** Begum R. (2017) Revisiting Aryl Amidine Synthesis Using Metal Amide and/or Ammonia Gas: Novel Molecules and Their Biological Evaluation. *Int J Rapid Communication of Synthetic Organic Chemistry* doi.org/10.1080/00397911.2017.1330959. **(IF:1.06)**

4. **Singh M**, Mansuri MS, Parasrampuria MA, Begum R. (2016) Interleukin 1-α: A modulator of melanocyte homeostasis in vitiligo. *Biochem Anal Biochem 5:273*.**(IF 2.6)**

5. Mansuri MS, **Singh M**, Begum R. (2016) miRNA signatures and transcriptional regulation of their target genes in vitiligo. *J Dermatol Sci*. pii: S0923-1811(16)30150-5.**(IF: 3.739)**

6. Jubin T, Kadam A, Gani AR, **Singh M**, Dwivedi M, Begum R. (2016) Poly ADP-ribose polymerase-1: Beyond transcription and towards differentiation. Semin. CellDev. Biol. 63:167-179. **(IF:5.18)**

7. Mansuri MS, Laddha NC, Dwivedi M, Patel D, Alex T, **Singh M**, Singh DD, Begum R. (2016) . Genetic variations (Arg5Pro and Leu6Pro) modulate the structure and activity of GPX1 and genetic risk for vitiligo. *Exp. Dermatol*. 25(8):654-657. **(IF: 2.675)**

8. Patel H, Mansuri MS, **Singh M,** Begum R, Shastri M, Misra A. (2016) Association of Cytotoxic T-Lymphocyte Antigen 4 (CTLA4) and Thyroglobulin (TG) Genetic Variants with Autoimmune Hypothyroidism. *PLoS One*. 11:e0149441. **(IF:3.23)**

9. Shah E, Upadhyay P, **Singh M**, Mansuri MS, Begum R, Dand NV, Soni HP. (2016) EDTA Capped Iron Oxide Nanoparticles Magnetic Micelles: Drug Delivery Vehiclefor Treatment of Chronic Myeloid Leukemia and T1-T2 Dual Contrast Agent forMagnetic Resonance Imaging. New J. Chem. 40:9507-9519 **(IF:3.277)**

10. Mansuri MS, **Singh M**, Dwivedi M, Laddha NC, Marfatia YS and Begum R (2014).miRNA profiling revealed differentially expressed miRNA signatures from skin ofnon-segmental vitiligo patients. *Brit. J. Dermatol.* 1:1263-7.**(IF 4.2)**

11. Mansuri MS, **Singh M**, Jadeja SD, Gani AR, Patel R, Dwivedi M, Laddha NC,Ansarullah, Ramachandran AV and Begum R. (2014) Could ER Stress be a Majorlink between Oxidative Stress and Autoimmunity in Vitiligo? *Pigmentary Disorders*1:1-14.

12. Laddha NC, Dwivedi M, Mansuri MS, **Singh M**, Patel HH, Agarwal N, Shah AM,and Begum R (2014). Association of Neuropeptide Y (NPY), Interleukin-1β (IL1B)Genetic Variants and Correlation of IL1B Transcript Levels with VitiligoSusceptibility.*PLoS ONE.* 9: e107020. **(IF 4.1)**

13. Laddha NC, Dwivedi M, Mansuri MS, **Singh M**, Gani AR, Yeola AP, Panchal VN, Khan F, Dave DJ, Patel A, Madhavan SE, Gupta R, Marfatia Z, Marfatia YS, Begum R. (2014).Role of oxidative stress and autoimmunity in onset and progression of vitiligo. *Exp Dermatol. 23*: 352-353.**(IF 4.1)**

Manuscript Under Communicated

- Interleukin 1 Receptor Antagonist (*IL1RN*) intron 2 VNTR polymorphism in Vitiligo patients: A Genotype-phenotype correlation study from Gujarat. Mala Singh, Mohmmad Shoab Mansuri, Shahnawaz D. Jadeja, Yogesh S. Marfatia and Rasheedunnisa Begum

- Role of Interleukin 6 (IL 6) in melanocyte homeostasis and investigation of its genetic variants and transcript levels in Vitiligo. Mala Singh, Mohmmad Shoab Mansuri, Shahnawaz D. Jadeja, Chandni Shah, Atul Shah and Rasheedunnisa Begum

Oral and Poster Presentations

1. **Singh M**, Mansuri MS, Shah A, Begum R. "Insights of epidermal cytokine imbalance in vitiligo pathogenesis" at the Science Conclave 2017 of The M.S. University of Baroda, Vadodara, on 28th February, 2017***(Awarded best oral presentation).***

2. **Singh M,** Mansuri MS, Jadeja SD, Mondal A, Parasrampuria M, Begum R. "Role of Micropthalmia Associated Transcription Factor-M (MITF-M) in Melanocyte Biology" at National Symposium on "Omics…to Structural Basis of Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 30th Sept. and 1st Oct. 2016. ***(Received the Second Prize for Oral presentation).***

3. **Singh M,** Mansuri MS, Jadeja SD, Basantani M, Marfatia YS, Begum R. "Role of Tumor Necrosis Factor-alpha (TNFα) in the melanocyte biology", at International Conference on Genomic Medicine in Skin Research, 24-25th June, 2016 held at New Delhi.

4. **Singh M,** Mansuri MS, Jadeja SD, Patel H, Khan F, Marfatia YS, Begum R. "Involvement of Interleukin 1 in Vitiligo pathogenesis," at 2nd International Genomic Medicine Conference, 29th November-3rd December, 2015 held at Saudi Arabia (IGMC), CEGMR, King Abdulaziz University, Jeddah.

5. **Singh M,** Mansuri MS, Laddha NC, Dwivedi M, Basantani M, Begum R. "*In vitro* effect of Interleukin 6 (IL-6) on human melanocytes and association of *IL6* -572 G/C polymorphism with vitiligo susceptibility" at the Master Class on Vitiligo and Pigmentary Disorders and 2nd Annual meeting of Vitiligo Academy of India, 28th – 30th November, 2014 held at Amritsar, India.

6. **Singh M,** Mansuri MS, Laddha NC, Dwivedi M, Sonawane S, Begum R."Association of Interleukin 10 (IL10) -819 C/T Promoter Polymorphism with Vitiligo Susceptibility in Gujarat Population" at the Conference on Genomics in Health and Diseases, 22nd- 23rd August, 2014 held at Central Research Centre Of Unani Medicine, Hyderabad, India. ***(Received the Second Prize).***

7. **Singh M,** Mansuri MS, Raval NP, Laddha NC, Dwivedi M, Begum R. "Association of Interleukin 6 -572 G/C And -174 G/C promoter polymorphisms with Vitiligo Susceptibility in Gujarat population" at the International Conference

on XXII International Pigment Cell Conference, 4th -7th September, 2014 held at Singapore.

8. **Singh M,** Mansuri MS, Laddha NC, Dwivedi M, Marfatia YS, Begum R. "Involvement of Cytokines in Vitiligo Pathogenesis" International Conference on Human Genetics and 39th Annual Meeting of the Indian Society of Human Genetics, 22nd - 25th January, 2014 held at the Ahmedabad Management Association, Ahmedabad, India.

9. **Singh M,** Mansuri MS, Laddha NC, Dwivedi M, Marfatia YS, Begum R. "*In vitro* effect of immune regulatory cytokines on vitiligo pathogenesis," in "2nd International Genomic Medicine Conference in Saudi Arabia (IGMC)", 23rd-24th November, 2013, CEGMR, King Abdulaziz University, Jeddah

10. Pararampuria M, **Singh M,** Mansuri MS, Jadeja SD, Modi H, Begum R. "In-situ analysis of TNFR1 in Vitiligo patients and controls of Gujarat population" at National Symposium on "Omics…to Structural Basis of Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 30th Sept. and 1st Oct. 2016.

11. Jadeja SD, Mansuri MS, **Singh M,** Patel H, Patel K, Begum R. "Analysis of MTHFR SNPs, Homocysteine and Vitamin B12 levels in Vitiligo cases and controls from Gujarat" at National Symposium on "Omics…to Structural Basis of Diseases" held at The M. S. University of Baroda, Vadodara, Gujarat, India on 30th Sept. and 1st Oct. 2016.

12. Mansuri MS, **Singh M,** Jadeja SD, Vyas V, Begum R. "Role of G6PD and H_2O_2 induced Oxidative stress in Melanocytes" at International Conference on Genomic Medicine in Skin Research at CSIR-IGIB, New Delhi, India on 24th and 25th June 2016.

13. Jadeja SD, Mansuri MS, **Singh M,** Patel K, Begum R. "Association of XBP-116 C/G SNP and elevated Homocysteine levels in Gujarat Vitiligo patients- Possible implication of Homocysteine induced ER stress in Vitiligo" at International Conference on Genomic Medicine in Skin Research at CSIR-IGIB, New Delhi, India on 24th and 25th June 2016.

14. **Singh M,** Parasrampuria M, Mansuri MS, Basantani M, Begum R. "*In vitro* effect of Tumor Necrosis Factor-alpha (TNF-α) on human melanocytes" at Three day National Symposium on Emerging Trends in Biochemical Sciences, 29-31st December, 2014 held at The M. S. University of Baroda, Vadodara, India.

15. Mansuri MS, **Singh M,** Laddha NC, Dwivedi M, Bhalara S, Vyas V, Begum R.“ Glucose -6- phosphate dehydrogenase (G6PD) polymorphisms and hsa-miR-1 in vitiligo susceptibility: A Genotype – phenotype correlation study” at the Master Class on Vitiligo and Pigmentary Disorders and 2nd Annual meeting of Vitiligo Academy of India, 28th – 30th November, 2014 held at Amritsar, India. ***(*Received the Second Prize for Oral presentation)***

16. Jadeja SD, Mansuri MS, **Singh M,** Ansarullah, Patel H, Begum R. “Investigating association of two genetic variants of MTHFR (677 C/T and 1298 A/C) with Vitiligo Susceptibility in Gujarat Population” at the Master Class on Vitiligo and Pigmentary Disorders and 2nd Annual meeting of Vitiligo Academy of India, 28th – 30th November, 2014 held at Amritsar, India.

17. Bharti A, Gupta R, Mansuri MS, **Singh M,** Begum R, Marfatia YS**. “**Koebner’s Phenomenon in Vitiligo and its association with Oxidative stress and Antimelanocyte activity”at the Master Class on Vitiligo and Pigmentary Disorders and 2nd Annual meeting of Vitiligo Academy of India, 28th – 30th November, 2014 held at Amritsar, India. ***(*Received the Third Prize for Oral Presentation)***

18. Mansuri MS, **Singh M,** Laddha NC, Dwivedi M, Patel D, Singh DD, Begum R. “Glutathione Peroxidase 1 (GPX1) in Vitiligo Susceptibility: Structural and Functional analysis” at the 2nd Foundation day Celebration, Dr. Vikram Sarabhai Science Centre for Interdisciplinary Research and Integrated Teaching Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India, on 30th September 2014. ***(*Received the First Prize)***

19. Mansuri MS, **Singh M,** Laddha NC, Dwivedi M, Bhalara S, Marfatia YS, Begum R. “Identification of potential microRNAs responsible for pathogenesis of vitiligo” at the International Conference on XXII International Pigment Cell Conference, 4th -7th September, 2014 held at Singapore. ***(*Received the IFPCS Travel Award for Poster Presentation).***

20. Jadeja SD, Mansuri MS, **Singh M**, Laddha NC, Dwivedi M, Begum R. “Association of LMP7 and TAP1 polymorphisms with Vitiligo susceptibility in Gujarat population” at the International Conference on XXII International Pigment Cell Conference, 4th -7th September, 2014 held at Singapore. ***(*Received the ICMR Travel Award for Poster Presentation).***

21. Mansuri MS, **Singh M,** Laddha NC, Dwivedi M ,Vasan K, Patel D, Singh DD, Begum R. "Biochemical, Genetic and Bioinformatics Analyses of Glutathione Peroxidase 1 (GPX1) in Vitiligo Susceptibility" at the Conference on Genomics in Health and Diseases, 22nd-23rd August, 2014 held at Central Research Centre Of Unani Medicine, Hyderabad, India.

22. Mansuri MS, **Singh M,** Laddha NC, Dwivedi M, Marfatia YS, Begum R."Skin miRNA Profiling Reveals Differentially Expressed miRNA Signatures From Non-Segmental Vitiligo Patients". *Molecular Cytogenetics* 2014, 7 (Suppl 1): P118.

23. Mansuri MS, **Singh M,** Laddha NC, Dwivedi M, Marfatia YS, Begum R. "Skin miRNA Profiling Reveals Differentially Expressed miRNA Signatures From Non-Segmental Vitiligo Patients" International Conference on Human Genetics and 39th Annual Meeting of the Indian Society of Human Genetics, 22nd - 25th January, 2014 held at the Ahmedabad Management Association, Ahmedabad, India.

24. Shah K, Laddha NC, Dwivedi M, Mansuri MS, **Singh M,** Patel H, Agrawal N, Shah AM, Shah BJ and Begum R"Genetic association of Neuropeptide-Y (NPY) and Interleukin1b (IL1B) polymorphisms with vitiligo susceptibility." HGM ICG-2013 Conference on "Genetics and genomics of Human health and global sustainability", 13th -18th April, 2013, Singapore. (Poster presentation)

Synopsis

Introduction:

Melanocytes are specialized skin cells, residing at the basal layer of epidermis, producing protective skin darkening pigment 'melanin' which provides protection against various kinds of genotoxic stress (Lin and Fisher, 2007). Melanocytes, the neural crest derived cells are present in various tissues other than epidermis for e.g. retinal epithelium, mucosa, cochlea (ear), iris (eye), and mesencephalon (brain), adipocytes etc. (Cichorek *et al.,* 2013; Yamaguchi *et al.,* 2014). Loss of functional melanocytes may lead to various kinds of disorders for e.g. vitiligo, albinism, oculocutaneous albinism etc. (Yamaguchi *et al.,* 2014).

Vitiligo is an acquired hypomelanotic pigmentary disorder characterized by the presence of circumscribed depigmented macules in the skin, caused due to loss of functional melanocytes. Studies have revealed a worldwide incidence ranging from 0.04-2.16 % (Kruger *et al.,* 2012). In India, it affects ~0.5 % of population and the states of Gujarat and Rajasthan have the highest incidence rate of ~8.8% (Sehgal and Srivastava, 2007). Earlier we have reported 21.93% of Gujarat vitiligo patients exhibit positive family history and 13.68% patients have first degree relative affected implicating importance for genetic predisposition for vitiligo susceptibility (Shajil *et al.,* 2006). The etiology of vitiligo remains obscure despite being in focused debate for more than six decades and hence, it is important to unravel the underlying pathomechanisms of vitiligo (Laddha *et al.,* 2013; Mansuri *et al.,* 2014). Various hypotheses have been put forward for explaining vitiligo pathomechanism i.e., autoimmune, neural and oxidative stress which alone or in various combinations contribute towards vitiligo precipitation. The inheritance pattern of vitiligo does not follow the simple Mendelian pattern of an autosomal dominant or autosomal recessive or X- linked inheritance. Therefore, it has been proposed that vitiligo is a polygenic disease influenced by a set of recessive alleles occurring at several unlinked autosomal loci that collectively confer the vitiligo phenotype (Nath *et al.,* 1994).

Increasing evidences including our previous studies propose that genetic polymorphisms of *TNFA, IL4, IFNG* and *ICAM1, IL1B, TNFB, SOD, CAT, NLRP1, CTLA4, MYG* and *GPX1* might be playing a crucial role in vitiligo susceptibility. Genetic studies on vitiligo suggest that the genetic variants of genes that are involved in oxidative stress and immune regulation could be responsible for conferring susceptibility or protection towards vitiligo (Laddha *et al.,* 2012; Imran *et al.,* 2012; Dwivedi *et al.,* 2013; Laddha *et al.,* 2014; Laddha *et al.,* 2013; Laddha *et al.,* 2013; Shajil *et al.,* 2007; Dwivedi *et al.,* 2013; Dwivedi *et al.,* 2011; Mansuri *et al.,* 2016). A key component in the inflammatory response is the increased production of pro-inflammatory

cytokines such as IL1β, IFNγ and TNFα which are found to be increased in vitiligo (Laddha *et al.*, 2014; Dwivedi *et al.*, 2013; Laddha *et al.*, 2012). A single dominant pathway appears unlikely to account for all cases of melanocyte loss in vitiligo and apparently, a complex interaction of genetic, environmental, biochemical and immunological events is likely to generate a permissive milieu. It is most likely that loss of melanocytes in vitiligo occurs through a combination of pathogenic mechanisms that act in concert.

To date the most accepted hypothesis is selective autoimmunity towards melanocytes therefore generalized vitiligo is also included in the list of autoimmune disorders (van den Boorn *et al.*, 2011, Mosenson *et al.*, 2013). Circulating autoantibodies that are present in the sera of vitiligo patients, and autoreactive T lymphocytes are found to recognize melanocyte antigens (Naughton *et al.*, 1983). Immunohistochemical studies have confirmed the presence of infiltrating T cells (Le Poole *et al.*, 1996). T cell infiltrates with a predominant presence of $CD8^+$ T cells are detected in generalized vitiligo (Abdel-Naser *et al.*, 1994; Badri *et al.*, 1993). Our studies also showed that ~75% of Gujarat vitiligo patients have antimelanocyte antibodies in their circulation and oxidative stress acts as an initial trigger for onset of vitiligo and autoimmunity is responsible for disease progression (Laddha *et al.*, 2014). Imbalance between pro and anti-inflammatory cytokines is well documented in vitiliginous skin and circulation. These immune-regulatory molecules are capable to initiate melanocyte apoptosis and are mainly produced by cells of immune system and/or surrounding keratinocytes. In recent years, a complex melanogenic cytokine network between skin cells which regulate melanocyte activity has been demonstrated. Epidermal melanin unit is a structural and functional unit in epidermis where single melanocyte is surrounded by ~36 keratinocytes. (Nordlund, 2007). Keratinocytes secrete additional cytokines, such as IL6 and TNFα, which function as paracrine inhibitors of growth and proliferation of melanocyte (Swope *et al.*, 1991). Altered levels of keratinocyte derived mediators have been recently described in vitiligo epidermis, suggesting an important role of epidermal cytokines in vitiligo pathogenesis (Moretti *et al.*, 2002; Lee *et al.*, 2005). Thus, cytokine imbalance has been well documented in vitiligo patients; however, their exact mode of action on melanocyte biology is not well explored.

PROPOSED OBJECTIVES:

(1) Isolation and culture establishment of primary normal human melanocytes from epidermal human skin.

(2) To study the transcript levels of the immune regulator genes: *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* in vitiliginous and control skin.

(3) To study the dose dependent effect of the interleukins: TNFα, IL1α, IL6 and IL10 on *in vitro* cultured melanocyte cell death, expression of melanin synthesis genes and immunoregulatory genes.

(4) Effect of receptor activation of TNFR1, IL1R1, IL6R and IL10R upon treatment of TNFα, IL1α, IL6 and IL 10 respectively on *in vitro* cultured melanocytes.

(5) To study the role of PARP upon H_2O_2 stimulation of *in vitro* cultured melanocytes.

(6) Genotyping of candidate genes: *IL1RN* intron *2 VNTR, IL6* and *IL10* in vitiligo patients and controls from Gujarat population with possible genotype-phenotype correlation.

Study Subjects:

The present study focuses on Gujarat population and vitiligo patients included in the study refer to S.S.G. Hospital, Vadodara, Gujarat, India. The diagnosis of vitiligo was clinically based on the presence of depigmented patches on the skin. Ethnically age and sex-matched unaffected individuals were also included in the study. None of the healthy individuals or their relatives had any evidence of vitiligo and any other autoimmune disease. The study plan was approved by the Institutional Ethical Committee for Human Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. The importance of the study was explained to all participants and written consent was obtained from all patients and controls.

Objective 1:

Isolation and culture establishment of primary normal human melanocytes from epidermal human skin

Vitiligo is a skin disorder caused due to selective destruction of epidermal melanocytes of unknown etiology. Cytokine imbalance is documented in vitiligo patients, but their exact mode of action on melanocyte is not well explored. Therefore, to study the effect of candidate cytokines on melanocyte biology it is very essential to establish the *in vitro* system as the *in vivo* or animal models system is not apt to study the role of cytokines *per se*. Moreover, cytokines affect melanocyte viability, their respective receptors activation and to address other parameters to

explore melanocyte biology in relation to cytokine imbalance, required designing of an ideal *in vitro* system. In our preliminary standardization experiments we have used melanoma cell lines SKMel28 (low melanin producing Caucasian origin cell line), G361 (melanin producing cell line) as well as transformed primary melanocytes PIG1, PIG3V derived from healthy individuals and vitiligo patient respectively. Our results showed that primary cultured normal human melanocytes (NHM) showed increased sensitivity and resemblance as compared to other *in vitro* models. The difference can be attributed due to incorporation of mutations during the procedure of immortalization. Therefore, NHM is a better model for studying the effect of various immnoregulatory molecules on melanocytes. The NHM culture system used for our studies is free of phorbol 12-myristate 13-acetate (PMA) and serum which minimizes the artifact of cytokine mediated sensitive studies.

Culture establishment of primary normal human melanocytes (NHM): Melanocytes were isolated from human skin samples and cultured successfully using the standard protocol with slight modifications (Im and Park ,1992; Czajkowski *et al.,* 2007). Briefly, the epidermis was separated from the dermis after an overnight incubation of skin biopsies in 0.25% Dispase II protease at 4°C. In order to separate epidermal cells, the epidermis was incubated in Trypsin Solution. Melanocyte-keratinocyte mixed population starts appearing around 4-9 days. Melanocytes were purified from keratinocytes by two rounds of differential trypsinisation, which is based on the sturdier and adherent property of keratinocytes as compared to melanocytes. Additionally, melanocytes were given G418 treatment for gradual removal of fibroblasts to obtain melanocyte and were further split in ratio of 1:3, when cell confluency reached ~80%. Media was replenished after every 48-72 hrs; melanocytes cultured up to fifth passage were used for experiments. The pure culture of melanocytes was confirmed by L-DOPA staining (Iijima *et al.,* 1957).

Objective 2:

To study the transcript levels of the immune regulator genes: *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* in vitiliginous and control skin

Skin samples stored in RNA preservative solution were used for RNA isolation and further converted to first strand cDNA for gene expression studies. Expression levels of *TNFA, IL1A, IL1B, IL4, IL6, IL10, IL1R1, IL1RN* and *IFNG* were monitored and analyzed between lesional vs. control, non- lesional vs. control and non- lesional vs. lesional skin (n=12) as stated in Table1.

Table 1: Transcripts levels analysis of candidate genes in vitiligo patients and controls (n=12)

Gene	Lesional skin vs. Control (*p* value)	Non lesional skin vs. Control (*p* value)	Non -Lesional vs. lesional skin (*p* value)
TNFA	0.0246 ↑	0.0467 ↑	0.6749 ns
IL1A	0.6000 ns	0.4004 ns	0.2886 ns
IL1B	0.5085 ns	0.0290 ↑	0.0021 ↑
IL4	0.2886 ns	0.2152 ns	0.9669 ns
IL6	0.5473 ns	0.5697 ns	0.9511 ns
IL10	0.0357 ↓	0.5565 ns	0.0980
IL1R1	0.8186 ns	0.2418 ns	0.8180 ns
IL1RN	0.2147 ns	0.8604 ns	0.4080 ns
IFNG	0.0231 ↑	0.0138 ↑	0.0808 ns

Increased levels of pro-inflammatory cytokines in the skin microenvironment of vitiligo patients were found as compared to healthy controls. Our results showed imbalance between pro and anti-inflammatory cytokines in the skin samples of vitiligo patients as compared to controls. *IL1B* expression level was higher in non lesional as compared to lesional skin suggesting its important role in disease progression. Expression levels of *TNFA, IFNG* was found to be increased in lesional as well as non lesional skin of vitiligo patients as compared to controls. Whereas, non significant changes in the expression levels of *IL1A, IL4, IL6, IL1R1*and *IL1RN* was observed between lesional and non lesional skin of vitiligo patients as well as in comparison to controls. Additionally, decreased level of *IL10* an anti inflammatory cytokine in lesional skin of vitiligo patients was observed. Overall, these skin based results provide evidence of cytokine imbalance in the microenvironment of skin.

Objective 3:

To study the dose dependent effect of the interleukins: TNFα, IL1α, IL6 and IL10 on *in vitro* cultured melanocyte cell death, expression of melanin synthesis genes and immunoregulatory genes

NHM between 3-5 passages were used for MTT assay (Mosmann, 1983) to monitor the dose dependent effect of various cytokines on *in vitro* cultured melanocytes.

Dose dependent effect of TNFα on melanocytes

NHM were treated with different doses of TNFα (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=4). The NHM did not show any difference in morphology and viability up to 24 hrs. After 48 hrs of treatment all doses ≥10ng/ml exhibited significant decrease in melanocyte viability (p<0.0001 for all treatments). TNFα inhibits significantly growth and proliferation of NHM in a dose and time dependent manner. Morphological observation revealed cellular vacuolization at higher magnification, tapering of cells and shedding of dendrites, cell distortion and clustering was observed upon exogenous TNFα. Additionally, we found significant synergism of TNFα with other pro inflammatory cytokines IL1α, IL6 and H_2O_2 (which is prevalent in the skin micro environment of vitiligo); on the other hand, IL10 an anti inflammatory cytokine was proficient to significantly rescue cytotoxicity caused due to TNFα.

Dose dependent effect of IL1α on melanocytes

NHM were treated with different doses of IL1α (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM did not show any difference in morphology and viability up to 24 hrs. Also, there was no significant morphological change observed in NHM even after 48 hrs of IL1α treatment. However, 100 ng/ml of IL1α treated NHM showed significant decrease in viability (p=0.0210) after 48 hrs of treatment, as compared to control. However, 10 ng/ml & 50ng/ml doses of IL1α did not show any significant difference (p=0.6658 and p=0.9301 respectively) in cell viability. The effect of IL1α was found to be cytostatic on NHM (Singh *et al.*, 2016).

Dose dependent effect of IL6 on melanocytes

NHM were treated with different doses of IL6 (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM did not show any difference in morphology and viability up to 24 hrs. Also, there was decrease in NHM confluency when observed after 48 hrs of IL6 exogenous stimulation and significant decrease in cell viability with 10, 50, 100 ng/ml IL6 stimulation (p<0.005 for all treatments).

Dose dependent effect of IL10 on melanocytes

IL10 an anti-inflammatory cytokine didn't have significant effect on melanocyte.NHM were treated with different doses of IL10 (0, 10, 50, 100 ng/ml) and observed after 24 and 48 hrs for morphology and viability (n=3). The NHM showed non-significant difference in morphology and viability up to 48 hrs of IL10 stimulation (p>0.05 for all treatments).

Overall, among the studied cytokines, TNFα exhibits the most potent response. Further, we tried to monitor detailed response of TNFα on melanocytes. Exogenous stimulation of TNFα on melanocytes caused significantly decreased viability with significant alteration in following parameters: increased cellular and mitochondrial ROS levels; ~20% decrease in mitochondrial complex1 activity; decrease in melanin content via shedding of dendrites and down regulation of *MITF-M, TYR* and increased *TNFR1, IL6, ICAM1* expression while, *TNFR2* levels remained unaltered. Upon TNFα stimulation, LC3I-II conversion at 12 hrs and caspase-8 activation at 48 hrs were observed, which disappeared at 48 hrs and 24 hrs respectively. Pro inflammatory cytokines, TNFα, IL1α and IL6 caused a dose dependent decrease in cell viability of melanocytes. Anti- inflammatory cytokine IL10 did not reveal significant alteration in growth and viability of melanocytes. All cytokines in synergism with H_2O_2 (which is higher in skin microenvironment of vitiligo) can lead to enhanced compromised state of melanocytes, effecting overall survival and homeostasis of melanocytes.

Objective 4:

Effect of receptor activation of TNFR1, IL1R1, IL6R and IL10R upon treatment of TNFα, IL1α, IL6 and IL 10 respectively on *in vitro* cultured melanocytes

TNF receptors expression upon TNFα treatment to NHM

TNFα mediates its action *via* its two receptors TNFR1 and TNFR2, TNFR1 is present on most of the cells but the presence of TNFR2 is restricted to few cell lineages. TNFR1 has death domain and may be responsible for apoptosis, on the contrary TNFR2 lacks death domain. Melanocytes have both the receptors of TNFα i.e., TNFR1 as well as TNFR2. There was significant upregulation of *TNFR1* transcript levels (p=0.024; n=3) whereas there was non-significant difference in the transcript levels of *TNFR2* upon TNFα stimulation (10ng/ml). TNFR1 serves as the major mediator of TNFα induced signaling pathways. Also our confocal microscopy and flow cytometry results suggest that the total membrane expression of TNFR1 is increased upon TNFα stimulation (p=0.004 & 17.2% increased expression as compared to controls respectively; n=3).

IL1R1 membrane expression upon IL1α treatment to NHM

Cytokines mediate their action *via* interacting with their respective receptors and hence we monitored the effect of IL1α on the membrane expression of IL1R1 (n=3). IL1α stimulation showed significant increase in membrane expression of IL1R1 upon 10 ng/ml (~25%) and 100 ng/ml (~22%) of IL1α treated NHM as compared to untreated controls. However, there was no significant difference in the transcript levels of *IL1R1* upon IL1α exogenous stimulation, suggesting involvement of post translational modifications in regulation of membrane expression of IL1R1 on NHM.

IL6R membrane expression upon IL6 treatment to NHM:

Significant upregulation of *IL6R* transcript levels upon exogenous IL6 (10ng/ml) stimulation was observed (n = 3; $p < 0.05$). Further, the membrane expression of IL6R upon exogenous IL6 (10ng/ml) stimulation was found to be increased (n =3; p=0.0262).

IL10R membrane expression upon IL10 treatment to NHM

The NHM cells were treated with IL10 (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 IL10 (n = 3; p=0.6432). Further, effect of IL10 on expression of IL10R on NHM was observed and non significant difference i.e. ~0.9% increase in membrane expression was observed as compared to untreated controls (n =3). Overall, all receptor expression studies indicate positive feedback regulation of receptors upon treatment with their respective cytokines.

Objective 5:

To study the role of PARP upon H_2O_2 stimulation of *in vitro* cultured melanocytes

Poly (ADP-ribose) polymerase (PARP) is an enzyme involved in DNA repair, genomic stability, apoptosis, gene transcription, proliferation, and autoimmunity. One of the major players of ROS-induced cell death is hyper-activation of PARP1 for the recruitment of repair enzymes. PARP1 acts as a co-activator of nuclear factor-κB (NFκB) and regulates NFκB dependent gene expressions. However, the role of PARP1 activation upon oxidative stress induction in melanocytes has not been elucidated yet. Therefore, we aimed to monitor the effect of PARP inhibitor (PJ34) on H_2O_2 treated NHM. NHM with or without pretreatment with PJ34 were exposed to H_2O_2 to monitor their rescue from H_2O_2 induced cell death using PJ34, a potent

PARP1 inhibitor. Further, cell viabilities were measured by trypan blue exclusion assay. Activation of PARP and oxidative stress-induced PARylation were assessed by Western blotting. Our results showed significant NHM death upon H_2O_2 treatment (p<0.0001) in a dose dependent manner (0, 5, 10, 25 μM H_2O_2; 30 min exposure). PJ34 treatment showed no toxicity to NHM (10 μM; 48 hrs (p<0.0001)). Inhibition of PARP1 (PJ34 10 μM; 4 hrs) showed significant rescue of NHM from (p<0.0001) induced death (p=0.0002) and decreased levels PARP cleavage and PARylation pattern as compared to untreated controls. These results suggest that PJ34 attenuates H_2O_2 induced NHM death via oxidative stress generation and therefore could be used as a potential drug in treatment modalities of vitiligo. The present study indicates that PARP1 might be playing a crucial role in melanocyte biology under oxidative stress condition in vitiligo pathogenesis.

Objective 6:

Genotyping of candidate genes: *IL1RN* intron *2 VNTR, IL6* and *IL10* in vitiligo patients and controls from Gujarat population with possible genotype-phenotype correlation

a) Genotyping of Interleukin 1 Receptor Antagonist (*IL1RN*) intron 2 VNTR polymorphism and determining the expression levels of *IL1RN* in Gujarat vitiligo patients and controls

The IL1RN is an important immunologic regulator that competes with IL1α and IL1β for the IL1R1 and IL1 RII receptor in target cells and act as its negative regulator with anti-inflammatory effects (Granowitz *et al.,* 1991). The gene sequence shows an 86-bp variable number tandem repeat (VNTR) in intron 2 of the *IL1RN* gene. This polymorphism has six alleles, comprising of one to six repeats of an 86-bp sequence. The four-repeat (*IL1RN**1) and two-repeat (*IL1RN**2) alleles are most common, while the other alleles occur at a combined frequency of less than 5% (Tarlow *et al.,* 1993; Vamvakopoulos *et al.,* 2002). The number of repeats may be of functional significance as these repeats contain putative binding sites for transcription factors which needs further investigation (Tarlow *et al.,* 1993). The role of *IL1RN* intron 2 VNTR (rs2234663) is well established with development of inflammatory disorders for several years (Fischer *et al.,* 1992; McIntyre *et al.,* 1991; Xu *et al.,* 2011). The pro-inflammatory cytokine IL1β and its antagonist, IL1RN, are encoded by polymorphic genes (Wilkinson *et al.,* 1999). An association between polymorphism in the *IL1RN* intron 2 VNTR (rs2234663) and several other autoimmune disorders including vitiligo has been reported. To date, there is no report from India regarding the *IL1RN*

intron 2 VNTR (rs2234663) association with vitiligo. We have investigated the association between *IL1RN* intron 2 VNTR polymorphism and monitored *IL1RN* transcript levels from PBMCs, and performed possible genotype phenotype correlation in vitiligo patients and healthy controls from Gujarat population. 307 vitiligo patients and 316 controls were enrolled; genotyping was performed using polymerase chain reaction (PCR). Relative gene expression was measured in PBMCs (n=36) using real-time-PCR. Significant difference was observed in *IL1RN**1/2 genotype between active and stable vitiligo (p =0.0172). Also, *IL1RN**2/2 genotype and allele frequencies differed significantly between stable vitiligo and controls (p=0.0246) and (p=0.0046) respectively. Moreover, significant difference was observed for *IL1RN**A2 in active and stable vitiligo (p=0.0060).However, other camparisons showed non-significant association of genotype as well as allele frequencies. Also, non-significant difference for *IL1RN* expression (p=0.5962) was observed between pateints and controls. Interestingly, our genotype-phenotype correlation showed individuals with *IL1RN**2/2 showed higher *IL1RN* expression when compared to other major genotypes *IL1RN**1/2 (p=0.01) and *IL1RN**1/1 (p=0.03). Additionally, we observed *IL1RN**3/2 only in vitiligo patients while *IL1RN**5/2 was observed only in controls.

The present study demonstrates the association of allele as well as genotype of *IL1RN* intron 2 VNTR (A2) polymorphism with active vitiligo patients and increased *IL1RN* expression (allele 2 carriers), suggesting *IL1RN**A2 as a risk factor for progressive vitiligo in Gujarat population. Larger studies with different ethnicities are required to find out the impact of *IL1RN* VNTR polymorphism as a risk factor for developing vitiligo.

b) Genotyping of *IL6* -174 G/C (rs1800795); -572 G/C (rs1800796) promoter polymorphisms and determining the expression levels of *IL6* in Gujarat vitiligo patients and controls

IL6 is a multifunctional Th2 cytokine and increased levels of IL6 have been reported in the serum and lesional skin samples of vitiligo patients. The gene for human *IL6* has been mapped to chromosome 7p21. Increased levels of serum IL6 have been reported in vitiligo patients in Indian population (Singh *et al.*, 2012). IL6 secreted by neighboring keratinocytes in epidermal melanin unit is reported to be a paracrine inhibitor of growth and proliferation of melanocytes (Swope *et al.*, 1990). Several allelic variants have been identified in the *IL6* gene promoter region which regulates the expression of *IL6* (Fishman *et al.*, 1998; Terry *et al.*, 2000). IL6 induces and enhances ICAM-1 expression on melanocytes, which promotes melanaocyte-leukocyte attachment (Yohn *et al.*, 1990). IL6 stimulates the inflammatory and auto-immune processes in

many diseases such as diabetes, atherosclerosis, depression, Alzheimer's disease, systemic lupus erythematosus, multiple myeloma, prostate cancer, and rheumatoid arthritis.

Two promoter polymorphisms of IL6 -572G/C and -174 G/C have not been reported in vitiligo till date. But these polymorphisms have been found to be associated with various autoimmune disorders. Investigated polymorphisms are located adjacent to cis-acting regulatory elements involved in regulating *IL6* expression at the level of transcription, suggesting that they may influence the interaction of proteins with the DNA at these sites (Tanabe *et al.,* 1998). *IL6* -572 G/C (rs1800796) and -174 G/C (rs1800795) polymorphisms were genotyped in 322 vitiligo patients and 343 controls; 100 vitiligo patients and 100 controls respectively, using PCR-RFLP technique.

IL6 -174 G/C (rs1800795) promoter polymorphism was found to be monogenic for GG allele screened in 100 vitiligo patients and equal number of controls in Gujarat population. Due to occurrence of monogenic genotype the sample size was confined to 100 each for patients and controls. Three genotypes were identified in both patients and controls were: GG, GC and CC for *IL6* -572 G/C (rs1800796) promoter polymorphism. The heterozygous 'GC' genotype was found to be significantly higher in control group and is associated with vitiligo susceptibility (p=0.0336, OR=0.6416, CI=0.42-0.9678). In addition, other genotype CC frequencies did not differ significantly between vitiligo patients and controls for *IL6* -572 G/C SNP (p=0.4078, OR=0.8114, CI=0.49 – 1.331). Similarly, the allele frequency did not differ significantly between vitiligo patients and controls (p=0.5281, OR=0.9331, CI=0.75 – 1.157). Further, we analysed the vitiligo patient's data in subgroups based on gender, disease progression and type of vitiligo. Analysis based on male and female revealed that the increased frequency of the 'CC' genotype in male as compared to female vitiligo patients (22.0% versus 9.0%, p=0.0282). However, there was no significant difference between 'GC' genotype and minor allele frequency of 'C' (p=0.3322, OR=0.7781, CI=0.46-1.293 and p=0.1172, OR=1.286, CI=0.93-1.761 respectively).

Analysis based on disease progression revealed that the increased frequency of the minor allele 'C' occurred predominantly in patients with AV (43.0% versus 52.0%, p=0.0025) compared to controls suggesting the important role of 'C' allele in disease progression. However, there was no significant difference between SV patients and controls (p=0.0892). Interestingly, the 'GC' genotype was predominant in controls as compared to AV and SV patients (65.0% versus 57.0% and 50.0% respectively; p=0.0003 and 0.0020 respectively). However, we did not find significant association between other comparisons. Further, analysis based on type of vitiligo revealed that

the increased frequency of the minor allele 'C' occurred predominantly in patients with GV (43.0% versus 52.0%, p=0.0024) compared to controls. However, there was no significant difference between LV patients and controls (p=0.329). Interestingly, the 'GC' genotype was predominant in controls compared to GV patients (66.0% versus 54.0%, p<0.0001) suggesting the important role of 'C' allele in GV. The 'CC' genotype was predominant in controls compared to GV patients (19.0% versus 16.0%, p=0.0013). However, there was no significant difference in genotype and allele frequencies based on age of onset, family history of patients.

Relative gene expression analysis in 70 patients and 74 controls revealed a non-significant difference in expression of *IL6* transcripts in patients as compared to controls (Mean ΔCp – SEM: 12.29 ± 0.7436 versus 12.09 ± 0.6649; p =0.8423). The $2^{-\Delta\Delta Cp}$ analysis showed approximately 0.147-fold increase in the expression of *IL6* transcript levels in patients compared to controls.

Gujarat population is monogenic (allele G) for *IL6* -174 G/C polymorphism; other genotype phenotype correlation studies have shown association of higher transcript levels of *IL6* with G allele in this polymorphism. The -572 G/C polymorphism minor allele 'C' has significant difference in distribution in stratification of vitiligo patients data, whereas there was no significant difference in *IL6* transcript levels between vitiligo patients and controls.

c) Genotyping of *IL10*: -819 C/T (rs1800871), -592C/A (rs1800872) and -1082G/A (rs 1800896) promoter polymorphisms and determining the expression levels of *IL10* in Gujarat vitiligo patients and controls

IL10 acts as an anti-inflammatory cytokine and it has been reported (Abanmi *et al.* 2008) that low levels of *IL10* is governed by the promoter polymorphisms. Hence, it becomes pertinent to investigate *IL10* promoter polymorphisms and its levels in vitiligo patients. Previously, Zhao *et al.,* showed significant decrease in the expression of *IL10* in vitiligo patients as compared to controls (Zhao *et al*. 2010). We have investigated three polymorphisms in promoter region of *IL10* gene for their role in susceptibility to vitiligo, by a case-control study involving around 400 patients and healthy age matched controls by PCR-RFLP and ARMS-PCR techniques. Our results showed that genotype and allele frequencies of *IL10* -819 C/T (p<0.0001; p<0.0001 respectively), were significantly different between vitiligo patients and unaffected controls suggesting the significant association of -819T allele with vitiligo susceptibility. Additionally, the allele frequency of -1082 G/A differed significantly between vitiligo patients and controls (p=0.0311). However, the genotype and allele frequencies for *IL10* -592C/A (p= 0.5796; p= 0.7096

respectively) and genotype frequencies of *IL10* -1082G/A (p = 0.1445) did not differ significantly between vitiligo patients and controls suggesting the non-association of these SNPs with vitiligo susceptibility. The LD analysis revealed that the three promoter polymorphisms investigated in the *IL10* gene were in low to moderate LD association; -819 C/T and -592 C/A polymorphisms were in moderate LD association (D'= 0.516 and r^2 = 0.186). The haplotype ATC (p=1.45 e^{-008}) was found to be less frequent in vitiligo patients, suggesting its crucial role in disease protection. Whereas, ACC (p=0.196 X 10^{-3}), GCC (p=0.021), GTA (p= 0.019) haplotypes were more found to be significantly associated with patients, suggesting their importance in vitiligo susceptibility. However, ACA (p=0.3401) and GCA (p=0.546) haplotypes were not found to be associated with vitiligo. Further, transcript as well as protein levels of IL10 will be estimated from PBMCs and plasma respectively in vitiligo patients and controls, and the results will be discussed in thesis.

The present study supports the autoimmune hypothesis of vitiligo pathogenesis with strong association of *IL10* -819C/T polymorphism in vitiligo patients thus, suggesting the crucial role of *IL10* in vitiligo pathogenesis. The present study will pave the pathway for understanding the higher prevalence of vitiligo in Gujarat population.

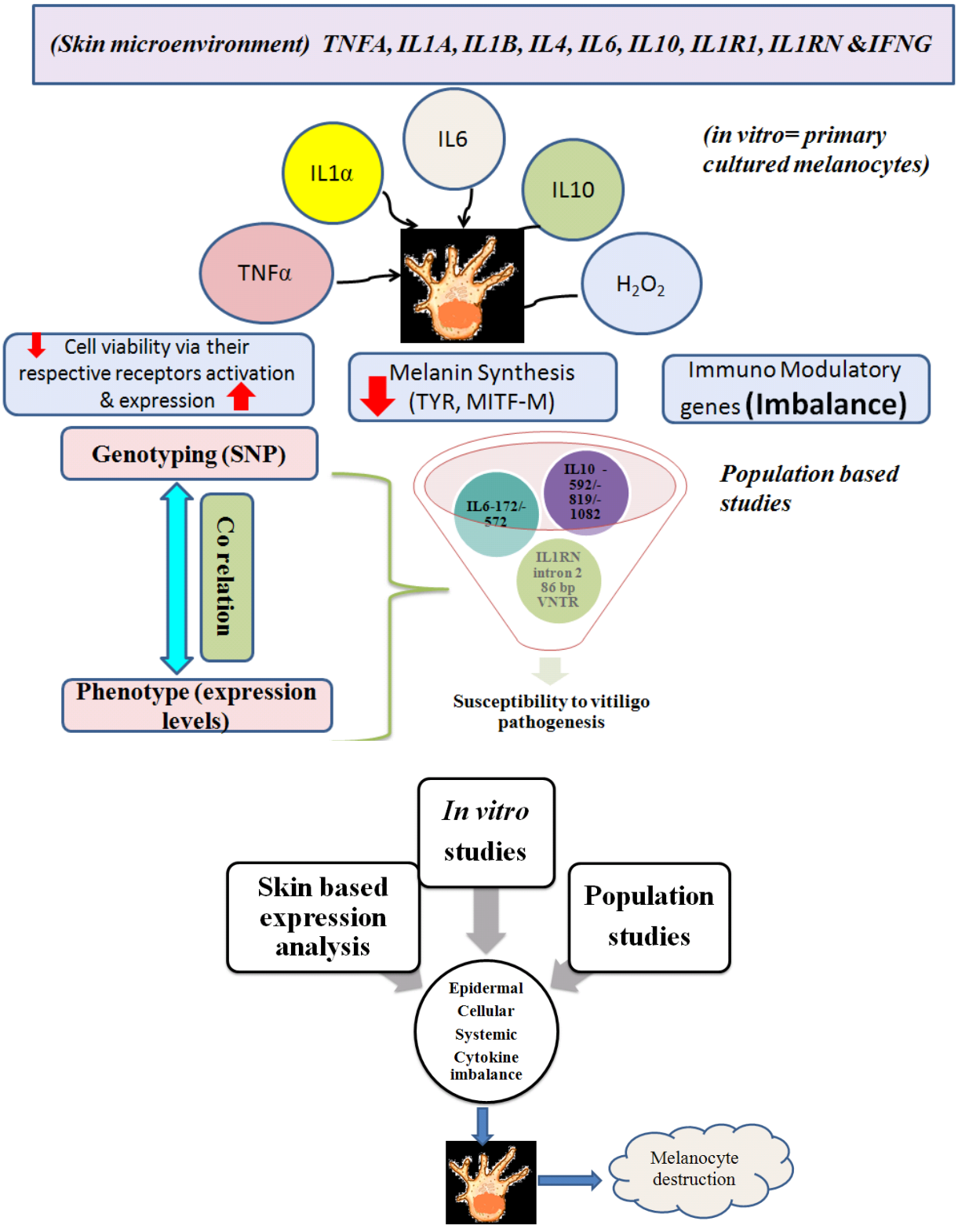

Figure 1: Role of cytokine imbalance in vitiligo pathogenesis: Increased levels of pro inflammatory cytokines *TNFA*, *IFNG* and *IL1B*; decreased levels of anti inflammatory cytokine

IL10 was observed in the skin microenvironment if vitiligo patients as compared to controls. Pro inflammatory cytokines: TNFα, IL1α, IL6 as well as H_2O_2 decreased melanocytes viability alone as well as in synergism. Additionally, the pro inflammatory cytokines altered expression of melanin synthesis genes (*TYR, MITF-M*), cell adhesion molecule (*ICAM-1*) as well as other immunoregulatory genes. On the contrary, IL10 was capable to rescue TNFα induced cytotoxicity in melanocytes. Population based study indicated the importance of genetic variants in vitiligo predisposition. Analysis of single nucleotide polymorphisms at promoter sites (*IL6, IL10*) as well as VNTR polymorphisms (*IL1RN)* of addressed cytokines revealed association of a few studied genetic variants with vitiligo susceptibility in Gujarat population. The genotype-phenotype analysis will be discussed in the thesis for holistic view of addressed polymorphisms. Overall, cytokine imbalance at tissue, cellular and genetic levels may aggravate the compromised state of melanocytes, advocating autoimmune mediated disease progression concept.

Conclusion: The present study suggests the cytokine imbalance and its involvement in vitiligo pathogenesis. Our skin based studies showed cytokine imbalance of pro- and anti inflammatory cytokines in the microenvironment of vitiligo. Expression levels of *TNFA, IFNG* were found to be increased in lesional and non lesional skin of vitiligo patients as compared to controls. Whereas, non significant changes in the expression levels of *IL1A, IL4, IL6, IL1R1*and *IL1RN* were observed in lesional skin compared to non lesional skin of vitiligo patients, as well as control skin. In addition, decreased levels of *IL10* (anti inflammatory cytokine) were observed in lesional skin of vitiligo patients compared to controls skin. Candidate cytokines TNFα, IL1α, IL6 and IL10 were further used for *in vitro* studies on primary normal human melanocytes (NHM). Pro inflammatory cytokines TNFα, IL1α, IL6 as well as H_2O_2 stress (which is higher in vitiligo skin) showed significant decrease in NHM viability. Interestingly, NHM upon H_2O_2 treatment showed PARP1 activation and increased PARylation of proteins which could be intercepted by PARP inhibitor (PJ34). The cytokines mediate their action *via* increased expression of the respective receptors on melanocytes. TNFα and IL1α also showed decreased expression of melanin synthesis genes *TYR, MITF* and melanin content upon exogenous stimulation of melanocytes. Increased expression of *ICAM* was observed which could act as a link between cytokines and T-cells; and T cells among others play a key role in the pathogenesis of generalized vitiligo. Further, to study genetic predisposition of vitiligo patients in Gujarat population, we investigated the promoter polymorphisms of *IL10* -819 C/T (rs1800871), -592C/A (rs1800872) and -1082G/A (rs 1800896); the promoter polymorphisms of *IL6* -174 G/C (rs1800795) -572 G/C (rs1800796) and *IL1RN*

intron 2 VNTR (rs2234663) polymorphism and performed possible genotype -phenotype correlation. Overall, our studies suggest compromised state of melanocytes due to cytokine imbalance that could lead to melanocyte loss in vitiligo pathogenesis (Figure 1).

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