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.

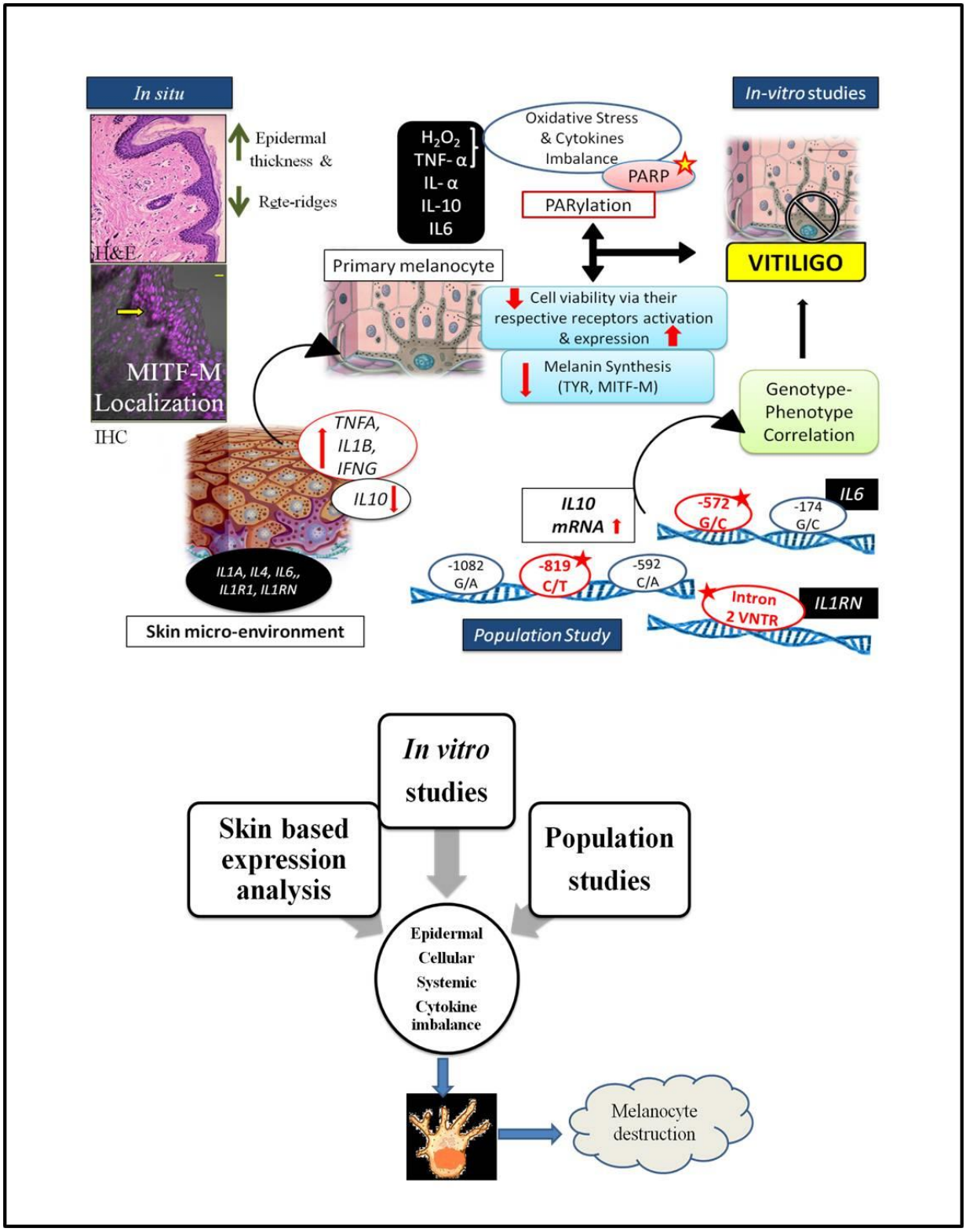
In situ
Epidermal thickness &
Rete-ridges
H&E
MITF-M Localization
IHC
TNFA, IL1B, IFNG
IL10
IL1A, IL4, IL6,, IL1R1, IL1RN
Skin micro-environment
H2O2 TNF-α IL-α IL-10 IL6
Oxidative Stress & Cytokines Imbalance
PARP
PARylation
Primary melanocyte
Cell viability via their respective receptors activation & expression
Melanin Synthesis (TYR, MITF-M)
In-vitro studies
VITILIGO
Genotype-Phenotype Correlation
IL10 mRNA
IL6
-572 G/C
-174 G/C
-1082 G/A
-819 C/T
-592 C/A
Intron 2 VNTR
IL1RN
Population Study
In vitro studies
Skin based expression analysis
Population studies
Epidermal
Cellular
Systemic
Cytokine imbalance
Melanocyte destruction

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.