CHAPTER I

INTRODUCTION

"I look to a day when people will not be judged by the color of their skin, but by the content of their character."

Martin Luther King, Jr.

Vitiligo is an acquired, progressive, multifactorial, depigmenting disorder characterized by the appearance of circumscribed white macules in the skin due to chronic, progressive loss of functional melanocytes in the epidermis (Guerra *et al.*, 2010). The selective destruction of melanocytes results in the development of depigmented patches. The disease can have devastating consequences on an individual's relationships with others and internal feelings of self-worth. Immediate recognition of an individual's difference from the norm is through their appearance. Not only can appearance indicate an underlying difference but it is a source of deviance in itself as vitiligo is a cosmetically disfiguring condition. As such vitiligo is hardly a disease of medical significance but there is more of a social stigma attached to it because of cosmetic reasons. Although it might be viewed as minor disorder, these patches gradually increase in size and cause lot of psychological stress in the patient, self esteem and social interactions, particularly in patients with deeply pigmented skin (Kent *et al.*, 1996). Young women face more social stigma and suffer due to matrimonial problems.

Based on a few dermatological outpatient records the prevalence of vitiligo is found to be 0.5 to 1% of the world population (Taieb, 2007), 0.5 to 2.5 % in India (Handa *et al.*, 1999) and the states of Gujarat & Rajasthan have the highest prevalence i.e ~8.8% (Valia *et al.*, 1996). Vitiligo affects all with no predilection for gender or race, and usually starts in childhood or young adulthood. Manifestation begins before 20 years of age in 50% of the cases, while in 25% of the cases the onset is before 14 years of age (Kakourou, 2009). Familial cases of vitiligo are common indicating a hereditary factor; 6%-38% of vitiligo patients have family members with the disease (Ortonne *et al.*, 1983).

Though vitiligo has been extensively addressed in the past six decades, its etiology is still being debated (Taieb, 2000). Many possible causes of vitiligo have been

proposed, including stress, infections, mutations, neural factors, melatonin receptor dysfunction, and impaired melanocyte migration and/or proliferation. In addition, the accumulation of toxic intermediate products of melanin synthesis (Pawelek *et al.*, 1980; Moellmann *et al.*, 1982) the breakdown of free radical defense (Nordlund *et al.*, 1982) and the build-up of excessive quantities of hydrogen peroxide (Schallreuter *et al.*, 1991; Schallreuter *et al.*, 1994) have all been suggested to result in the self-destruction of pigment cells. An autoimmune etiology has also been proposed, and this is supported by the frequent association of vitiligo with autoimmune diseases (Ochi *et al.*, 1969; Macaron *et al.*, 1977) together with studies demonstrating that many vitiligo patients have autoantibodies and autoreactive T cells against melanocyte antigens (Naughton *et al.*, 1983; Bystryn *et al.*,1985). It is possible that these different causal factors can act independently or together to yield the same effect - namely, the disappearance of melanocytes from the skin (Le Poole *et al.*, 1993). Vitiligo is a complex, polygenic disorder (Strömberg *et al.*, 2008). Gene expression studies, allelic association studies of candidate genes and genome-wide linkage analyses are being used to discover new genes, and these studies have begun to shed light on the mechanisms of vitiligo pathogenesis (Spritz, 2008).

1.1 STRUCTURE OF SKIN

The skin is the largest body organ and functions as a metabolically active biological barrier separating internal homeostasis from the external environment. Depending on anatomic localization and environmental influences, the skin shows remarkable functional and structural diversity (Slominski and Wortsman, 2000). The dermal-epidermal junction is undulating in section; ridges of the epidermis, known as rete ridges, project into the dermis (Figure 1). The junction provides mechanical support for the epidermis and acts as a partial barrier against exchange of cells and large molecules. Below the dermis is a fatty layer, the panniculus adiposus, usually designated as 'subcutaneous'. This is separated from the rest of the body by a vestigial layer of striated muscle, the panniculus carnosus. The superficial epidermis is a stratified epithelium largely composed of keratinocytes that are formed by division of cells in the basal layer, and give rise to several distinguishable layers as they move outwards and progressively differentiate. Within the epidermis, there are

everal other cell populations, namely melanocytes, which donate pigment to the keratinocytes; Langerhans cells, which have immunological functions; and Merkel cells. The hair follicles comprise pockets of epithelium that are continuous with the superficial epidermis. They undergo intermittent activity throughout life. During the active phase, the follicle envelops at its base a small papilla of dermis. A bundle of smooth muscle fibres, the arrector pili, extends at an angle between the surface of the dermis and a point in the follicle wall. Above the insertion, the holocrine sebaceous gland opens by a short neck into the pilary canal, and some follicles in certain areas of the body, notably the axilla, have, in addition, an apocrine gland. Also derived from the epidermis and opening directly to the skin surface, are the eccrine sweat glands, present in every region of the body in densities of 100–600/cm^2. The basis of the dermis is a supporting matrix or ground substance in which polysaccharides and proteins are linked to produce macromolecules with a remarkable capacity for retaining water. Within and associated with this matrix are two kinds of protein fibres: collagen, which has great tensile strength and forms the major constituent of the dermis, and elastin, which makes up only a small proportion of the bulk. The cellular constituents of the dermis include fibroblasts, mast cells and histiocytes (monocytes/macrophages). The dermis has a very rich blood supply, although no vessels pass through the dermal-epidermal junction. The motor innervation of the skin is autonomic, and includes a cholinergic component to the eccrine sweat glands and adrenergic components to both the eccrine and apocrine glands, to the smooth muscle and the arterioles and to the arrector pili muscle. The sensory nerve endings are of several kinds: some are free, some terminate in hair follicles and others have expanded tips.

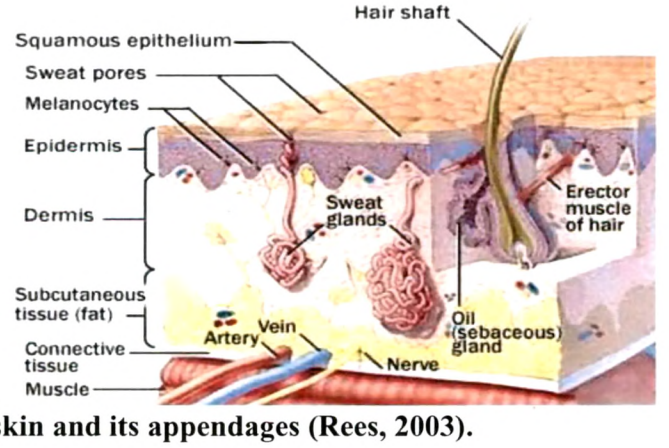

Figure 1: The skin and its appendages (Rees, 2003).

1.1.1 Colour of the skin:

Skin color is primarily due to the presence of a pigment called melanin. Both light and dark complexioned people have this pigment. Under normal conditions it is not the number of melanocytes in the skin that determine the degree of pigmentation but their level of activity. Skin color is also affected by red cells in blood flowing close to the skin. To a lesser extent, the color is affected by the fat under the skin and carotene a reddish-orange pigment in the skin.

1.1.2 Melanocytes:

The melanocytes are neural crest derived cells that migrate via mesenchyme into the epidermis and hair follicles during embryogenesis (Bolognia and Orlow, 2003). Apart form the skin, melanocytes are known to be present in other areas such as retinal pigment epithelium, uveal tract, inner ear and leptomeninges.

In the skin melanocytes reside in the basal layer of the epidermis and in the matrix of the hair follicle. They derive from the melanoblasts that originate from the neural crest from where they migrate during embryogenesis. Melanocytes are highly dendritic and these dendrites project into the malphigian layer of the epidermis where they transfer the melanosomes to keratinocytes (Jimbow *et al.,* 1999). Each epidermal melanocyte secretes melanosomes into approximately 36 keratinocytes in the neighborhood and this entire unit is called epidermal melanin unit (Figure 2).

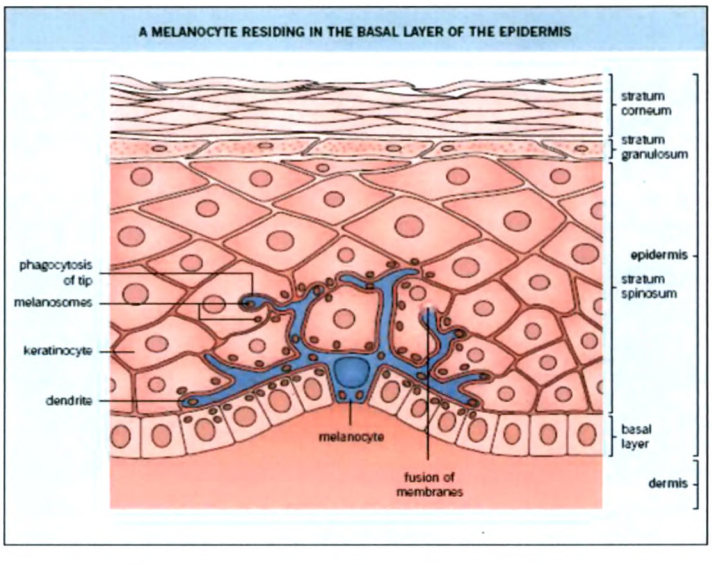

Figure 2. Epidermal melanin unit (Bolognia, Jorizzo, and Schaffer, *Dermatology*, 3rd edition).

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

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

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

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

Stage IV, with 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).

1.1.3 Melanogenesis:

Pheomelanin and eumelanin are the two pigments formed during melanogenesis. Pheomelanin and eumelanin differ not only in colour but also in the size, shape and packaging of their granules (Slominski *et al.*, 2004). Both melanins derive from a common tyrosinase-dependent pathway with the same precursor, tyrosine (Figure 3).The obligatory step is hydroxylation of tyrosine to dopaquinone, from which L-DOPA can also be derived (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) and TRP2 (also known as dopachrome tautomerase). Pheomelanin is derived from conjugation by thiol-containing cysteine or glutathione. As a result, pheomelanin is more photolabile and can produce, among its by-products, hydrogen peroxide, superoxide and hydroxyl radicals, all known triggers of oxidative stress, which can cause DNA damage. Individual melanocytes typically synthesize both eumelanins and pheomelanins, 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.

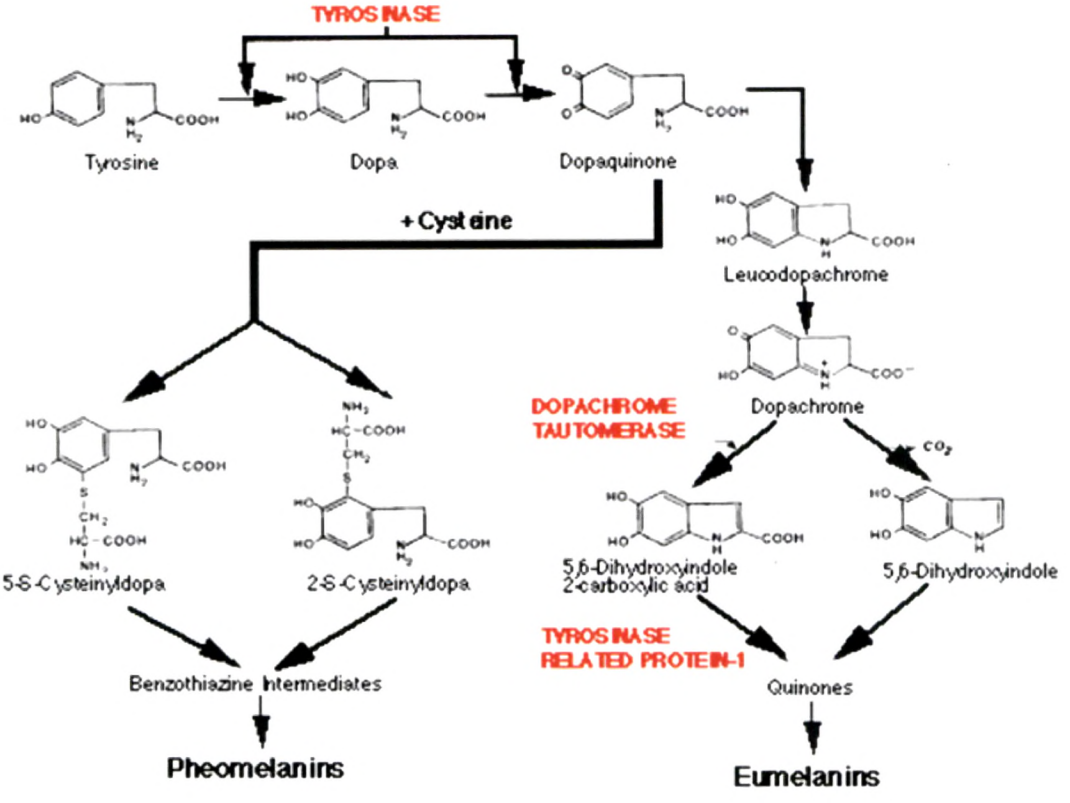

Figure 3. Melanin synthesis pathway.

Once melanin is produced, the melanosomes are transferred into the neighboring keratinocytes. The size of these organelles and their numbers are important in determining skin pigmentation. The melanosomes in 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. Seiberg *et al.*, 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).

1.1.4 Role of melanocytes:

- Melanocytes are responsible for skin, hair and eye color by manufacturing melanin in melanosomes and mediating their transfer to neighboring cells via dendritic processes.
- Melanocytes are responsible for sun tanning, as UV irradiation leads to increased melanin production and transfer of melanosomes to keratinocytes to protect against UV-induced DNA damage.
- Melanocyte stem cells reside in the bulge of the hair follicles and loss of the stem cell population gives hair graying.
- Melanocytes located in the stria vascularis are required for hearing and their loss can lead to deafness. (Hearing *et al.*, 2000).

1.1.5 Melanocyte immunobiology:

Melanosomes contain enzymes and structural components required for effective melanization. MART-1 appears to lay the groundwork for the deposition of the melanosomal matrix, assisted by gp100 (Hoashi *et al.*, 2005). These two molecules are also considered as the most immunogenic antigens of the melanocytic cells and T cells infiltrating melanoma tumors are most frequently reactive with either of these antigens (Kawakami *et al.*, 1997).

The subsequent arrival of tyrosinase and tyrosinase related enzymes TRP-1 and TRP-2 completes the arsenal of melanosomal enzymes. The melanosome is unique in particular because it is transferred to neighboring cells in a process that remains poorly understood till date. This process is of particular interest to immunologists. Organelle transfer has otherwise been described only for exosomes, small lipid vesicles serving to discard or transfer membrane proteins. Exosomes are considered important for Dendritic Cell activation and the induction of immune responsiveness to antigens contained within these organelles (Taieb *et al.*, 2005). Interestingly,

organelles transferred from melanocytes to keratinocytes are in fact equivalent to lysosomes in other cells. It is well possible that lysosome transfer can likewise occur between cells as well but cannot be easily observed for lack of a marker as readily detectable as melanin. The transfer of lysosome-like organelles may allow for sharing of antigens destined for presentation in the context of MHC class II. It should be noted that ripe melanosomes do not contain intact melanogenic enzymes as shown by melanocyte fractionation (Orlow *et al.,* 1993). But peptide fragments may be generated within the acidic environment of the melanosome. Resulting peptides may retain their immunogenicity when transferred to dendritic cells. Thus peptides derived from gp100, MART-1 and other melanosomal proteins, may be transferred to infiltrating dendritic cells in the absence of melanocyte death within the skin.

Melanocyte immunogenicity has also been ascribed to their Langerhans cell-like morphology and distribution. Melanocytes synthesize primary cytokines, and are thus capable of eliciting a local immune response (Kruger-Krasagakes *et al.,* 1995). Moreover, melanocytes are capable of phagocytosis and can process and present antigens in the context of MHC class II to $CD4^+$ proliferative and cytotoxic T cells (Le Poole *et al.,* 1993, Le Poole *et al.,* 1993). As melanocytes lack the mobility of Langerhans cells and other professional antigen presenting cells, it is conceivable that antigen presentation within the skin renders the melanocyte a 'sitting duck' for an immune attack. Of course, this situation will arise only when the melanocyte expresses MHC class II molecules, which happens to be the case only in vitiligo and in melanoma (Overwijk *et al.,* 2000).

1.2 TYPES OF VITILIGO

Vitiligo is most often classified clinically according to the extent and distribution of depigmentation (Figure 4) (Gawkrodger et al., 1998). 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 et al., 2000) because, compared with focal and segmental vitiligo non-segmental forms show a later age of onset, a stronger association with autoimmunity and unstable results following autologous grafting.

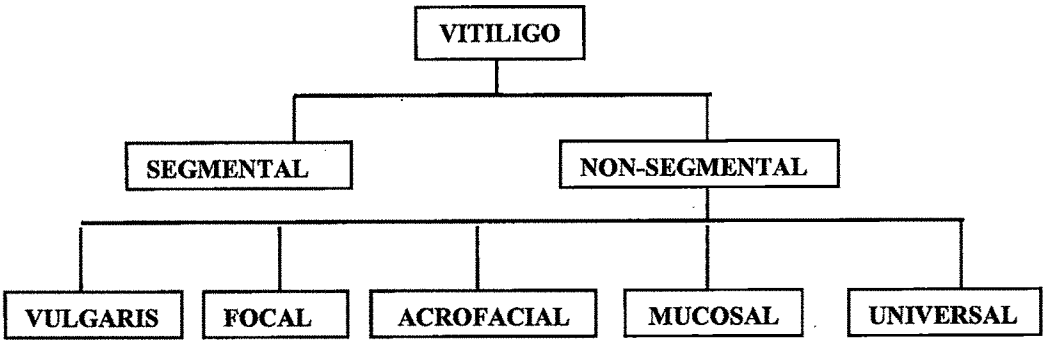

Figure 4: Classification of vitiligo (Dave *et al.*, 2002).

1.2.1 Segmental/unilateral-

It is often present in childhood and occurs in dermatomal, asymmetric distribution with one or more macules localized to one area of the body. It is rarely associated with autoimmune disorders and responds well to autologous grafting (Kemp *et al.*, 2001).

1.2.2 Focal vitiligo-

It describes one or more depigmented patches localized in a discrete area with autoimmune disorders and responds well to autologous grafting (Kemp *et al.*, 2001).

1.2.3 Symmetrical/bilateral vitiligo-

This is the most common type of vitiligo and is often referred to as generalized. It is characterized by a bilateral, symmetrical depigmentation with a widespread distribution of many macules in a random pattern. Many parts of body can be affected, including the face (particularly periorificial areas), the neck, torso, hand and legs. A latter age of onset is normal for this clinical subclass and it is often associated with autoimmunity. Unstable results are evident following autologous grafting in patients with this type of vitiligo (Kemp *et al.*, 2001).

1.2.4 Acrofacial vitiligo-

Vitiligo of this type is characterized by depigmentation of the distal fingers and facial orifices- the latter in a circumferential pattern. It often forms with symmetrical vitiligo (Kemp *et al.*, 2001).

1.2.5 Universal vitiligo-

This type of vitiligo is characterized by loss of pigmentation over the entire body, but is rare (Kemp *et al.*, 2001).

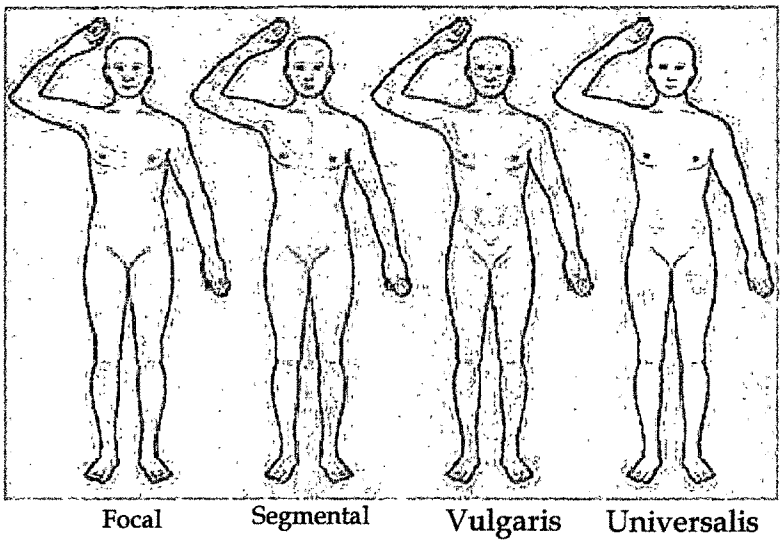

Figure 5: Clinical types of vitiligo

1.3 Causes of vitiligo:

Vitiligo involves complex interaction of environmental and genetic factors that ultimately contribute to melanocyte destruction, resulting in the characteristic depigmented lesions (Spritz, 2008).There is a gradual loss of melanin pigment from the skin layers which results in white patches. It is difficult to precisely define the triggering factors for vitiligo. Nevertheless, it is essential to elicit the details of the history of emotional stress, drug intake, infections, trauma/injury (Koebner phenomenon) existent prior to the development of vitiligo lesions. Figure 6 shows various probable triggering factors in the natural history/ development of vitiligo. A better understanding of these factors may prove to be helpful in the management of vitiligo.

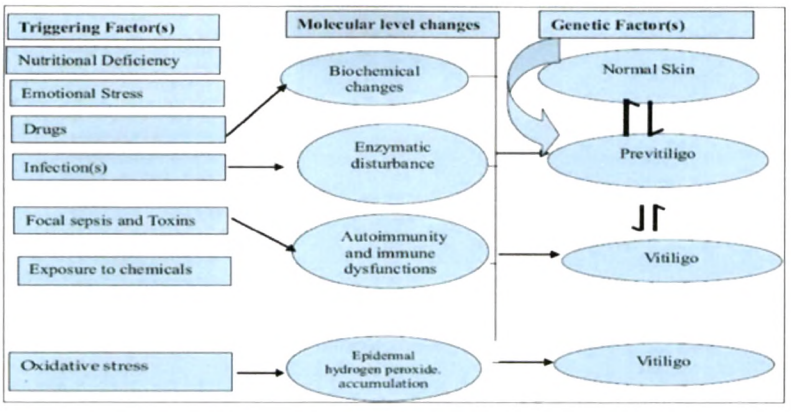

Figure 6: The triggering factors in vitiligo.

1.4 Pathogenesis of vitiligo:

Vitiligo is an idiopathic disease. Many triggering factors for vitiligo have been proposed. As patients are not born with the disease, it is thought that an initiating event, such as illness, stress, UV exposure or injury may trigger depigmentation.
Various hypotheses have been proposed explaining the etiology of vitiligo and the most important theories are:

- Oxidative stress
- Neurochemical
- Autoimmune
- Genetic

It is possible that these different causal factors can act independently or together to yield the same effect i.e. disappearance of melanocytes from the skin (Le Poole *et al.*, 1993).

1.4.1 Oxidative Stress and Vitiligo:

Oxidative stress is a major form of assault on the skin. Skin exposure to ionizing and UV radiation and/or xenobiotics/drugs generates ROS in excessive quantity. It overwhelms tissue antioxidants and other oxidant-degrading pathways. Uncontrolled release of ROS and increased lipid peroxidation are seen in multiple skin disorders such as atopic dermatitis, psoriasis, acne vulgaris, pemphigus vulgaris (PV), lichen

planus, alopecia areata and vitiligo (Yesilova *et al.*, 2012). Oxidative stress has been implicated in the initial pathogenic event in melanocyte destruction (Schallreuter *et al* 1999a; Maresca *et al.,* 1997) marked by accumulation of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al.*, 1999a; Schallreuter *et al.*, 2001). Defective recycling of tetrahydrobiopterin in vitiligo epidermis is related with the intracellular production of H_2O_2 (Schallreuter *et al* 1994; Schallreuter *et al.*, 1999b). In addition, a compromised antioxidant status with a significant reduction in catalase activity and increase in SOD activity has been demonstrated in both lesional and non-lesional epidermis (Schallreuter *et al.*, 1991; Sravani *et al.*, 2009) as well as in melanocytes (Maresca *et al.*, 1997). Interestingly, antioxidant imbalance has also been observed in the peripheral blood mononuclear cells of active vitiligo patients. This was correlated with an increased intracellular production of reactive oxygen species due to mitochondrial impairment (Dell'Anna *et al.*, 2001), supporting the concept of a possible systemic oxidative stress in vitiligo.

In normal cellular processes, O_2^- radicals are converted into H_2O_2 due to the action of SOD enzyme which in turn converted to oxygen and water by the action of catalase and glutathione peroxidase. As a result cell maintains its normal integrity and function. Sometimes equilibrium of antioxidants and oxidants gets imbalanced which leads to accumulation of free radicals resulting into accumulation of oxidative stress (Figure 7). This accumulated oxidative stress causes DNA damage (Mohamed and Salem, 2009), lipid and protein peroxidation .Many proteins and peptides, results in altered or even complete loss of functionality due to H_2O_2-mediated oxidation.

The inhibition of thioredoxin reductase, a free radical scavenger located in the membrane of melanocytes, also seems to contribute to the generation of oxidative stress in the vitiligo epidermis (Schallreuter and Pittelkow, 1988). Further, increased amount of superoxide radicals generated due to elevated levels of extracellular calcium levels has been shown to inhibit tyrosinase (Schallreuter *et al.*, 1996b). Several sources have been documented for the unusual production/accumulation of epidermal H_2O_2 (Passi *et al.*, 1998; Schallreuter *et al.*, 1991; 1996a; 1999a; Rokos *et al.*, 2002). Since even millimolar levels of H_2O_2 can inactivate catalase (Aronoff 1965; Schallreuter *et al.*, 1991; Dell'Anna *et al.*, 2001) and glutathione peroxidase (Beazley

et al., 1999; Agrawal *et al.*, 2004), such high levels of accumulation is likely to have detrimental effects (Figure 8, pathways 1,2 and 4). Contextually, higher concentration of H_2O_2 by its inhibitory effect on 4 α-carbinolamine dehydratase is shown to disrupt recycling of the essential cofactor (6R)- L - erythro- 5,6,7,8 tetrahydrobiopterin ($6BH_4$) for aromatic amino acid hydroxylases as well as nitric oxide synthases (Hasse *et al.*, 2004). Further, Giovannelli *et al.*, 2004 have shown significantly higher level of oxidative DNA damage in mononuclear leukocytes in active vitiligo patients compared to controls.

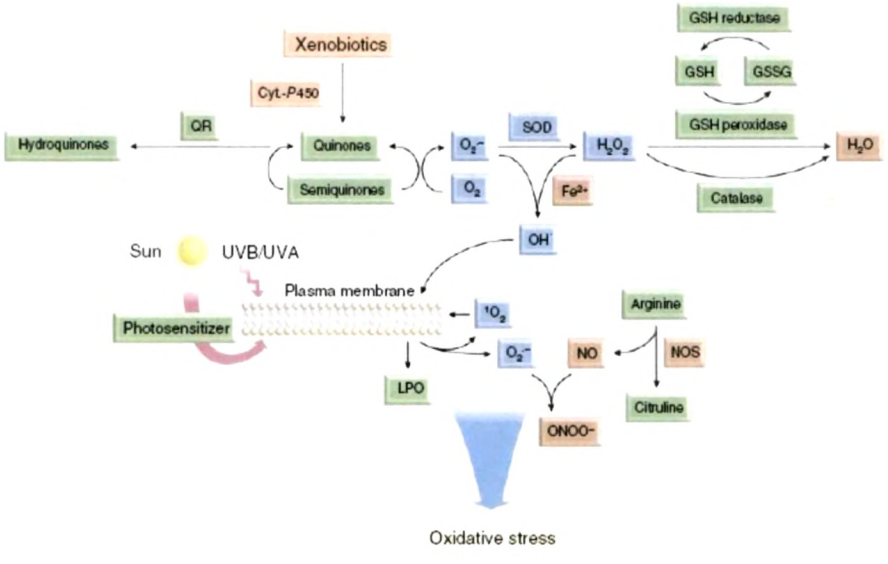

Figure 7: Reactive oxygen species generation leading to oxidative stress.

We have reported systemic oxidative stress in vitiligo patients due to an imbalance in enzymatic and non-enzymatic antioxidant systems (Agrawal *et al.*, 2004). Our observations suggest different mechanisms of generation of oxidative stress in different clinical types of vitiligo (Shajil and Begum, 2006). Whereas low levels of catalase may contribute to the generation of oxidative stress in segmental vitiligo, generation of oxidative stress in non-segmental vitiligo appears attributable to lower levels of glutathione peroxidase and reduced glutathione (Shajil and Begum, 2006). Moreover, our studies on neurochemical basis of vitiligo have documented significantly decreased acetylcholine esterase (AChE) activity (Shajil *et al.*, 2006). This could be due to H_2O_2 mediated oxidation of AChE, which emphasizes the role of oxidative stress in precipitating vitiligo (Schallreuter *et al.*, 2004). Natarajan *et al.*, 2010 have also shown the involvement of Nrf2-dependent Phase II detoxification

pathway, considering its importance in the regulation of epidermal skin homeostasis. The role of enhanced Nrf2 effectors seems to be the prime protective function conferred by this pathway on skin homeostasis. Apart from the protective response, phase II genes could also be interestingly responsible for sustaining depigmented lesions in vitiligo patches. Thus, this study demonstrated the involvement of Nrf2 and phase II genes in homeostatic mechanisms of vitiligo skin and moreover, lesional skin from vitiligo patients recorded higher levels of oxidative stress compared to non-lesional pigmented skin (Natarajan *et al.*, 2010). Eskandani *et al.*, 2010 have reported increased DNA damage in leucocytes and lowered levels of tyrosinase activity in lesional skin of vitiligo patients compared to their non-lesional skin. Tyrosinase is an important sensitive enzyme in pigmentation process as a range of factors can influence its activity including oxidative agents such as H_2O_2. In presence of DOPA substrate and 3-methylbenzothiazolinone-2-hydrazone (MBTH), H_2O_2 can function as an inhibitor of tyrosinase or, the presence of H_2O_2 and DOPA substrate can generate a secondary complex that can bind and inhibit the enzyme. This study suggested a meaningful correlation between increased oxidative stress and decreased tyrosinase activity (Eskandani *et al.*, 2010). In addition, an important role for oxidative stress in pathogenesis of vitiligo in experimental mice, suggesting that melanocyte damage in vitiligo might be linked to generalized oxidative stress (Jalel *et al.*, 2009).

Thus high ROS/RNS in melanocytes may cause the cell to undergo defective apoptosis and release of aberrated proteins from the cell. Failure of phagocytosis of these apoptotic cells causes expression of auto-antigens. These auto-antigens are presented by MHC to T-cells leading to autoimmunity (Kühtreiber *et al.*, 2003). TNF-α is known as a paracrine inhibitor of melanocyte growth and increased levels of TNF-α cause maturation of dendritic cells and thus results in to development of autoimmunity (Clemens *et al.*, 2000). The intracellular levels of H_2O_2 and other ROS also increase in several cellular systems in response to external stimuli and cytokines such as TNF-α and TGF β1 (transforming growth factor β1) (Celia *et al.*, 2001). High ROS also increases the levels of cytokines, including IL-2 which upregulates the expression of anti-apoptotic protein, Bcl-2 thereby making T-cells resistant to apoptosis (Figure 9, pathway 2). These cytokines are potent inhibitors of melanogenesis in B16 melanoma cells and human melanocytes (Swope *et al.*, 1991; Martínez-Esparza *et al.*, 1997; Martínez-Esparza *et al.*, 1998). In addition, the

generation of ROS/RNS and/or the resulting increase in lipid peroxidation products have been proposed for hair graying (Nordlund and Abdel-Malek, 1988) and several pathological conditions, like vitiligo (Passi *et al.*, 1998).

1.4.2 Neural Factors and vitiligo:

Melanocytes are neural crest derived cells giving them an embryological link to the nervous system (Reedy *et al.*, 1998). Neurochemical mediators that are secreted by the nerve endings such as norepinephrine and acetylcholine are toxic to melanocytes (Figure 8, pathway 3). Different studies on vitiligo patients showed higher levels of plasma and urinary catecholamines and their metabolites especially at the onset and in the active stage of the disease (Morrone *et al.*, 1992; Orecchia *et al.*, 1994; Cucchi *et al.*, 2000; Cucchi *et al.*, 2003). An increased release of catecholamines from the autonomic nerve endings in the microenvironment of melanocytes causes melanocyte death and ultimately resulting in to vitiliginous lesions (Figure 8, pathway 3).
Norepinephrine has both direct and indirect melanocytotoxic actions which include its interaction with cellular sulfhydryl groups, enzyme inhibition, impairing mitochondrial calcium uptake, and forming some cytotoxic products including free radicals (Figure 9, pathway 1). High concentrations of norepinephrine and its metabolites in vitiligo patients may be due to a reduction in phenylethanolamine-N-methyl transferase (PNMT) activity and an increase in tyrosine hydroxylase (TH) activity (Schallreuter *et al.*, 1994). These enzymes play a key role in production of L-DOPA form, L-tyrosine. Also (6R)-5,6,7,8-tetrahydrobiopterin (6BH4), the rate limiting cofactor/electron donor for TH is increased due to decreased 4a-hydroxy-6BH4 dehydratase (DH) activity in vitiligo patients (Schallreuter *et al.*, 2001). There is also a defective recycling of $6BH_4$ which leads to increased non-enzymatic production of $7BH_4$, an isomer, concomitant with an increased production of H_2O_2. Presence of $7BH_4$ in the epidermis seems to initiate the process of depigmentation in vitiligo patients by blocking the supply of L-tyrosine to melanocytes. These alterations seem to cause melanocyte destruction in vitiligo (Schallreuter *et al.*, 1994). The indirect effects include activating α-receptors of the arterioles, causing a severe vasoconstriction, and thereby producing toxic oxygen radicals caused by hypoxia. Increased levels of norepinephrine also appear to induce another catecholamine degrading enzyme, monoamine oxidase (MAO) (Bindoli *et al.*, 1992). Keratinocytes

and melanocytes in the depigmented skin exhibit increased monoamine oxidase-A activity which further causes keratinocytes to produce 4-fold more norepinephrine and 6.5-fold less epinephrine than control keratinocytes (Schallreuter *et al.*, 1996a). Norepinephrine is reported to be toxic to melanocytes. The increased MAO-A activity favors the formation of hydrogen peroxide, which is toxic to melanocytes (Schallreuter *et al.*, 1996a). Moreover, the induced damage to melanocytes is not buffered by low epidermal catalase levels (Schallreuter *et al.*, 1991).

A derangement of the enzyme dealing with catabolism of adrenergic transmitters namely catechol-o-methyl transferase (COMT) is also reported. COMT normally prevents the formation of toxic ortho quinones during melanin synthesis. Vitiligo patients show higher epidermal COMT activity, probably induced in the tissues by elevated levels of catecholamines secreted by keratinocytes or by nerve endings (Le Poole *et al.*, 1994).

In addition, neurochemical mediators such as acetylcholine secreted by the nerve endings cause the destruction of melanocytes. Acetylcholine esterase activity is found to be lowered in vitiliginous skin during depigmentation (Iyengar, 1989). Our study also reported decreased acetylcholine esterase activity in vitiligo patients and suggested that acetylcholine esterase may be inactivated due to high systemic oxidative stress in these patients (Shajil *et al.*, 2006).

Aberrations in beta-endorphin and met-enkephalin secretion are also reported in vitiligo patients (Mozzanica *et al.*, 1992). In vitiligo, the levels of met-enkephalin levels are found to be higher. It is suggested that this abnormality may be correlated with the emotional stress, which precipitates vitiligo in some patients. Abnormalities of neuropeptides are observed in perilesional skin and blood of vitiligo patients (Al'Abadie *et al.*, 1994). The neuropeptide Y (NPY) is released by either exogenous stimulus like trauma (e.g. Koebner phenomenon) or by endogenous stimuli (e.g. stress) (Al' Abadie *et al.*, 1994) and this altered balance of neuropeptides in vitiliginous skin supports a role for the nervous system in the pathogenesis of vitiligo (Liu *et al.*, 1996). Neuropeptides are also reported to have immunoregulatory effects (Covelli and Jirillo, 1988). Caixia *et al.*, 1999 showed that the levels of NPY in the plasma of vitiligo patients were found to be significantly higher than the normal

controls. The levels of NPY from skin lesions were significantly higher than those from uninvolved skin in both the local type and segmental types of vitiligo. NPY could evoke the secretion of IFN- γ and IL- 2 (Figure 8, pathway 2) suggesting that NPY might be involved in the cell mediated immunological mechanism, which plays a role in the melanocyte destruction in vitiligo (Caixia *et al.*, 1999).

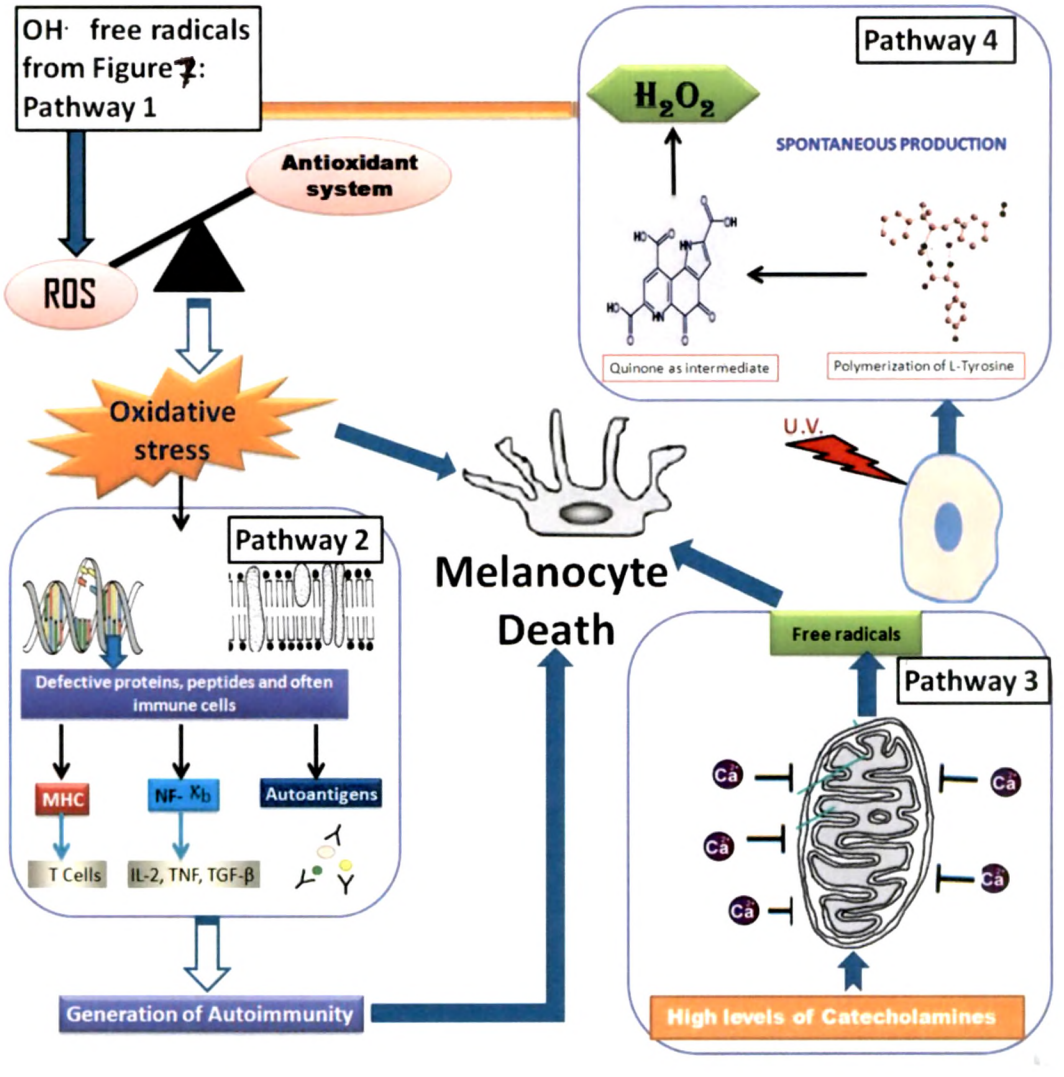

Figure 8: Different pathways for melanocyte destruction:

1. Generation of ROS by various metabolic processes. 2. Imbalance in ROS generation and antioxidant system leads to accumulation of free radicals resulting in oxidative stress. This accumulation causes DNA damage, synthesis of defective proteins and membrane disintegration which provokes immune system resulting in autoimmunity. 3. Increased catecholamines inhibits mitochondrial calcium uptake

which results in free radicals generation 4. Exposure to UV radiation, leads in spontaneous production of quinones in melanocytes which in turn results in ROS generation.

1.4.3 Autoimmunity and vitiligo:

Different hypotheses have been proposed to explain this disorder (Halder and Chappell, 2009). Nevertheless, there is strong evidence that vitiligo is preferentially an autoimmune disease. The association with autoimmune conditions such as Addison's disease, hypothyroidism, pernicious anemia and systemic lupus erythematosus; presence of some alleles of MHC II antigens and other autoimmune-susceptibility genes; detection of organ-specific antibodies (anti-ANA, anti-gastric parietal cells) in the serum of patients with vitiligo; positive response to topical immunosuppressant therapy (topical steroids and tacrolimus); studies in animal models of vitiligo; the participation of immune cells in the demonstration of auto reactive T cells; and the presence of antibodies against different antigens of melanocytes; all these aspects support autoimmune hypothesis (Ongenae *et al.*, 2003; Passeron and Ortonne, 2005). It has also been proposed that two major mechanisms are related to autoimmune vitiligo, an antibody-based dominant mechanism in diffuse vitiligo and a T-cell-based dominant mechanism in localized disease (Michelsen, 2010). Therefore, the role played by cellular and humoral immune responses in the pathogenesis of vitiligo has been further discussed.

1.4.3.1 Autoantibodies and antigens:

Different circulating antibodies to melanocytes, which are uncommon in healthy individuals, have been found in the sera of vitiligo patients. These antibodies seem to be related with the extent of disease, and are present in more than 90% of the patients with greater depigmentation and in 50% of the patients with minimal lesions (Abu Tahir *et al.*, 2010). Various methods have been used for their detection. Characterization of these antibodies has demonstrated that they belong to the IgG class, involving the subclasses IgG1, IgG2 and IgG3. Interestingly, IgG and C3 deposits have also sporadically been observed in the basal membrane zone of lesional skin, which correlates with the observation that the binding of IgG to cultured

melanocytes increases with disease activity and extent. Furthermore, studies have found that IgA levels of anti- pigment cell membrane antibodies are associated with disease activity, suggesting a close relationship with anti-melanocyte IgA antibody levels (Ongenae *et al.*, 2003; Kemp *et al.*, 2007). These findings together, may be linked to the alterations in immunoglobulins showed in vitiligo. A recent study of the serum immunoglobulin profile of vitiligo patients showed that IgG and IgA levels are significantly decreased ($p=0.05$) with no changes in IgM, compared to controls (Ali *et al.*, 2010), indicating that IgG anti-melanocyte antibodies could have an important role in this disorder, as they can induce melanocyte damage *in vitro* by a complement-mediated mechanism and antibody-dependent cellular cytotoxicity. In addition, IgG anti-melanocyte antibodies may play an active role in the stimulation and inappropriate expression of HLA-DR and induction of ICAM-1 on melanocytes, and also an increased IL-8 production. Thus, MHC II molecules expressed on melanocytes can present antigens to $CD4^+$ cells, allowing an immune response, and ICAM-1 may play an essential part in immunological and inflammatory reactions resulting in melanocytotoxicity (Li *et al.*, 2000). Ruiz- Argüelles *et al.* (2007), studied 15 patients with vitiligo and found that 93% patients showed antibodies to a 75 kDa melanocyte protein in serum samples, and apoptotic markers in vitiligo skin biopsies. When melanocytes were cultured with purified IgG from vitiligo patients, apoptosis was induced and the cells showed a higher proportion of intracellular IgG suggesting that antibodies directed to melanocyte-specific antigens may enter the melanocytes and induce apoptosis.
A few specific autoantigens identified include tyrosinase, a melanocytic enzyme; tyrosinase-related protein (TRP) 1 /gp75 and 2; the melanosomal matrix protein gp100 (Pmel17) and Melan A/MART 1 etc. as shown in Table 1.

In addition, a recent study using phage display identified B-cell autoantigens i.e., antigens to gamma-enolase (8%), alpha-enolase (9%), heat-shock protein 90 (13%), osteopontin (4%), ubiquitin-conjugating enzyme (15%), translation-initiation factor 2 (6%), and GTP-binding protein, Rab38 (15%), in patients with non-segmental vitiligo while in contrast, patients with segmental vitiligo had no reactivity to these autoantibodies (Waterman *et al.*, 2010). However, none of these proteins have been identified as the major autoantigen in vitiligo.

Table 1: Antigens recognized by autoantibodies of vitiligo patients.

Autoantigens	Reference
Tyrosinase	Song *et al.*, 1994, Baharav *et al.*, 1996; Xie *et al.*, 1999; Kemp *et al.*, 1997
TRP 1	Kemp *et al.*, 1998b
TRP 2	Okamoto *et al.*, 1998; Kemp *et al.*, 1997
Pmel 17	Kemp *et al.*, 1998a
Melan A/MART 1	Waterman *et al.*, 2002
MCHR 1	Waterman *et al.*, 2002
SOX 9	Hedstrand *et al.*, 2001
SOX 10	Hedstrand *et al.*, 2001

1.4.3.2 T cell participation:

Skin biopsies of vitiligo patients have shown that inflammatory cells are prominent in perilesional areas, and one of the first clues for the participation of cellular immunity in the pathogenesis of vitiligo was the discovery of T cell infiltration in the margins of active vitiligo skin lesions associated with areas of melanocyte loss. This perilesional infiltration consists of $CD8^+$ and $CD4^+$ T cells, often with an increased $CD8^+/CD4^+$ ratio (Le Poole *et al.*, 2004). An *in vitro* study analyzing T cells from perilesional and nonlesional skin biopsies from vitiligo patients found that both $CD8^+$ and $CD4^+$ T cells in perilesional areas exhibit a predominantly Type-1-like cytokine secretion profile, with secretion of TNF-α and IFN-γ. In particular, IFN-γ enhances T-cell trafficking to the skin by increasing ICAM-1 expression (Wańkowicz-Kalińska *et al.*, 2003). Another important observation is expression of the cutaneous lymphocyte-association antigen (CLA) by $CD8^+$ cells, which is a skin homing receptor that could recruit T cells from peripheral circulation to the affected skin (Ogg *et al.*, 1998). High frequencies

of Melan A/MART 1 specific $CD8^+$ T cells in perilesional skin and peripheral blood have been detected; these cytotoxic T cells showed *in vitro* anti-melanocyte cytotoxic activity and expressed skin-homing capacity, which seems to correlate with disease extension and severity (Palermo *et al.*, 2001). Van den Boorn *et al.* (2009) in a study with a skin explant model, investigated *in situ* activities of perilesional $CD8^+$ T cells in the effector phase of depigmenation and found that $CD8^+$ T cells could infiltrate normally pigmented skin explants and eradicate melanocytes. In the same study, melanocyte apoptosis was accompanied by suprabasal keratinocyte apoptosis, and perilesional cytotoxic T cells did not induce apoptosis in lesional skin devoid of melanocytes, which indicates their melanocyte-specific cytotoxic activity and skin- homing capacity. The capacity of cytotoxic cells for melanocyte damage has also been observed in an experimental murine model of vitiligo. It was found that melanocytes can be destroyed by $CD8^+$ T cells that recognize a single H2-Kb-binding peptide derived from tyrosinase-related protein 2 (TRP-2) (Steitz *et al.*, 2004). Additionally, the presence of granzyme B and perforin expressed by CLA/HLA-DR/$CD8^+$ T cells at the perilesional epidermal–dermal junction of vitiliginous skin shows the major role of T cells in melanocyte death in vitiligo (van den Wijngaard *et al.*, 2000; Oyarbide-Valencia *et al.*, 2006).Moreover, it is proposed that the patchy, symmetrical distribution of lesions is caused by autoimmune melanocyte damage due to clones of lymphocytes with affinities for specific skin areas (al Badri *et al.*, 1993). This reactivity of $CD8^+$ T cells towards melanocytes observed in vitiligo patients could be used in the clinical application in the treatment of melanoma (Oyarbide-Valencia *et al.*, 2006).

Autoimmune diseases are often associated with malfunction of peripheral $CD4^+$ cells, and therefore $CD4^+$ T cells may be involved in the pathogenesis of vitiligo. It is suggested that peripheral tolerance of $CD8^+$ cells recognizing melanocyte self- antigens are regulated in two steps: firstly, an induction phase where the stimulation and expansion of autoreactive $CD8^+$ cells depend on $CD4^+$ T cells in the lymphoid system and, secondly, an effector phase where autoimmune destruction of pigmented cells depends on local inflammation, with the migration of T cells supporting the effector functions (Steitz *et al.*, 2005). Severe autoimmune vitiligo may develop after genetic removal of the cytotoxic T lymphocyte-associated

antigen 4 (CTLA-4), an important immunomodulatory molecule to the T cell tolerance expressed in $CD8^+$ and $CD4^+$ T cells. It has been shown that removal of CTLA-4 induces the activation and acquisition of effector functions in $CD8^+$ cells, but only in the presence of dysregulated $CD4^+$ T cells (Gattinoni *et al.*, 2006). Furthermore, a correlation between idiopathic $CD4^+$ T lymphocytopenia (ICTL) and autoimmune vitiligo has been reported, indicating that an abnormal response of $CD4^+$ T lymphocytes is involved in this disease (Yamauchi *et al.*, 2002). Abnormal expression of MHC class II molecules by perilesional melanocytes and increased numbers of expressed intercellular adhesion molecule ICAM-1, which play an important part in antigen presentation and $CD4^+$ T cell activation, have been documented (al Badri *et al.*, 1993). Likewise, studies have indicated that marginal skin from patients with progressive generalized vitiligo shows a down-regulated expression of CDw60 molecule by $CD4^+$ T cell type 2 (Le Poole *et al.*, 2003) and recent study of the polymorphism of the $CD4^+$A4 allele has shown a modest association with the development of vitiligo (Zamani *et al.*, 2010).

The hypothesis that depigmentation can progress in the absence of regulatory T cells (Treg) is established. Reduced numbers of Treg cells appear in non-lesional, perilesional and lesional vitiligo skins, and a reduced expression of the skin homing chemokine CCL22 in vitiligo skin has been observed by immunohistochemistry. This may explain the failure of circulating Treg cells and their reduction of skin homing due to the failure of functionality, which might perpetuate reactivity against melanocytes in vitiligo (Klarquist *et al.*, 2010).

Abnormalities in circulating T cells have been reported with inconsistent results, and there is no simple explanation for the differences. T cells have been reported to be normal, decreased or increased, and which may be attributable to various factors including the study techniques used to determine T-cell subpopulations, disease characteristics, the study population and previous therapy. A recent study by Pichler *et al.* (2009) evaluating lymphocyte fractions from peripheral blood in a cohort of patients with vitiligo found an elevated $CD4^+/CD8^+$ T cell ratio with a disturbed balance of cytotoxic/suppressor and helper/inducer T cells in peripheral blood. However, the absolute and relative counts of lymphocyte subtypes were normal. Another study found significantly

increased $CD3^+$ counts in generalized vitiligo and increased memory ($CD45RO^+$) T cells in acrofacial vitiligo compared with other vitiligo types, and raised percentages of $CD8^+$ T cells in vitiligo patients compared with controls (Basak *et al.*, 2008). Other studies have found decreased percentages of $CD4^+$ T cells (Grimes *et al.*, 1986), lower total lymphocytes, increased percentages of activated ($CD3^+$ HLA-DR^+) T cells and decreased total naive T cells ($CD4^+CD45RA^+$) (Mahmoud *et al.*, 2002) in vitiligo patients. Taken together, these findings suggest that an important disturbance of cell-mediated immunity takes place in this disorder. These are also situations where T cell responses to melanocytes prevail. In conclusion, a role for melanocytes within the Skin Immune System cannot be overlooked as we assess the etiology of vitiligo. However, the reasons for these inconsistent findings are not clear and require further study.

Skin infiltrating T cells can be isolated from tissue biopsies of interest and grown in bulk. T cells isolated from vitiligo skin are highly reactive with melanoma-associated antigens (Das *et al.*, 2001). High affinity TCRs, once expressed in PBMC from melanoma patients, can more effectively target the tumor (Duval *et al.*, 2006). It is well possible that high affinity T cells previously escaped clonal deletion in skin-draining lymph nodes and were inadvertently allowed to enter the circulation, emigrating to the skin to inflict damage to the melanocyte population (Palermo *et al.*, 2005). An ongoing autoimmune response may be allowed to further develop and mature in the absence of functional Tregs (Dejaco *et al.*, 2006). These cells actively mediate suppression of the immune system generally by secreting IL-10 and TGF-β to prevent autoimmunity (Dejaco *et al.*, 2006). In the absence of regulatory T cells, cytotoxic T cells with increasing affinity for their targets enter the skin and continuously proliferate and migrate towards novel target cells, causing depigmentation. This unfortunate event of disfiguring depigmented skin patches left in vitiligo patients may turn out to be a blessing in disguise if the T cell receptors expressed by high affinity T cells provide an effective treatment for melanoma patients.

1.4.3.2.1 Regulatory T cells (Tregs) in Autoimmune Diseases:

No definite surface marker is currently available for human Tregs. The high constitutive surface expression of the IL-2 receptor alpha chain (CD25) is generally

considered as a characteristic feature of the majority of human Tregs, and regulatory activity is enriched in $CD4^+$ T cells expressing the highest levels of CD25 ($CD4^+$ $CD25^{hi}$ T cells) (Baecher-Allan *et al.,* 2001; de Kleer *et al.,* 2004; Makita *et al.,* 2004; Cao *et al.,* 2003; Baecher-Allan *et al.,* 2005). Upon activation of T cells and independently of their regulatory capacity, most of these markers become up-regulated and have therefore only limited specificity to identify Tregs (Shevach *et al.,* 2004).

Intracellular expression of FOXP3 is currently considered as the most specific marker for human Tregs (von Boehmer *et al.,* 2005; Picca *et al.,* 2005; Sakaguchi *et al.,* 2005). Human FOXP3 is localized on the X chromosome encoding 'scurfin', which binds to the IL-2 promotor and the granulocyte–macrophage colony-stimulating factor enhancer near the nuclear factor of activated T cell (NFAT) sites. FOXP3 represses these genes, thus reducing IL-2 production by $CD4^+$ T cells. From the clinical perspective, a mutation of this transcription factor is strongly linked to immune dysregulation. Patients with a mutated FOXP3 gene encounter autoimmune polyendocrinopathy (especially type 1 diabetes mellitus and hypothyroidism) and enteropathy [immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome] (Wildin *et al.,* 2002). Studies have all supported the role of FOXP3 as a 'master control gene' in the development and functioning of Tregs (Sakaguchi *et al.,* 2005).

1.4.3.2.1.1 Levels of Tregs in autoimmune diseases:

Although several efforts have been made to combine different surface markers for a more specific characterization of Tregs (Ruprecht *et al.,* 2005, Koenen *et al.,* 2005) gating on $CD4^+$ $CD25^{hi}$ T cells is usually preferred to define Tregs. The level of circulating $CD4^+$ $CD25^{hi}$ T cells out of the $CD4^+$ T cell pool in healthy humans ranges from 0.6% to 7.9%. In patients with autoimmune diseases, reduced levels of circulating $CD4^+$ $CD25^{hi}$ T cells were described, specifically in individuals with juvenile idiopathic arthritis (Cao *et al* ., 2004; de Kleer *et al.,* 2004), psoriatic arthritis (de Kleer *et al.,* 2004), hepatitis C virus (HCV) associated mixed cryoglobulinaemia (Boyer *et al.*,2004), autoimmune liver disease (Longhi *et al.,* 2004), systemic lupus erythematosus (Liu *et al.,* 2004, Crispin *et al.,* 2003) and Kawasaki disease (Furuno *et al.,* 2004). Lower levels of circulating $CD4^+$ $CD25^{hi}$ T

cells also correlate with a higher disease activity or poorer prognosis (de Kleer *et al.*, 2004, Boyer *et al.*, 2004, Longhi *et al.*, 2004, Crispin *et al.*, 2003, Furuno *et al.*, 2004). It has been proposed that the reduced levels may be caused by the impaired proliferation of peripheral $CD4^+$ $CD25^{hi}$ T cells, as observed *in vitro* (Viglietta *et al.*, 2004, Longhi *et al.*, 2004). Thereby, the balance between pro-inflammatory and regulatory T cells would be disturbed, leading to the breakdown of self-tolerance. However, further studies are required to confirm these results *in vivo* and to exclude any bias of these experiments owing to contamination with anergic effector T cells expressing high levels of CD25 (Shevach *et al.*, 2004).

1.4.3.2.1.2 Different developmental stages of Tregs and Tregs homing:

The observation that murine Tregs expressing FOXP3 constitute several phenotypically and functionally distinct subsets (Belkaid *et al.*, 2005, Fontenot *et al.*, 2005, Huehn *et al.*, 2004) led to the concept of developmental stages of Tregs. In brief, one subgroup of $CD4^+$ $CD25^+$ Tregs expresses high surface levels of CD62L and the chemokine receptor CCR7, and preferentially homes to antigen-draining lymph nodes (similarly to naive T cells), where they efficiently inhibit induction of inflammatory reactions (Fontenot *et al.*, 2005, Huehn *et al.*, 2004, Siegmund *et al.*, 2005).

1.4.3.2.1.3 Suppressive mechanisms of Tregs:

The mechanisms used by Tregs to suppress immune responses are still unresolved. In brief, inhibition is contact dependent, and transwell assays and supernatants of Tregs revealed no suppressive effects. After activation, human Tregs may directly kill activated $CD4^+$ and $CD8^+$ T cells in a perforin or granzyme dependent manner *in vitro* (Grossman *et al.*, 2004). Although evidence for such Tregs mediated cytotoxicity is lacking *in vivo*, the observation of patients with mutations in the perforin gene and who suffer from haemophagocytic lymphohistiocytosis (HLH) indicates a critical involvement of perforin in the regulation of immune responses (Stepp *et al.*,1999). These patients have an overactive immune system and immune responses to infections are not down-regulated after the cessation of an infection. Untreated HLH patients then develop end organ damage from lymphocyte infiltration and macrophage activation. Perforin-deficient mice develop a disease similar to HLH after exposure to viruses (Badovinac *et al.*, 2003). Perforin

polymorphisms may thus predispose to a prolonged activation of the immune system during infections, which could provoke the breakdown of tolerance. Another effector mechanism of suppression has been proposed with reverse signalling through cross-linking B7 (CD80 and CD86) on the cell surface of antigen presentin cells or activated T cells. This signalling is mediated by CTLA-4 expressed on Tregs (Birebent *et al.*, 2004). However, as murine Tregs with target deletion of genes encoding CTLA-4 are suppressive *in vitro*, a non-redundant role of CTLA-4 in the suppressive process is unlikely (Tang *et al.*, 2004). Whether cytokines modulate the T_{reg} mediated suppression of immune responses is also unclear. Both TGF-β and IL-10 have been linked to this effect in murine colitis and type 1 diabetes, although *in vitro* blockade of TGF-β or IL-10 does not totally abrogate suppression (Shevach *et al.*, 2002; Green *et al.*, 2003; Asseman *et al.*,1999).

The signals that facilitate or direct peripheral T_{reg} formation remain elusive, but may involve costimulatory molecules such as CTLA-4, cytokines such as TGF-β, and dendritic cells (Sakaguchi *et al.*, 2004; Sakaguchi *et al.*, 2005).

1.4.3.2.2 Imbalanced immune homeostasis and autoimmunity:

Normal maintenance of the homeostatic equilibrium is achieved through thymic T-cell generation with subsequent development of peripheral T cells and cell death. Under conditions of an extremely disturbed immune system, such as lymphopenia or acute depletion of lymphocytes, T cells undergo peripheral proliferation in the absence of foreign antigen stimulation and can restore the size of the peripheral T-cell compartment independently of the thymic output of naive T cells (Jameson *et al.*, 2002). As lymphopenic states are common throughout life, for example, during viral infections, lymphopenia-induced proliferation may be the primary mechanism to restore the T-cell pool in aged individuals with reduced thymic function (Khoruts *et al.*, 2005).

Although mechanisms that regulate this proliferation are still under investigation, it became apparent that the proliferative capacity of individual T cells correlates with their avidity for self-ligands (Khoruts *et al.*, 2001). Indeed, in patients with rheumatoid arthritis, a contraction of TCR diversity and oligoclonal T-cell expansion

further support this concept (Koetz *et al.*, 2000; Utsinger *et al.*, 1976; Rivero *et al.*, 1978; Iannone *et al.*, 1996; Heimann *et al.*, 1986; Wagner *et al.*, 1998).

1.4.3.2.3 Breakdown of tolerance by environmental factors:

Depletion of $CD25^+$ cells in mice eliminates a high proportion of circulating T_{regs} and activated T cells with severe lymphopenia, but is not sufficient to induce autoimmunity without administration of strong adjuvants. This observation supports the multifactorial pathogenesis of autoimmunity (McHugh *et al.*, 2002). Environmental factors, such as microbial infections or drug metabolism, have long been suspected to trigger the onset of autoimmune diseases by antigen-specific and non-specific effects. Molecular mimicry is one possible mechanism used by microbes to break immune tolerance (von Herrath *et al.*, 2003).The underlying concept is that infectious agents share one or more epitopes with various self components. An alternative hypothesis is that infectious agents cause bystander activation of immune cells with autoaggressive potential. Thus, infections cause transient lymphopenia and organ damage with a release of autoantigens, favouring the proliferation of T cells bearing an autoreactive TCR (Christen *et al.*, 2004). In addition, they provoke the presentation of self-antigens, together with cytokines and costimulatory molecules, as a 'danger' signal (Matzinger *et al.*, 1994).These danger signals are important for inducing an effective immune response against microbes by activating not only naive T cells but also attenuating Tregs mediated suppression (Takahashi *et al.*, 1998, Christen *et al.*, 2004, Horwitz *et al.*, 1998). In the event of disturbed tolerance mechanisms with impaired Tregs generation and bias of non-regulatory T cells towards autoreactivity, infections may initiate or even trigger 'pre-existing' autoimmunity. Genetic polymorphisms of molecules influencing Tregs generation or activation, such as IL-2, CTLA-4 or CD28 (Encinas *et al.*, 1999, Atabani *et al.*, 2005), the timing of infection and the magnitude of inflammation may be additional factors involved in the exacerbation of autoimmunity (Horwitz *et al.*, 1998).

1.4.3.2.4 Tregs -mediated therapeutic approaches for the future:

Strategies to support Tregs in autoimmune diseases are considered an intriguing new approach for using to suppress the inflammatory process, by manipulating both the function and number of Tregs. It is believed that protocols for such manipulation have

the therapeutic potential to induce tolerance in patients with autoimmune diseases, because in mice with collagen-induced arthritis, depletion of Tregs caused rapid progression, but early joint damage could be reversed by the transfer of isolated and *ex vivo* proliferated Tregs (Morgan *et al.*, 2003). Other animal models of autoimmunity show similar results (Kohm *et al.*, 2002). Monoclonally expanded Tregs, which specifically target autoantigens, may even provide more efficient suppression, (Tarbell *et al.*, 2004) and protocols for the *in vitro* expansion of human Tregs are already available (Hoffmann *et al.*, 2004). Alternatively, transfection of nonregulatory T cells with FOXP3 could generate antigen specific Tregs with increased suppressive activity that target sites of inflammation (Yagi *et al.*, 2004, Oswald-Richter *et al.*, 2004, Hori *et al.*, 2003). Transfection of polyclonally expanded Tregs with genes encoding TCR-α and β chains or genes encoding a chimeric antigen MHC-CD3-f molecule might also result in significant numbers of potent, highly directed, antigen specific Tregs (Mekala *et al.*, 2005).

Other approaches to increase Tregs function or numbers without *ex vivo* manipulation include administration of cytokines (such as TGF-β) that favour Tregs activity and survival. TGF-β may also convert non-regulatory T cells into Tregs (Wan *et al.*, 2005) and TGF-β gene therapy was found to induce Tregs in syngenic islet transplanted non-obese diabetic mice, blocking islet destructive autoimmunity (Luo *et al.*, 2005). Tolerance can also be induced by short-term treatment with monoclonal antibodies against costimulatory molecules, adhesion molecules or the TCR complex (Waldmann *et al.*, 1998, Lehmann *et al.*, 1997, Beyersdorf *et al.*, 2005). There are, however, technical difficulties regarding reliable identification of Tregs and the generation of sufficient numbers of Tregs for therapeutic purposes (Bluestone *et al.*, 2005). T cells with retroviral expression of FOXP3 have recently been shown to be less suppressive than freshly isolated $CD4^+$ $CD25^+$ Tregs (Allan *et al.*, 2005) and may therefore be insufficient to down-regulate autoimmunity in humans.

1.4.3.3 B cell participation:

The role of B cells in the pathogenesis of vitiligo is unclear, while some studies have found no B cell infiltration in vitiligo skin lesions (Le Poole *et al.*, 1996). A study found B cell infiltration in juxtaposition to depigmented zones, supporting

the idea that the autoimmune phenomenon is mediated by a humoral mediator or is local to some areas of skin, suggesting that the depletion of melanocytes is mediated by locally secreted products (Argüelles *et al.*, 2007). Interestingly, another study found that the MCHR1 is a B cell autoantigen in vitiligo with the production of antibodies to this receptor (Gavalas *et al.*, 2009), confirming its participation. Therefore, blocking of this receptor may alter the signalling pathway that regulates melanocyte function, affecting pigment cell behavior (Kemp *et al.*, 2007).

1.4.3.4 Cytokines:

Various studies have investigated the presence and role of cytokines in the depigmentation process of vitiligo. Increased serum levels of soluble IL-2R have been associated with vitiligo activity, indicating T cell activation. Specifically, soluble IL-2R are significantly higher in focal type vitiligo compared with segmental or generalized types, and are higher in patients with less than a year of disease duration (Yeo *et al.*, 1999). In addition, elevated production of IL-6, a cytokine that induces ICAM-1 expression, facilitating leukocyte–melanocyte interactions, and IL-8, an attracting cytokine to neutrophils by mononuclear cells in vitiligo patients has been found (Yu *et al.*, 1997). Another study found decreased serum levels of transforming growth factor-β), which can diminish maturation of T_{reg} cells. Likewise, a correlation with the extension of vitiligo and levels of IL-17 has been demonstrated (Basak *et al.*, 2009). Studies found a significantly higher expression of pro-inflammatory cytokines with an inhibitory effect on pigmentation, such as IL-6 and TNF-α, in lesional and perilesional skins in vitiligo patients, but a lower expression of melanogenic mediators such as granulocyte-monocyte colony stimulating factor (GM-CSF), basic fibroblastic growth factor (bFGF), stem cell factor (SCF) and endothelin-1 (ET-1) (Moretti *et al.*,2002; Moretti *et al.*, 2009). TNF-α production might contribute to keratinocyte apoptosis, resulting in reduced release of melanogenic cytokines (Moretti *et al.*, 2009). In addition, a recent study demonstrated that the TNF-α -308G/A polymorphism is common in vitiligo patients and is associated with cytokine production (Namian *et al.*, 2009).

1.4.3.5 Participation of other cells:

Abnormalities in other cell types have been observed in vitiligo. In a recent study of peripheral blood mononuclear cells of vitiligo patients, genomic and gene specific DNA methylation levels were investigated to relate the changes to the expression of genes that regulate methylation and the IL-10 gene. The results showed increased genomic DNA methylation in mononuclear cells in vitiligo patients compared with controls ($p = 0.012$). Interestingly, IL-10 exression was decreased in vitiligo patients ($p = 0.030$) and an IL-10 enhancer region was shown to be hypermethylated ($p = 0.014$), demonstrating involvement of the autoimmunity-related genes (Zhao *et al.*, 2010). Likewise, increased macrophage infiltration in perilesional skin, probably due to clearing activity, and an increase in Langerhans cells in perilesional vitiliginous skin showing degenerative changes have been reported (Le Poole *et al.*, 1996), although the participation of Langerhans cells remains unclear. Dendritic cell involvement in vitiligo is also not clear, but they might recruit T cells in the epidermis, possibly carrying out a cytotoxic function mediating the killing of stress melanocytes contributing to depigmentation (Le Poole *et al.*, 2004). In addition, alterations in natural killer (NK) cells have been demonstrated in vitiligo. Studies have found higher NK cell activity with significantly increased percent- ages of the activatory receptors, $CD16^+CD56^+$, $CD3^+CD16^+CD56^+$ and a decrease in the inhibitory receptor $CD16^+CD158a^+$ in vitiligo patients (Basak *et al.*, 2008).

1.4.3.6 Defective apoptosis and generation of autoimmunity:

Many signals that may originate either endogenously or exogenously have been shown to influence life processes. These include hormones, immune killing, ROS, genetic and physical trauma, oncogene expression, etc. Vitiligo melanocytes may have an intrinsic defect leading to melanocyte death. These melanocytes demonstrate various abnormalities, including abnormal, rough endoplasmic reticulum and incompetent synthesis and processing of melanocytes. In addition, homing-receptor dysregulation has also been detected. Early apoptosis of melanocytes has also been suggested as a cause of reduced melanocyte survival (Ortonne and Bose, 1993; van den Wijngaard *et al.*, 2000).

The execution of Programmed Cell death (PCD) is often associated with characteristic morphological and biochemical changes. Apoptotic hallmarks include membrane blebbing, cell shrinkage, chromatin condensation, DNA cleavage and fragmentation, etc. (Mignotte and Vayssiere, 1998). During apoptotic breakdown many nuclear constituents are post-translationally modified altering antigenicity. It is therefore speculated that failure to achieve PCD and clear apoptotic cell fragments may be a key pathological factor leading to autoimmune disorders. Autoimmunity could result from a failure to kill an autoreactive cell or by inducing autoimmunity against apoptotically modified cellular constituents. Therefore, the process of apoptosis may provide a source of nuclear antigens to drive the autoantibody response and provide antigens in SLE. Reports have suggested that apoptosis is abnormal in autoimmune diseases and may play a role in the induction of autoimmunity. Studies on apoptosis and clearance of apoptotic cells in lupus have shed light on the development and course of the disease. During maturation of the immune system, apoptosis of autoreactive lymphocytes in the central lymphoid organs underlies the development of tolerance. Whenever apoptotic cells accumulate due to an increased rate of apoptosis, decreased elimination or both, tolerance can be broken. Disturbances in any one of the many factors that regulate the apoptotic process might change the balance in the immune system and may predispose for the development of autoimmune phenomenon (Bijl *et al.*, 2001).

Nitric Oxide (NO) is a reactive endogenous molecule with multiple functions including inflammation and immunity. Studies have shown that nitric oxide could inhibit the *de novo* attachment of melanocytes to extra cellular matrix (ECM) suggesting that NO induced aberrant perturbation of melanocyte - ECM interaction could be a reason for melanocyte loss in vitiliginous lesions. However, Ivanova *et al.*, showed that NO induced apoptosis mediated detachment of both normal melanocytes and vitiliginous melanocytes from fibronectin occurs in a similar mechanism, suggesting that non-lesional vitiliginous melanocytes are not characterized by an increased proneness to NO induced apoptosis (Ivanova *et al.*, 2005).

A few studies suggest that loss of melanocytes in vitiligo is the result of apoptosis (Huang *et al.*, 2002). Oxidative stress, which can induce apoptosis by cytochrome c mediated pathway of caspase activation (Simon *et al.*, 2000), may contribute to

melanocyte destruction in vitiligo lesions. During apoptosis, modification of melanocytic antigens through proteolysis, changes in the phosphorylation state and citrullination may give rise to potentially immunostimulatory forms of intracellular or membrane-associated autoantigens. Generally, the efficient clearance of apoptotic cells results in the exposure of intracellular self-antigens to the immune system under non-inflammatory conditions. It has been proposed that under these conditions circulating dendritic cell precursors take up apoptotic cells and travel to lymphoid organs, where they present autoantigens from apoptotic cells to T cells in the absence of costimulatory molecules, leading to tolerance induction.

However, under the proinflammatory cytokine and extracellular acidic environment of vitiligo lesions, or the release of proteases secondary to tissue injury (Koebner reaction), these modified autoantigens, which may also expose cryptic epitopes, may be processed by mature Langerhans cells and presented to T cells. Subsequently, the autoreactive $CD4^+$ T cells may stimulate autoreactive B cells to produce autoantibodies, whereas $CD8^+$ T cells may attack melanocytes directly. It is worth noting that efficient clearance of apoptotic cells is crucial for the avoidance of autoimmune responses to intracellular antigens (Casiano and Pacheco, 2006).

1.4.3.7 Interplay of oxidative stress and immune system:

Vitiligo pathogenesis is an extremely complex event involving both genetic susceptibility as well as environmental triggers. The two major theories of vitiligo pathogenesis include an autoimmune etiology for the disease and an oxidative stress mediated toxicity in the melanocyte. Although these two theories are often presented as mutually exclusive entities, it is likely that vitiligo pathogenesis may involve both oxidative stress and autoimmune events, for which there is variability within a patient. Reactive oxygen species are recognized as important signaling molecules within the cells of the immune system. This is, at least in part, because of the reversible activation of kinases, phosphatases and transcription factors by modification of critical thiol residues (Grifiths, 2005). In fact, free radicals are involved in specific early events of T-cell activation and antioxidants reduce T-cell proliferation, IL-2R expression and IL-2 production (Chaudhri *et al.*, 1998).

Reactive oxygen species are produced as byproducts of melanogenesis in melanocytes, and controlled by several redundant antioxidant enzymes such as catalase and glutathione peroxidase, both of which are decreased in the epidermis of vitiligo patients (Schallreuter, 1999a). Oxidative stress plays a very important role in the immune system, as phagocytic cells generate reactive oxygen intermediates such as superoxide, hydrogen peroxide and nitric oxide, which are toxic to many pathogens, and at the same time they can be toxic to the host as well. Given the role of oxidative stress in both melanogenesis and in the immune system it can be hypothesized that biochemical defects in the melanin biosynthesis pathway, as well as possible defects in patient's antioxidant enzymes, are responsible for the generation of reactive oxygen species in the epidermis of vitiligo patients (Casp *et al.*, 2002). Build up of ROS along with possible immune system defects allow for the inappropriate autoimmune response against normal melanocytes.

In autoimmune disorders the immune system aberrantly targets host cells for destruction, often creating a chronic or relapsing inflammatory milieu. The effects of chronic inflammation can be devastating on the host, eventually causing damage and/or destruction of the target organ. In this inflammatory environment, ROS can accumulate with a toxic effect on surrounding cells. This can explain the pathogenesis of inflammatory vitiligo (Buckley 1953). In this rare disorder a raised rim surrounds the depigmented lesion. The question that lies unanswered is what is causing this aberrant inflammatory response in autoimmunity and whether these ROS are a result of the chronic inflammation and autoimmunity, or part of the cause of the autoimmune response.

Oxidative processes enhance the reaction of the adaptive response. Oxidation of carbohydrates enhances the antibody response to co-administered co-antigens. Moreover, the administration of the Schiff base-forming agent tucaresol during immunization with protein antigen increased T-cell-dependent immune response. Direct modification of protein antigen has been demonstrated for the enhanced immune response (Kurien *et al.*, 2006).

Oxidative stress, produced through increased catecholamine release or from other sources such as toxic intermediates of melanin precursors, can also initiate, or at least

amplify, the autoimmune destruction of melanocytes as well. Cutaneous axon terminals and epidermal melanocytes make contact via chemical synapses in human skin. An increased release of catecholamines from the autonomic nerve endings in the microenvironment of melanocytes in the vitiliginous areas has been reported. Norepinephrine is known for having direct and indirect melanocytotoxic effects. Direct actions include interacting with cellular sulfhydryl groups, enzyme inhibition, impairing mitochondrial calcium uptake, and forming some cytotoxic products including free radicals. The indirect effects include activating α-receptors of the arterioles, causing a severe vasoconstriction, and thereby producing toxic oxygen radicals caused by hypoxia (Namazi, 2003).

Hypoxia induces extracellular acidosis. It is demonstrated that extracellular acidosis activates dendritic cells. It increases the acquisition of extracellular antigens for MHC class I-restricted presentation and the ability of antigen-pulsed dendritic cells to induce both specific CD8+ cytotoxic T lymphocytes and B-cell responses (Vermeulen *et al.*, 2004). It is also demonstrated that hypoxia contributes to the initiation of adaptive immune responses by dendritic cells, favoring the development of Th1 immunity (Martı´nez *et al.*, 2007). Moreover, there are several ways by which toxic oxygen radicals, besides having a direct melanocytotoxicity, can induce an autoimmune attack against melanocytes.

The melanogenic pathway involves the formation and polymerization of L-Tyrosine. Its first and rate-limiting step is the conversion of L-tyrosine into L-dopaquinone which involves O-quinone as an intermediate product. Exposure to UV radiation for longer time causes the spontaneous production of O-quinone that further leads to the formation of H_2O_2 as a by-product (Cervantes *et al.*, 2001) (Figure 8, pathway 4).
The structures of melanocytic macromolecules and small molecules, such as Melan-A and tyrosinase, may markedly change by acute or chronic oxidative stress and can act as antigens (neo-antigens). Neo-antigens with sufficient homology or identity to host antigenic proteins induce auto-reactivity. This phenomenon is referred to as 'molecular mimicry' (Kannan, 2006). Aldehydic products, mainly the 4-hydroxy-2-alkenals, form adducts with proteins and make them highly immunogenic (Kurien *et al.*, 2006). Hydroxyl radicals are also very highly reactive and could attack a wide range of targets. The presence of rheumatoid factors in the sera and lesions of vitiligo

patients can be explained by this mechanism (Vahedi Darmian *et al.*, 2004). Over time, chronic oxidative stress could generate several adducted and / or non-adducted molecules that would essentially act as a 'neo-antigens'. This is consistent with the slow maturation of auto-antibodies in the evolution of this disease. More than one neo-antigens / autoantigens are involved in amplifying the autoaggressive lymphocytes by a process referred to as 'antigen spreading'. This is an autoimmune reaction initially directed against a single autoantigen that spreads to other autoantigens, causing the T helper cells to recognize them (Kannan, 2006).

Westerhof *et al.* (2007) suggested that phenols / catechols, which are increased in vitiliginous areas, may serve as a preferred surrogate substrate of tyrosinase, competing with tyrosine and converting into reactive quinones. Such reactive quinones, whose production is enhanced by increased hydrogen peroxide in the vitiligo lesions, can covalently bind to tyrosinase (haptenation). This could give rise to a neo-antigen, carried by Langerhans cells to the regional lymph nodes and stimulating the proliferation of cytotoxic T cells (Song, 1997).

Moreover, a few studies suggest that Melan-A is the principal vitiligo autoantigen, as high frequencies of Melan-A specific $CD8^+$ cells were found in vitiligo and seem to be correlated with the extent and severity of the disease (Lang *et al.*, 2001; Ogg *et al.*, 1998).

During chronic oxidative stress, neo-antigens potentially cause tissue damage and release a plethora of sequestered auto-antigens. This process is referred to as the 'bystander effect'. Such an outburst of auto-antigens from the target tissue would potentially amplify the effect of the neo-antigens, leading to the breakdown of self-tolerance (Kannan, 2006).

Hypoxia directly induces mitochondrial reactive oxygen species, which subsequently activate NF-κB through activation of kinases (Chandel *et al.*, 2000) (Figure 3). On activation, NF-κB regulates the expression of almost 400 different genes, which include enzymes such as iNOS, cytokines (such as TNF-α, IL-1, and chemokines), and adhesion molecules (Ahn and Aggarwal, 2005). It deserves noting that TNF-α can increase mitochondrial reactive oxygen species production through an autocrine effect

on a cell membrane receptor (Santos-Rosa *et al.*, 1999). The possible cross talk between cellular and humoral immune mechanisms in vitiligo is given in the Figure 9.

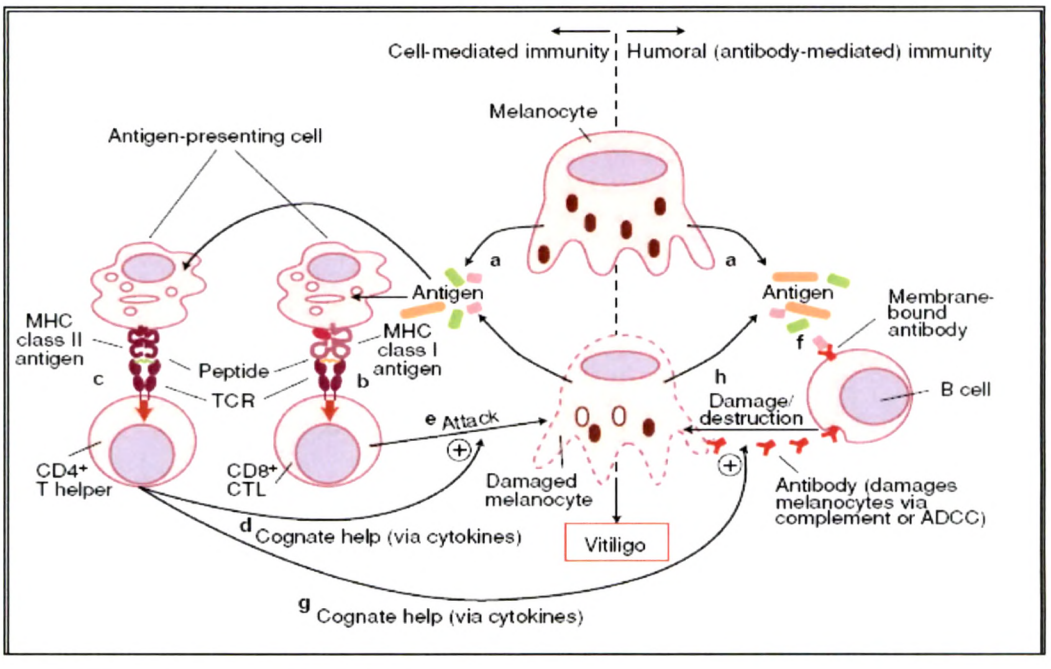

Figure 9. Possible cellular and humoral immune mechanisms in vitiligo (Kemp *et al.*, 2001).

1.4.4 Convergence Theory:

Several hypotheses on the mechanism of pathogenesis of vitiligo have been combined and formulated a convergence theory to explain the etiopathogenesis of vitiligo (Le Poole *et al* 1993). This theory states that stress, accumulation of toxic compounds, infection, autoimmunity, mutations, altered cellular environment and impaired melanocyte migration and proliferation can contribute to vitiligo pathogenesis in varying proportions.

According to the new hypothesis put forward by Dell'Anna and Picardo (Dell'Anna and Picardo 2006), a compromised membrane could render the cell sensitive to external and internal agents differentially. According to this hypothesis, the melanocytes present biochemical defects, probably due to a genetic background, affecting the structure and functionality of the membranes. A compromised

membrane could render the cell sensitive to external and internal agents differently (UV, cytokines, catechols, melanin intermediates, growth factor withdrawal) usually ineffective on cell activity and survival. The impaired arrangement of the lipids, involving fatty acids and cholesterol, may affect the transmembrane housing of proteins with enzymatic or receptor activities. The altered expression and release of transmembrane proteins could be basis for the exposure of 'new antigens' triggering an immune response (Broquet *et al* 2003; Kroll *et al* 2005). The final result could depend on the intensity or duration of the stimuli; a mild aggression leading to a reduction of ATP production impairs the adhesion function; a great stimulus acting as pro apoptotic agent affects mitochondrial cell survival check points; finally, a strong stress directly causing the necrotic death with an inflammatory, or at least lymphocytic infiltrate (Dell'Anna and Picardo 2006).

1.5 VITILIGO GENETICS:

Vitiligo is characterized by multiple susceptibility loci, incomplete penetrance, and genetic heterogeneity (Spritz *et al.*, 2008). The inheritance of vitiligo may involve genes associated with the biosynthesis of melanin, response to oxidative stress and regulation of autoimmunity. It is however not yet clear as to whether the abnormalities observed in neural pathways, oxidative stress and autoimmune events represent a cause or effect response of the disease. Nevertheless, it may involve both genetic and environmental factors. Recent studies suggest that genetic factors may play a major role in the pathogenesis of vitiligo. There is a positive family history in about 20% of cases and similar concordance in identical twins (Spritz, 2008). Shajil *et al* (2006) also reported that 21.93% of Gujarat vitiligo patients exhibited positive family history and 13.68% patients had at least one affected first-degree relative.

Vitiligo is a polygenic disease, and attempts have been made to identify genes involved in susceptibility include gene expression studies, genetic association studies of candidate genes and genome wide linkage analyses to discover new genes. Recent genome-wide association studies (GWAS), have identified generalized vitiligo susceptibility genes which involve immune regulation and immune targeting of melanocytes, suggesting that generalized vitiligo is a primary autoimmune disease

however, the biological triggers of the autoimmune process remain unknown (Spritz *et al.*, 2011).

Considerable progress has been made in the identification of candidate genes. Such studies have been carried out in different populations with differing susceptibility factors. Systematic studies in different populations would help identify candidate genes governing oxidative stress that contribute to the pathogenesis of vitiligo. As immune system also seems to play a major role in vitiligo, understanding the mechanism of immune responses involved and, identification of the potential antigen/s in a particular population would help in designing therapeutic regimens by neutralizing the specific cell types or masking the specific antigen/s that are involved in the pathogenesis of vitiligo. The mammalian skin pigmentation is possibly controlled by more than a hundred genes; these genes as well as the genes regulating oxidative stress and immune responses qualify as potential candidate genes for vitiligo.

The role of genetic factors in vitiligo was also considered early because of the frequent clustering of cases among close relatives (Stuttgen, 1950; Teindel, 1950), and eventual genetic epidemiological studies by Das *et al.* (1985) supported multifactorial, polygenic inheritance, which currently is termed ''complex disease''. In 1960s and 1970s, ABO, haptoglobin, erythrocyte enzymes, and various serum proteins were tested as genetic markers for vitiligo, with negative results. In the 1970s, a plethora of analyses of HLA in vitiligo were reported, with equivocal and conflicting findings. Some of the earliest genetic studies of vitiligo were carried out in India, of ABO blood groups (Kareemullah et al., 1977), α1-antitrypsin, and haptoglobin (Mujahid et al., 1990). Several candidate genes have been tested for genetic association with generalized vitiligo, including the *MHC, ACE, CAT, CTLA4, COMT, ESR, GCH1, MBL2, PTPN22,* and *VDR* (Spritz 2007, 2008). Most of these studies reported significant associations, although some yielded only marginal significance and several were not replicated by subsequent studies. Recently, a number of genes which play a role in vitiligo susceptibility, including *HLA, NALP1, XBP1, FOXP1, IL2RA* have been tested for genetic association with vitiligo (Spritz, 2010).

HLA molecules present peptides to T-cells, and it has been proposed that certain HLA haplotypes confer more efficient presentation of cognate autoantigen, thereby predisposing to autoimmunity; an example is HLADQB1*0301 (Gilhar *et al.,* 2007). *NALP1* is involved in the innate immune response to pathogens. Recent fine-mapping studies showed associations with chromosomes 7 and 9 (Jin *et al.,* 2009). Numerous other candidate genes and susceptibility loci bear ongoing scrutiny, including *CAT*, *GST*, *COMT*, *ACE*, mannose-binding lectin 2 and *XBP1* (Casp *et al.*,2002;Onay *et al.*, 2007; Liu *et al.,* 2009; Ren *et al.,* 2009). A recent genome-wide association study by Jin *et al.* (2010) using European white subjects and controls showed significant associations of generalized vitiligo with the following loci, which have been previously linked with autoimmune diseases: HLA class I and II molecules, *PTPN22*, *LPP*, *IL2RA*, *UBASH3A* and *C1QTNF6*. Two additional immune-related loci identified were *RERE* and *GZMB*. The HLA class I association occurred in the regions between *HLA-A* and *HCG9*, consistent with previous reports of strong associations with the HLA-A*02 allele, and the HLA class II gene association occurred in the region between *HLADRB1* and *HLADQA1*, in keeping with known associations to the HLA-DRB1*04 allele. With the exception of *PTPN22*, the associations were similar whether patients had vitiligo alone or vitiligo as well as another autoimmune disease. An important association with a non-immune related gene, tyrosinase was identified. Tyrosinase is a melanocyte enzyme that catalyzes the rate limiting step in melanin biosynthesis and is a putative target autoantigen in vitiligo. Interestingly, certain *TYR* SNPs are associated with melanoma risk, and some of these are in linkage disequilibrium with vitiligo. Vitiligo *TYR* SNPs could be more antigenic than melanoma *TYR* SNPs, thereby conferring protection from melanoma through immune surveillance (Jin *et al.*, 2010; Bishop *et al.*, 2009).

1.5.1 SINGLE NUCLEOTIDE POLYMORPHISMS

Humans are 99.9% genetically identical (Venter *et al.,* 2001) and the most common type of genetic variability found in humans is in the form of Single Nucleotide Polymorphisms (SNPs). A SNP refers to a single base change in DNA. These SNPs occur when there are two or more possible nucleotides are seen at a specific mapped location in the genome, where in the least frequent allele has an abundance of 1% or

more (Brookes *et al.,* 1999). An International Single Nucleotide Polymorphism Consortium (ISNPC) has currently identified over 6 million SNPs, approximately one at every 1-2 kilobase. SNPs may occur in non-coding regions as well as in coding regions. Some missense polymorphisms are more conservative than others e.g. a change in the codon CUU (leucine) to AUU (isoleucine) would have minimal structural impact, whereas modification of CAU (histidine) to CCU (proline) would be expected to have dramatic structural and/or functional influence on the protein.

SNPs act as potential useful markers for the gene mapping studies, particularly for identifying genes involved in complex diseases (Chakravarti *et al.*, 2001). But the knowledge of frequency and distribution of these SNPs across ethnically diverse populations is essential in order to know their usefulness as markers for gene mapping studies. Additionally, the density of SNPs needed for mapping complex diseases will likely vary across populations with distinct demographic histories (Tishkoff and Verrelli 2003).

The Common Disease/ Common Variant hypothesis states that common genetic disorders are affected by common disease susceptibility alleles at a few loci that are at high frequency across ethnically diverse populations e.g. the APOE ε 4 allele is associated with increased risk for Alzheimer's disease (Chakravarti *et al.,* 1999; Goldstein and Chikhi 2002). Thus, these alleles might arise prior to population differentiation. Alternatively, some complex diseases may be influenced by rare susceptibility alleles at many loci. If these disease predisposing alleles are geographically distributed due to mutation, drift, or regional specific selection pressure, then characterizing SNP diversity, haplotype structure and linkage disequilibrium across a broad range of ethnically diverse populations is of particular importance for identifying disease predisposing alleles (Tishkoff and Williams 2002).

1.5.1.1 SNP analysis:

SNP analysis techniques fall into two distinct classes:

I. SNP Identification: Detection of novel polymorphisms

II. SNP Genotyping: Identifying specific allele in a known population.

1.5.1.1.1 SNP identification methods:

The identification and characterization of large numbers of SNPs are necessary before their use as genetic tools. The following four methods are commonly used for SNP detection (Gray *et al.*, 2000).

1.5.1.1.1.1 SSCP detection:

For single strand conformation polymorphism (SSCP) detection, the DNA fragment spanning the putative SNP is PCR amplified, denatured and run on denaturing polyacrylamide gel. During the gel run, the single-stranded fragments adopt secondary structures according to their sequences. Fragments bearing SNPs are identified by their aberrant migration pattern and are further confirmed by sequencing. Although SSCP is a widely used and relatively simple technique, it gives a variable success rate for SNP detection, typically ranging from 70 to 95%. It is labor intensive and has relatively low throughput, although higher capacity methods are under development using capillary-rather than gel based detection (Orita *et al.,* 1989).

1.5.1.1.1.2 Heteroduplex analysis:

This relies on the detection of a heteroduplex formed during reannealing of the denatured strands of a PCR product derived from an individual heterozygous for the SNP. The heteroduplex can be detected as a band shift on a gel, or by differential retention on a HPLC column. HPLC has rapidly become a popular method for heteroduplex-based SNP detection due to simplicity, low cost and high rate of detection i.e. 95-100% (Lichten and Fox 1983).

1.5.1.1.1.3 Direct DNA sequencing:

The favored high-throughput method for SNP detection is direct DNA sequencing. SNPs may be detected *in silico* at the DNA sequence level. The wealth of redundant sequence data deposited in public databases in recent years, in particular expressed sequence tag (EST) sequences, allows SNPs to be detected by comparing multiple versions of the same sequence from different sources.

1.5.1.1.1.4 Variant detector arrays (VDA):

VDA technology is a relatively recent addition to the high throughput tools available for SNP detection. This technique allows the identification of SNPs by hybridization

of a PCR product to oligonucleotides arrayed on a glass chip and measuring the difference in hybridization strength between matched and mismatched oligonucleotides. The VDA detection allows rapid scanning of large amounts of DNA sequences (Wang *et al.,* 1998).

1.5.1.1.1.5 High Resolution Melting (HRM):

High Resolution Melting (HRM) is a novel, homogeneous, close-tube, post-PCR method, enabling analysis of genetic variations (SNPs, mutations, methylations) in PCR amplicons. HRM characterizes nucleic acid samples based on their disassociation (melting) behavior. Samples can be discriminated according to their sequence, length, GC content or strand complementarity. Even single base changes such as SNPs (single nucleotide polymorphisms) can be readily identified (Reed *et al.*, 2004).

1.5.1.1.2 SNP GENOTYPING METHODS:

SNP genotyping involves two components (Chen and Sullivan 2003) i.e. a method for discrimination between alternate alleles and a method for reporting the presence of the allele or alleles in the given DNA sample.

A typical genotyping protocol consists of the following steps.

1. Target fragment amplification by PCR.
2. Allelic discrimination reaction can be carried out by either of the following methods: primer extension, pyrosequencing, hybridization and sequence specific cleavage.
3. Allele specific product identification can be done by either of the following ways. Fluorescence resonance energy transfer (FRET), electrophoresis, microarray and mass spectroscopy.
4. Taqman assay for SNP genotyping: The TaqMan genotyping assay combines hybridization and 5' nuclease activity of polymerase coupled with fluorescence detection. It allows screening, association, candidate region, candidate gene, and finemapping studies

1.5.1.2 Association studies:

SNP based studies can be performed mainly for two purposes:

a. Direct testing of a SNP with functional consequence for association with a disease trait.
b. Using a SNP as a marker for linkage disequilibrium.

1.6 Candiate genes associated with vitiligo susceptibility:

The complex genetics of vitiligo involves multiple susceptibility loci, genetic heterogeneity and incomplete penetrance with gene-gene and gene-environment interactions (Zhang, 2005). A few genes that are identified to contribute to vitiligo susceptibility are given in the Table 2.

Table 2. Genes that contribute to vitiligo susceptibility.

Gene	Method	SNP	Reference
ACE	*Candidate gene association*	rs1799752	Jin *et al.*, 2004a
AIRE	*Candidate gene association*	rs1800521	Nagamine *et al.*,1997
ASIP	*Candidate gene association*	rs6058017	Na *et al.*, 2003
CAT	*Candidate gene association*	rs769217 rs7943316	Casp *et al.* 2002, Gavalas *et al.* 2006, Park *et al.* 2006.
CD4	*Candidate gene association*	CD4 pentanucleotide repeat	Zamani *et al.*,2009 Kristiansen et al., 2004
CLEC11A	*Candidate gene association*	rs7246355	Lan *et al.*,2009
COMT	*Candidate gene association*	rs4680	Tursen *et al.*,2002
CTLA4	*Candidate gene association*	rs231775	Kemp *et al.*, 1999
C12orf10	*Candidate gene association*	rs7975232	Philips *et al.*,2010
DDR1			
EDN1	*Candidate gene association*	rs2267641	de Castro *et al.*,2010
		rs2071942-	Kim *et al.*,2007

	Candidate gene association	rs5370	Jin *et al.*, 2004b
ESR1			Li *et al.*,2008
FAS	*Candidate gene association*	rs2234693	Li M *et al.*,2009
	Candidate gene association	rs2234767	Alkhateeb et al., 2005
FOXD3		rs41285370	Hori *et al.*,2003
FOXP3	*Genome-wide linkage*	rs11798415	Uhm *et al.*,2007
	Defective in IPEX syndrome	rs2071487	
GSTM1			Liu *et al.*,2009
GSTT1	*Candidate gene association*	rs2234953	
IL1RN	*Candidate gene association*	IL1RN VNTR	Pehlivan *et al.*,2009
IL10	*Candidate gene association*	rs689466	Abanmi *et al.*,2008
	Candidate gene association	rs1800872; rs1800871	
KITLG		rs11104947	Lan *et al.*,2009
MC1R	*Candidate gene association*	rs2228479	Na *et al.*, 2003
MBL2	*Candidate gene association*	rs6721961	Onay *et al.*,2007
NFE2L2	*Candidate gene association*	rs36901	Guan *et al.*,2008
NALP1	*Candidate gene association*	rs6502867	Jin *et al.*, 2007
PDGFRA-KIT	*Candidate gene association*	rs689466	Chen *et al.*, 2005
PTPN22	*Candidate gene association*	rs2476601	Canton *et al.*, 2005
PTGS2	*DNA sequencing*	rs7574865	Li K *et al.*,2009
STAT4	*Candidate gene association*	rs1135216	Hu *et al.*,2010
TAP1	*Candidate gene association*	rs2005061	Casp *et al.*,2003
TGFBR2	*Candidate gene association*	rs1800629	Yun *et al.*,2010
TNF	*Candidate gene association*	rs3806933	D'Alfonso *et l.*,1994
			Pociot *et al.*, 1993

TSLP	*Candidate gene association*	rs1043784	
TXNDC5	*Candidate gene association*	rs1458836-	Birlea *et al.*,2011
UVRAG	*Candidate gene association*	rs7933235	Jeong *et al.*,2010a
VDR	*Candidate gene association*	rs7975232	Jeong *et al.*,2010b
XBP	*Candidate gene association*	rs2269577	Birlea *et al.*,2006
	Candidate gene association	rs2269577	Birlea *et al.*,2011

1.6.1 *AIRE*

Vitiligo is commonly associated with autoimmune polyglandular syndrome type I (APS I) (Ahonen *et al.*,1990) and mutation in *AIRE* gene causes this disease. *AIRE* gene is normally expressed in immune related organs such as thymus and lymph nodes. The function of AIRE protein is to act as a transcription factor (Nagamine *et al.*, 1997). Mutation analysis has identified two mutations in this gene in Swiss and Finnish APS I patients (Nagamine *et al.*, 1997).

1.6.2 *CAT*

Catalase converts hydrogen peroxide to water and thereby prevents the cell damage from highly reactive oxygen derived radicals. The *CAT* gene is considered as a candidate gene because of the reduction in catalase activity and concomitant accumulation of H_2O_2 is observed in the epidermis of vitiligo patients (Schallreuter *et al.*, 1991). An association has been established between vitiligo and a SNP in exon 9 of *CAT* gene (Casp *et al.*, 2002; Gavalas *et al.*, 2004). It has been reported that C/T heterozygotes are more frequent among vitiligo patients than controls. The C allele is transmitted more frequently to patients than controls, which suggests that linked mutations in or near the *CAT* gene may contribute to a quantitative deficiency of catalase activity in vitiligo patients and the accumulation of H_2O_2.

1.6.3 *COMT*

In melanocytes, COMT prevents the formation of toxic o-quinones during melanin synthesis (Pavel *et al.*, 1983). It was found that epidermal homogenates from vitiligo patients expressed altered levels of COMT activity than homogenates from healthy controls (Le Poole *et al.*, 1994). A common biallelic polymorphism in the *COMT*

gene that determines high and low enzyme activity has been associated with neuropsychiatric disorders (Karayiorgou *et al.,* 1997). *COMT* polymorphism has not been detected in vitiligo patients compared to controls. However, *COMT*- LL (low activity homozygote) genotype was found to be significantly associated with acrofacial vitiligo (Tursen *et al.,* 2002).

1.6.4 *LMP* and *TAP*

Genes within the class II region of the major histocompatibility complex (MHC) are reported to be associated with several autoimmune diseases (Tanaka *et al.,* 1998; 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 protein polypeptide 2 and 7 (LMP 2 and 7) and transporter associated with antigen processing protein 1 (TAP 1). Casp *et al.,* (2003) showed genetic association of early onset of vitiligo with the *TAP1* gene. Moreover alleles from heterozygous parents were disequilibratedly transmitted to affected offspring for the *TAP1* gene, as well as for the closely linked *LMP2* and *LMP7* genes (Casp *et al.,* 2003).

1.6.5 *MC1R* and *ASIP*

Polymorphism studies in *MCIR* and *ASIP* revealed that G274A and A488G represented abundant forms of the SNPs of the *MCIR* in Korean population. The frequency of the A allele of G274A was higher in vitiligo patients; however this SNP was not statistically significant.The patients who carried both the SNPs of *MCIR* and *ASIP* were prone to vitiligo (Na *et al.,* 2003).

1.6.6 ESR1

It was reported that high estrogen levels in the serum was associated with increased skin pigmentation (Shahrad and Marks 1977). Successful treatment of vitiligo was shown with the steroid- thyroid hormone mixture containing estrogen (Nagai *et al.,* 2000; Ichimiya *et al.,* 1999). It was shown that *ESRI* (Estrogen receptor gene 1) intron 1 C/T polymorphism was associated with female or generalized vitiligo patients.

ESR1 gene may be a possible risk factor for the female or generalized type of vitiligo (Jin *et al.*, 2004b).

1.6.7 *KIT*

KIT encodes for a tyrosine kinase receptor named c-kit expressed on the surface of melanocytes, mast cells, germ cells and hematopoietic stem cells (Grabbe *et al.*, 1994). The c-kit ligand, SCF (stem cell factor) is involved in the proliferation and survival of melanoblasts and may be associated with the dysfunction and/or loss of melanocytes (Nishikawa *et al.*, 1991). The expression of c-kit and its down stream effector microphthalmia associated transcription factor (MITF) is reduced in vitiligo epidermis (Norris *et al.*, 1996; Kitamura *et al.*, 2004). It was also observed that vitiligo had remained stable for many years after treatment with tyrosine kinase inhibitors (Passeron and Ortonne 2005). Moreover, several cases of vitiliginous depigmentation occurring after treatment with new tyrosine kinase inhibitors (STI-571 and SU 11428) are reported (Raanani *et al.*, 2002). BCL2 is a MITF dependent KIT transcriptional target in melanocytes (McGill *et al.*, 2002) and a decrease in *BCL 2* expression in melanocytes increases their susceptibility to apoptosis. Interestingly, SCF strongly protects melanocytes form TNF related apoptosis inducing ligand (TRAIL) (Larribere *et al.*, 2004). SCF/c – KIT thus brings new interesting potential clues regarding the physio-pathology of vitiligo.

1.6.8 *FOXD3*

Forkhead box D3 (*FOXD3*) is a transcription factor that suppresses melanoblast development from neural crest (Kos *et al.*, 2001). Therefore dysregulated expression might harm melanocytes. FOXD3 also regulates endodermal differentiation including thyroid, pancreas, adrenal gland and gut (Guo *et al.*, 2002). Also other FOX factors are involved in autoimmune syndromes (Jonsson and Peng 2005). Mutations in *FOXD3* leading to elevated FOXD3 transcription is recently reported in one AIS 1 linked family (Alkhateeb *et al.*, 2005).

1.6.9 *TNFA*

TNFA expression may depend on polymorphisms in the *TNFA* promoter region or a linkage association with the HLA genotype (D'Alfonso *et al.,* 1994; Pociot *et al.,* 1993). In this respect, the -308 allele is associated with the HLA A1, B8, DR3, DR4 and the DQ2 haplotypes; the DR2 is associated with low TNFα responses; and the DR3 and DR4 genotypes are associated with high TNFα production (Wilson *et al.,* 1997; Yucesoy *et al.,* 2001). The increased HLA associations in vitiligo patients were: HLA-A2, A31, B13, B27, B56, B60, CW6, DR4, DR5, DR7, DR53 and DQ3 (Zhang *et al.,* 2005). Therefore, the increased production of TNFα could contribute to the increased incidence of vitiligo observed in individuals with DR4 haplotype. However, a study in the Turkish population shows that -308 *TNFα* promoter polymorphism has no significant influence on vitiligo susceptibility and clinical manifestations (Yazici *et al.,* 2006).

1.6.10 *CD4*

The *CD4* gene plays an important role in the cell-mediated immune response and its association with type 1 diabetes mellitus has been previously reported. Zamani *et al.,* (2009) reported an association with a pentanucleotide variable number of tandem repeats polymorphism (VNTR) with vitiligo.

1.6.11 *CLEC11A*

C-type Lectin Domain Family 11, Member A *(CLEC11A)* is one of the relevant keratinocyte-growth related genes and its role has been implicated in the pathogenesis of vitiligo vulgaris (Lan *et al.,* 2009).

1.6.12 *DDR1* (Discoidin domain receptor tyrosine kinase 1)

Receptor tyrosine kinases play a key role in the communication of cells with their microenvironment. These kinases are involved in the regulation of cell growth, differentiation and metabolism. The protein encoded by this gene belongs to a subfamily of tyrosine kinase receptors. Sakuma *et al.* (1996) cloned genomic DNA of the *DDR1* gene. The gene contains 15 exons spanning approximately 9 kb. The

promoter region of the gene contains a consensus binding site for p53. Silva de Castro *et al.* (2010), reported association of *DDR1* gene with vitiligo. However, Kim *et al.* (2010) found no association of vitiligo with *DDR1*.

1.6.13 *EDN1* (Endothelin-1)

Endothelin-1, which is expressed by keratinocytes, has paracrine effects on melanocytes, influencing their homeostasis, proliferation and pigmentation. It is thought to play a role in the skin response to 311-nm, narrow-band ultraviolet irradiation. Interestingly, the haplotype frequencies of *EDN1* polymorphisms differed significantly between vitiligo patients and healthy controls. When analysed according to clinical type, the haplotype frequencies in the focal and segmental clinical types differed significantly from healthy controls (Kim *et al.*, 2007)

1.6.14 *FAS*

The FAS/FASLG system plays a key role in regulating apoptosis. Previous findings have shown that CD4-dependent destruction of melanocytes is partially inhibited by blocking FAS-FASLG interactions in autoimmune vitiligo. Functional polymorphisms of the FAS and FASLG genes can alter their transcriptional activities. Li *et al.* (2008) and Li *et al.* (2009) reported an association of vitiligo with the *FAS* gene.

1.6.15 *FBXO11-MSH6*

The involvement of FBXO11 (previously, VIT1) in vitiligo was suggested on the basis of differential expression analysis (Le Poole *et al.*, 2001). Putative mutations in the adjacent MSH6 gene were reported in a single patient with early-onset colorectal cancer, systemic lupus erythematosus, and vitiligo (Rahner *et al.*, 2008).

1.6.16 *FOXP3*

FOXP3, a member of the fork-winged helix family of transcription factors, plays an important role in the development and function of naturally occurring CD4 (186940)-positive/CD25 (IL2RA; 147730)-positive T regulatory cells (Tregs). Tregs are

involved in active suppression of inappropriate immune responses. Due to similarities between the autoimmunity and inflammation produced by manipulation of CD25-positive/CD4-positive regulatory T (Tr, or Treg) cells and those induced by genetic defects in the *FOXP3* gene, Hori *et al.* (2003) investigated the contribution of Foxp3 to the development and/or function of Tr cells in mice. *FOXP3* is the defective gene in the X-linked recessive immunodysregulation, polyendocrinopathy, and enteropathy multiple autoimmune disease syndrome and vitiligo.

1.6.17 *GSTM* and *GSTT*1

The glutathione S-transferases (GSTs) are a family of enzymes responsible for the metabolism of a broad range of xenobiotics and carcinogens (Mannervik, 1985). Uhm *et al.* (2007) reported an association with a *GSTM1* INDEL polymorphism with vitiligo. Liu *et al.* (2009) reported an association with a *GSTT1* INDEL polymorphism with vitiligo however, another study showed no association of the same marker with vitiligo (Uhm *et al.*, 2007).

1.6.18 *IL1RN* (IL1 receptor antagonist)

IL1RN is a protein that binds to IL1 receptors (IL1R1) and inhibits the binding of IL1-alpha and IL1-beta (IL1B). As a consequence, the biological activity of these two cytokines is neutralized in physiological and pathophysiological immune and inflammatory responses. IL1RN was the first-to-be described, naturally occurring cytokine or hormone-like molecule to function as a specific receptor antagonist (Arend, 1991). Pehlivan *et al.* (2009) reported an association with an *IL1RN* intronic VNTR polymorphism, however, this result was rendered not significant by appropriate multiple-testing correction.

1.6.19 *IL10* (Interleukin 10)

IL10 family of cytokines produced by activated macrophages that targets various leukocytes and mainly represses excessive inflammatory responses. This family of cytokines also plays a role in epithelial cell proliferation, anti-apoptotic responses, tissue remodeling and healing. As IL 10 acts as an anti- inflammatory cytokine and inhibits the production of several cytokines such as IFN- γ from Th1 cells, the low

levels of this cytokine is been associated with autoimmune diseases. Abanmi *et al.* (2008) reported association of vitiligo with *IL10*.

1.6.20 *PDGFRA-Kit* (Platelet-derived growth factor receptor alpha)

The *PDGFRA* gene is a proto-oncogene that maps to 4q12. It belongs to the human type III family of transmembrane receptors, with an intrinsic tyrosine kinase component. The PDGFRA protein has been shown to be important for several cellular and tissue processes, such as proliferation, apoptosis, chemotaxis, melanogenesis, hematopoiesis and gametogenesis (Mol *et al.*, 2003). Several reports have documented PDGFRA in regulation of pigmentation. PDGF has been shown to be important for the differentiation and survival of melanocytes during embryonic development (Adamyeko *et al.*, 2009). These reports lead us to speculate that the *PDGFRA* gene may be a candidate susceptibility gene of vitiligo mapped to the region of 4q12.

1.6.21 *TSLP* (Thymic stromal lymphopoietin)

TSLP induces naïve CD4+ T cells to produce Th2 cytokines. In addition, to low production of Th2 cytokines, strong Th1 response, which plays an important role in vitiligo development, has been induced by blockade of TSLP or TSLP receptor.Cheong *et al.* (2009) reported an association with the TSLP 50-flanking SNP rs3806933 (-847C4T).

1.6.22 *UVRAG* (UV radiation resistance-associated gene)

Teitz *et al.* (1990) identified a cDNA that partially complements the ultraviolet (UV) sensitivity of xeroderma pigmentosum complementation group C (278720) cells. Perelman *et al.* (1997) named this gene *UVRAG* and reported that the 4.0 kb *UVRAG* mRNA encodes a predicted 648-amino acid protein. Jeong *et al.* (2010b) reported association with a haplotype defined by SNPs rs7933235 and rs1458836 in vitiligo.

1.6.23 *VDR* (Vitamin D receptor)

VDR is an intracellular hormone receptor that specifically binds 1,25 (OH)2D3 and mediates its effects. In 31 GV cases and 33 controls, Birlea *et al.* (2006) reported an association with the VDR restricted fragment length polymorphism rs7975232.

1.6.24 *XBP1* (X box-binding protein 1)

XBP1, is a protein which in humans is encoded by the XBP1 gene.(Liou et al.,1990) The XBP1 gene is located on chromosome 22 .The XBP1 protein is a transcription factor that regulates the expression of genes important to the proper functioning of the immune system and in the cellular stress response (Yoshida *et al.*, 2006).Spritz *et al.* (2004) detected linkage of GV to microsatellites at 22q11–q11.22 and Liang *et al.* (2007) at 22q12 in Chinese families. Ren *et al.* (2009) tested XBP1 as a positional/biological candidate gene within the linkage interval.

1.7 LINKAGE AND ASSOCIATION STUDIES:

Familial clustering and linkage disequilibrium studies showed that genetic factors predispose vitiligo although a clear transmission pattern and cosegregation of vitiligo with specific mutations have not been demonstrated.

1.7.1 HLA associations:

The frequent association of vitiligo with other autoimmune diseases has prompted the studies of HLA association with vitiligo predisposition. The *HLA* loci are strongly linked to other loci in the major histocompatibility region of chromosome 6p. Therefore, it may be that vitiligo associated *HLA* alleles are not disease causing but are genetic markers that are usually co inherited in the population (i.e. in strong linkage disequilibrium) with the actual disease allele at another locus within the major histocompatibility region (Zhang et al 2005). Linkage disequilibrium studies in different populations have consistently showed a significant association between the HLA system and vitiligo predisposition. There are several studies on the association between vitiligo and HLA complex. HLA subtypes vary with racial/ethnic background.

Association of *MHC* alleles with a disease gains importance because of the antigen-presenting function of the MHC. Recent genome wide association studies have implicated the role of MHC in vitiligo (Jin *et al.*, 2010, Quan *et al.*, 2010) where several SNPs in the *MHC* region were significantly associated with the disease. However, the authors imputed the Human Leukocyte Antigen (HLA) class-I alleles based on the linkage disequilibrium (LD) of HLA alleles with the specific SNPs, but could not impute HLA class-II alleles due to limitations of HLA allele imputation in the CHB (Chinese Han from Beijing, China) samples (Quan *et al.*, 2010). The association of multiple HLA class I and class II antigens have been suggested for vitiligo (de Vijlder et al., 2004, Zhang *et al.*, 2004a, Zhang *et al.*, 2004b) although no consensus could be reached, due to distinct ethnic groups (Orozco-Topete *et al.*, 2005, Tastan *et al.*, 2004, Zamani *et al.*, 2001) small sample sizes and low resolution typing methods used to identify the HLA antigens. For example, HLA-DR4 is increased in blacks, HLA-B13 in Moroccan Jews, and HLA-B35 in Yemenite Jews. An association of HLA-B13 with anti-thyroid antibodies has been reported (Rezaei *et al.*, 2007). However, using high resolution typing methods and large number of samples, Singh *et al.*, 2012 suggested a consistent increase of A*33:01, B*44:03, and DRB1*07:01 in both initial and replication studies implicating these alleles as possible markers of vitiligo in North India and Gujarat. These data apparently suggest auto reactive $CD4^+$ T-helper cells to be restricted by HLA-DRB1*07:01 and the auto reactive $CD8^+$ cytotoxic T cells by HLA-A*33:01, A*02:01, B*44:03, and B*57:01 in the Indian population. Previous studies in Caucasians showed association of generalized vitiligo with both MHC class I (specifically, HLA-A*02:01) (Jin *et al.*, 2010; Jin *et al.*, 2012) and class II loci (Jin *et al.*, 2010), whereas studies carried out in Chinese show no apparent association in the class I or II regions and instead favor association in the class III region (Quan *et al.*, 2010). Together, these findings indicate that the principal MHC genetic associations with generalized vitiligo differ among different populations, and may in part mediate differing prevalence of this autoimmune disease in different groups around the world. The HLA association studies reported till now are listed in Table 3.

Table 3. HLA associations reported in vitiligo.

1.7.1.1 Positive association	Negative association	Reference
HLA-A*33:01, HLA-A*02:01, HLA-B*44:03, HLA-DRB1*07:01	DRB1*03:01	Singh *et al.*, 2012
HLA-A*02:01	-	Jin *et al.*, 2012
DRB1*07:01	-	Ren *et al.*, 2009
HLA-A2	-	Liu *et al.*, 2007
DRB1*04-DQB1*0301	DRB1*15-DQB1*0602	Fain *et al.*, 2006
DQA1*0302,*0601, DQB1*0303, *0503	*0503 DQA1*0501	Yang *et al.*, 2005
A*2501, A*30, B*13, B*27, Cw*0602	A*66	Zhang *et al.*, 2004
DR4, DR53	DR3	de Vijlder *et al.*, 2004
DR3, DR4, DR7	-	Tastan *et al.*, 2004
DRB4*0101, DQB1*0303	-	Zamani *et al.*, 2001
DRB1*0701, DQB1*0201, DPB1*1601	-	Buc *et al.*, 1998
A2, A10, A30 + A31, B13, B15	A28, B46	Wang *et al.*, 2000
A2, Dw7	-	Buc *et al.*, 1996
B21, Cw6, DR53	A19, DR52	Al-Fouzan *et al.*, 1995
DR6	DQ2	Valsecchi *et al.*, 1995
Bw6, DR7	-	Venkataram *et al.*, 1995
DR6	Cw7	Venneker *et al.*, 1993
B46, A31, Cw4	-	Ando *et al.*, 1993
DR12, A2	-	Schallreuter *et al.*, 1993
A30, Cw6, DQ3	C4AQ0	Orecchia *et al.*, 1992
DR1	-	Poloy *et al.*, 1991

A30, Cw6, B27, DR7	DR1, DR3	Finco *et al.*, 1991
A2, A3	-	Dai *et al.*, 1990
DR4, DQ3	-	Dunston and Halder,1990
DR4	-	Foley *et al.*, 1983
BW35	-	Metzker *et al.*, 1980
A1, A2, A31	A10	Kachru *et al.*, 1978
Cw* 0602	-	Xia *et al.*, 2006

1.8 GENOME WIDE ASSOCIATION STUDIES (GWAS)

Genome wide linkage scans involve the typing of families using polymorphic markers that are positioned across the whole genome, followed by calculating the degree of linkage of the marker to a disease trait. Positional candidate genes can be identified by examining the regions around the peaks of linkage that are obtained by the study. Several genome wide linkage analyses of vitiligo have been performed and multiple linkages to vitiligo have been identified (Nath *et al.*, 2001; Fain *et al.*, 2003; Alkhateeb *et al.*, 2002; Spritz *et al.*, 2004). The susceptibility loci identified by genome wide linkage analyses are given in Table 4.

The most important recent vitiligo developments were two large-scale genomewide association studies of generalized vitiligo, one in Caucasians (Jin *et al.*, 2010) and the other in Chinese (Quan *et al.*, 2010), which together identified and confirmed at least 16 different loci that contribute to generalized vitiligo susceptibility. Jin *et al.*, (2010 a,b) carried out a GWAS of GV in non-Hispanic white subjects, identifying and confirming different loci that contribute to GV risk, including *FOXP1, MYH15, CCR6, ICA1, TBC1D2, IKZF4, SH2B3* almost all of which have immunoregulatory functions (Jin *et al.*, 2010a, b). Two of these loci and one additional signal in the MHC were also identified in a Chinese GWAS of GV (Quan *et al.*, 2010).

All but one of these genes encode proteins involved in regulation of the immune system and/or have been genetically associated with susceptibility to other autoimmune diseases. The sole exception is *TYR*, encoding tyrosinase, the key enzyme of melanin biosynthesis and the principal vitiligo autoimmune antigen. In

Caucasians, a common *TYR* missense variant, R402Q, confers both relative protection from generalized vitiligo and relative susceptibility to malignant melanoma, by modulating the presentation of the TYR peptide by HLA-A2*01, thereby modulating recognition of melanocytes by the immune system. These genes together account for a relatively small fraction of the genetic risk of generalized vitiligo, indicating that many additional vitiligo susceptibility genes undoubtedly remain to be discovered.

Recently, Jin *et al.*, 2012 reported a large GWAS (450 individuals with vitiligo and 3,182 controls), an independent replication study (1,440 cases and 11,316 controls) and a meta-analysis (3,187 cases and 6,723 controls) identifying 13 additional vitiligo-associated loci. These include *OCA2-HERC2, MC1R*, a region near *TYR, IFIH1, CD80, CLNK, BACH2, SLA, CASP7, CD44, IKZF4, SH2B3* and *TOB2*. Most vitiligo susceptibility loci encode immunoregulatory proteins or melanocyte components that may mediate immune targeting and the relationships among vitiligo, melanoma and eye, skin and hair coloration.

Table 4. Susceptibility loci for vitiligo.

Susceptibility loci	Chromosomal Region	Reference
SLEV1	17p13	Spritz *et al.*, 2004, Nath *et al.*, 2001
AIS1	1p31.3-p32.2	Alkhateeb *et al.*, 2002
AIS2	7p	Spritz *et al.*, 2004
FOXP1	3p13	Jin *et al.*, 2010b
MYH15	3q13.13	Jin *et al.*, 2010b
CCR6	6q27	Jin *et al.*, 2010b
ICA1	7p21.3	Jin *et al.*, 2010b
TBC1D2	9q22.33	Jin *et al.*, 2010b
IKZF4	12q13.2	Jin *et al.*, 2010b
SH2B3	12q24.12	Jin *et al.*, 2010b
RNASET2	6q27	Quan *et al.*, 2010
FGFR1OP	6q27	Quan *et al.*, 2010
CCR6	6q27	Quan *et al.*, 2010
IFIH1	2q24.2	Jin *et al.*, 2012
CD80	3q13.33	Jin *et al.*, 2012
CLNK	4p16.1	Jin *et al.*, 2012
BACH2	6q15	Jin *et al.*, 2012
SLA	8q24.22	

CASP7	10q25.3	Jin *et al.*, 2012
CD44	11p13	Jin *et al.*, 2012
TYR	11q21	Jin *et al.*, 2012
OCA2-HERC2	15q12-13.1	Jin *et al.*, 2012
MC1R	16q24.3	Jin *et al.*, 2012
TICAM1	19p13.3	Jin *et al.*, 2012
TOB2	22q13.2	Jin *et al.*, 2012

1.9 Candidate genes addressesd in the present study:

1.9.1 *ACE* (Angiotensin converting enzyme):

ACE, a key enzyme in the renin-angiotensin system. The gene encoding *ACE* (or dipeptidyl carboxy peptidase 1: DCP1) is of 24 kb consisting of 26 exons, and is located on chromosome 17q23. It catalyzes the conversion of angiotensin I to angiotensin II in kidney through removal of two C-terminal residues. Activation of ACE is as explained in Figure 10.The ACE plays an important role in the physiology of the vasculature, blood pressure and inflammation, and it has also been found to be associated with several autoimmune disorders (Vuk-Pavlovic *et al.,* 1988, Scholzen *et al.,* 2003).

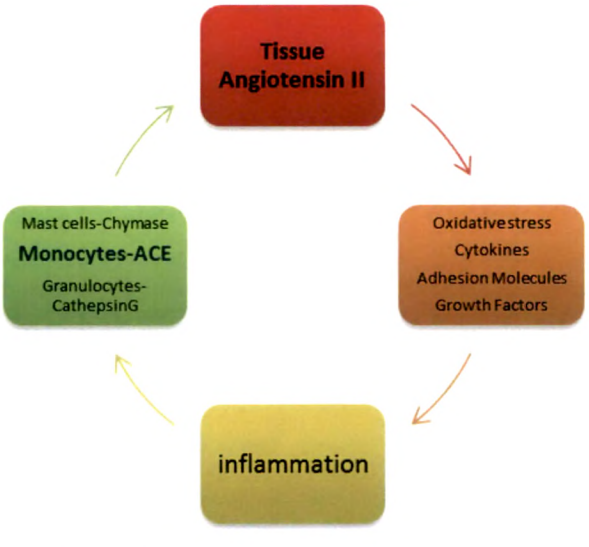

Figure 10. Activation of tissue ACE:

It creates a positive-feedback mechanism for tissue Ang II formation with subsequent induction of oxidative stress and inflammation. Activation of tissue ACE promotes release of cytokines and growth factors that increase vessel wall inflammation.

Inflammatory cells, in turn, release enzymes and other substances that generate Ang II. (Dzau *et al.*, 2001).

1.9.1.1 *ACE* I/D polymorphism:

An insertion/deletion (I/D) polymorphism of a 287-base pair repetitive sequence in intron 16 of the *ACE* gene (NCBI: AF118569; repeat region 14094–14381) has been associated with the development of vitiligo (Jin *et al.*, 2004; Deeba *et al.*, 2009; Tippisetty *et al.*, 2011). The insertion/deletion (I/D) polymorphism of the *ACE* gene accounts for a large proportion of the variability of serum ACE activity, D/D genotypes having the highest and I/I genotypes having the lowest ACE activity (Rigat *et al.*, 1990).

1.9.2 *CTLA4* (Cytotoxic T lymphocyte-associated antigen4):

CTLA-4, also known as CD152 is a type 1 transmembrane glycoprotein of the immunoglobin superfamily (Brunet *et al.*, 1987). The cytoplasmic portion of CTLA-4 lacks any intrinsic enzymatic activity (Baroja *et al.*, 2000). It contains a lysine-rich motif located proximal to the membrane (Baroja *et al.*, 2002). The *CTLA4* gene is located on chromosome 2 in humans (Dariavach *et al.*, 1988,). This gene consists of 4 exons (Dariavach *et al.*, 1988, Ling *et al.*, 1999) (Figure 11). The resulting transcript can undergo alternative splicing, which is different between human and mouse *CTLA4*. For human *CTLA4*, one can detect (a) full-length mRNA containing exons 1 to 4, (b) a transcript coding for soluble *CTLA4* (sCTLA-4) and (c) a transcript coding only for exons 1 and 4 (Brunet *et al.*, 1987, Dariavach *et al.*, 1988, Ling *et al.*, 1999, Ueda *et al.*, 2003, Vijayakrishnan *et al.*, 2004, Magistrelli *et al.*, 1999, Oaks *et al.*, 2000).

The *CTLA4* gene is mainly expressed in T cells upon activation (Perkins *et al.*, 1996), although it is constitutively expressed in the $CD4^+CD25^+$ regulatory T cell (Treg) subset (Lindsten *et al.*, 1993, Perkins *et al.*, 1996, Harper *et al.*, 1991, Takahashi *et al.*, 2000). Memory T cells have a relatively large pool of intracellular CTLA-4, which is rapidly expressed on the cell surface upon activation.The transcriptional regulation of *CTLA4* gene expression is only partially known. Sequence analysis of

the 5' upstream region of the *CTLA4* gene revealed several transcriptional regulatory elements, including sites for AP1, STAT, GATA1, NF-κB, Oct1, and an IL4 negative regulatory element (Ling *et al.,* 1999, Perkins *et al.,* 1996, Finn *et al.,* 1997). Also, *CTLA4* expression is upregulated by cAMP, suggesting a cAMP-dependent regulation of *CTLA4*gene transcription (Vendetti *et al.,* 2002).

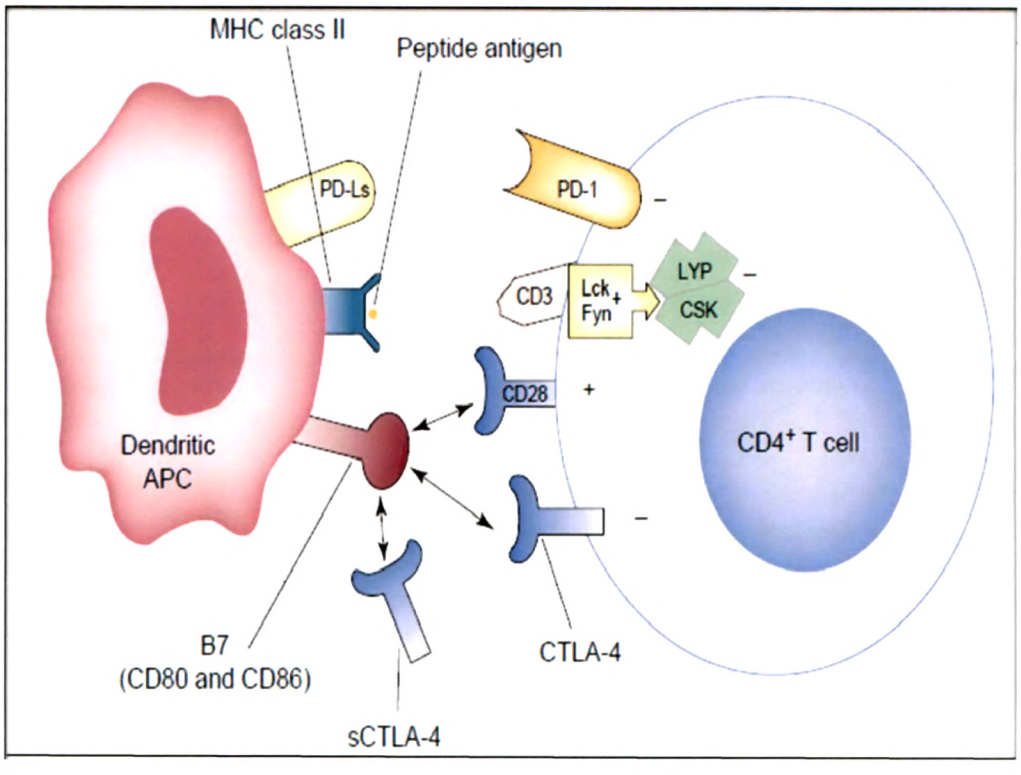

Figure 11: Interaction of CTLA4 and B7 molecules of Dendritic APC's.

(Kuby, *Immunology*, 6 *th Ed.*, 2007)

1.9.2.1 *CTLA4* polymorphisms:

Given the evolutionary conservation of *CTLA4* and its differential expression in T cell subsets, transcriptional or translational changes in its expression may have serious immunological consequences. This has justified the search for Polymorphisms and their link with immune-mediated diseases. To date, four main Polymorphisms of the *CTLA4* gene have been identified and studied in the context of autoimmune disorders. SNP's have been noted in human *CTLA4* at positions –1722, –1661, and –318, all in the regulatory/promoter region, and at position +49 in exon 1, which results in the substitution of an alanine for a threonine in the signal sequence of *CTLA4* (Nistico *et*

al., 1996, Deichmann *et al.,* 1996, Donner *et al.,* 1997, Wang *et al.,* 2002, Kristiansen *et al.,* 2000).

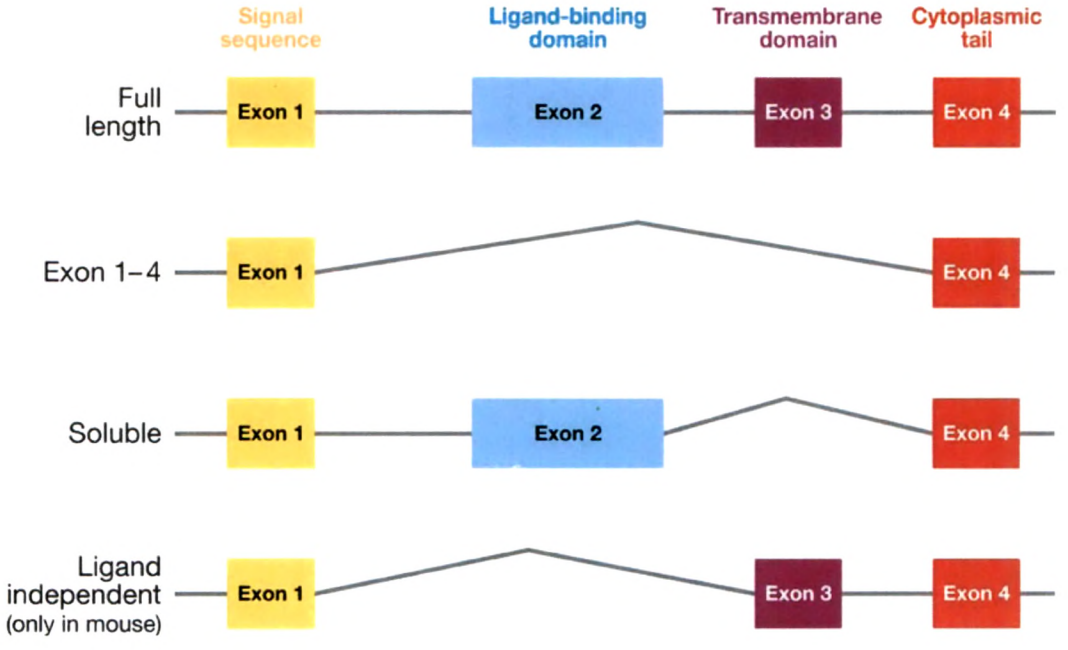

Figure 12: The *CTLA4* gene and its various splice variants.

The *CTLA4* gene consists of four exons that can give rise to four different splice variants. Each exon codes for a domain in the protein as indicated in the top of the figure. Humans express the full-length *CTLA4*, exon 1–4 *CTLA4*, and soluble *CTLA4*splice variants, whereas mice additionally express the ligand-independent *CTLA4*.

The A49G polymorphism is the only polymorphism that changes the primary amino acid sequence of *CTLA4*. In vitro studies of A49G *CTLA4* have shown that this mutant form of *CTLA4* is aberrantly processed in the endoplasmic reticulum, leading to reduced surface expression (Anjos *et al.,* 2002). In contrast, the C-318T polymorphism has been associated with higher promoter activity and subsequently increased *CTLA4* expression (Wang *et al.,* 2002). The other two Polymorphisms, T-1772C and A-1661G, are less characterized, and further investigation is required to define their effects on *CTLA4* expression and association with immunological pathologies. In addition, a dinucleotide repeat polymorphism in the 3UTR of *CTLA4*

has been identified (Huang *et al.,* 2000). Patients with longer AT repeats in the 3' UTR have T cells that have higher proliferative responses when stimulated with anti-CD3/anti- CD28 (Wang *et al.,* 2002). The correlation between all these polymorphisms of *CTLA4* and susceptibility to autoimmune disorders has been contentious, with some reports indicating strong correlation and others disputing such a correlation. More importantly, how these Polymorphisms affect CTLA4 function is unclear. They may affect CTLA4 processing and intracellular trafficking, or they may affect oligomerization and surface retention through indirect effects on CTLA4 posttranslation modifications.

Using the candidate gene approach, the role of CTLA4 has been investigated in various autoimmune diseases e.g. Graves disease, Hashimoto's thyroiditis (HT), Addison's disease (AD), Insulin-dependent diabetes mellitus (IDDM), Myasthenia gravis (MG), Multiple sclerosis (MS), Systemic lupus erythematosus (SLE), Rheumatoid arthritis (RA) including Vitiligo.

1.9.3 *PTPN22* (Protein tyrosine phosphatase non receptor 22):

The *PTPN22* gene, located on chromosome 1p13, encodes a lymphoid specific phosphatase (LYP), which is important in the negative control of T lymphocyte activation (Hill *et al.,* 2002). LYP is an intracellular PTP with a molecular weight of 110 kDa that contains an N- terminal catalytic domain and a non-catalytic C-terminus with 4 proline rich motifs. LYP physically bound through proline-rich motif to the SH3 domain of the Csk kinase, which is an important suppressor of T-cell receptor signaling kinases LCK and FYN that mediate T-cell activation (Cloutier *et al.,* 1999, Palacios *et al.,* 2004). The ability of Csk and LYP to inhibit T-cell receptor signaling requires their physical association (Cohen *et al.,* 1999).

Protein tyrosine phoshatases (PTPs) are critical regulators of T-cell signal transduction (Figure 13) (Bottini *et al.,* 2004, Smyth *et al.,* 2004). In conjunction with protein tyrosine kinases PTPs regulate the reversible phoshorylation of tyrosine residues and thereby play important roles in many different aspects of T-cell physiology (Alonso *et al.,* 2004). Abnormalities in tyrosine phosphorylation have been shown to be involved in the pathogenesis of numerous human diseases from

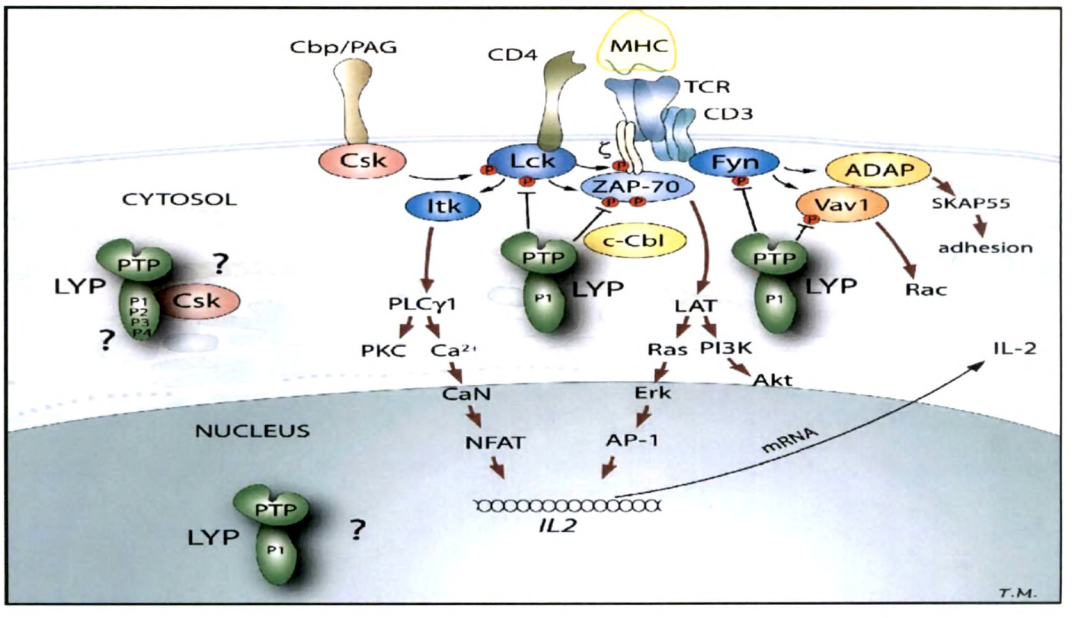

Figure 13: Role of *PTPN22* in T-cell receptor signaling (Bottini *et al.*, 2006)

1.9.3.1 *PTPN22* Polymorphism:

Recently, an association between the minor allele (T) of a missense single-nucleotide polymorphism [SNP; R620W (rs2476601, 1858C/T)] in the protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*) and susceptibility to generalized vitiligo has been described (Canton *et al.,* 2005). Besides its association with vitiligo the missense R620W polymorphism in the *PTPN22* gene at nucleotide 1858 (1858C/T) in codon 620 (620Arg→Trp) has been also associated with autoimmune diseases including type I diabetes mellitus, Graves' disease, systemic lupus erythematosus and rheumatoid arthritis (Bottini *et al.,* 2004, Smyth *et al.,* 2004, Onengut-Gumuscu *et al.,* 2004, Ladner *et al.,* 2004, Velaga *et al.,* 2004, Kyogoku *et al.,* 2004, Orozco *et al.,* 2005) suggesting a genetic predisposition towards generalized T-cell autoimmunity. Despite the association of *PTPN22* C1858T SNP with the multiple different autoimmune disorders, there are some autoimmune diseases such as psoriasis, IBD, MS, Addison's disease and Celiac diseases in which *PTPN22* C1858T SNP does not appear to play a role in susceptibility (Prescott NJ *et al.,* 2005, Martin MC *et al.,* 2005, Wagenleiter SE *et al.,* 2005, Matesanz F *et al.,* 2005, Ittah M *et al.,* 2005 and Gonzalez-Gay MA *et al.,* 2005).

The *PTPN22* 1858 C→T SNP changes the amino acid at position 620 from an Arginine(R) to a tryptophan (W) and disrupts the interaction between Lyp and Csk, avoiding the formation of the complex and therefore the suppression of T-cell activation. In vitro experiments have shown that the T-allele of *PTPN22* binds less efficiently to Csk than the C-allele does, suggesting that T-cells expressing the T-allele may be hyper responsive and consequently, individuals carrying this allele may be prone to autoimmunity (Cloutier JF *et al.,* 1999). The *PTPN22* 1858T variant has recently been described to result in a gain-of-function form of the enzyme leading to stronger suppression of the early T-cell activation process. T cells from individuals carrying the predisposing T-allele produced less interleukin-2 and encoded a phosphatase that had higher catalytic activity and thus represents a gain of function polymorphism (Vang *et al.,* 2005).

Experimental evidence suggests that the disease-associated LYP variant SNP lies within the first proline-rich domain of *PTPN22* and results in the substitution of tryptophan for arginine. Trp620 prevents the interaction of LYP with CSK . Consequently, the T-cell receptor-associated kinases might be able to induce T-cell activation in an uncontrolled manner and this may increase the overall reactivity of the immune system and may result in failure to delete autoreactive T cells during thymic selection or decreased activity of regulatory T cells. This in turn may predispose an individual to autoimmune disease.

1.9.4 *MBL* (Mannose binding lectin):

MBL is a serum protein produced in the liver, and is a key molecule in innate immunity. MBL, along with other molecules such as surfactant protein A (SP-A) and surfactant protein D (SP-D), is a member of the collectin family, the characteristic of which being possession of a carbohydrate recognition domain (CRD) and a collagenous domain [Holmskov *et al.,* 1994]. MBL is a trimer protein composed of 3 identical polypeptides with a molecular weight of around 32 kDa (228 amino acids). Each polypeptide consists of a CRD, a neck domain, a collagen domain and a cystein rich region. MBL binds to various organisms by its CRD, and excises an opsonin effect. The multimeric structure of MBL allows it to bind to various microorganisms including Gram positive and negative bacteria, mycobacterium, viruses and fungi.

The binding of MBL leads to agglutination of these microorganisms and will help their clearance by phagocytes. In addition, MBL activates the complement pathway (the lectin pathway) through mannose binding lectin associated serine proteases (MASPs). Therefore, MBL is important in host defense, especially in infancy, when the acquired immunity has not fully developed. Individuals lacking this protein may develop severe episodes of bacterial infections from early life [Koch *et al.,* 2001, Summerfield *et al.,* 1997]. MBL is also important when the immune system of an individual is compromised, such as when a patient is under immunosuppressive therapy, or receiving chemotherapies or bone marrow transplant [Mullighan *et al.,* 2004]. While MBL is an acute phase protein and its production is enhanced by inflammatory stimuli, Polymorphisms of the MBL gene is known to greatly influence serum MBL concentration. The MBL gene, located on the long arm of chromosome 10 at 10q11.2–q21, contains 4 exons [Sastry *et al.,* 1989].

1.9.4.1 *MBL2* Polymorphisms :

The functional *MBL2* gene is located on chromosome 10 (q11.2-q21) and comprises of four exons. Exon 1 encodes the signal peptide, a cysteine rich region and part of the glycine-rich collagenous region. Three functional single-nucleotide Polymorphisms (SNPs) in exon 1 of *MBL2* gene have been reported: codon 54 (GGC→GAC; designated B allele), codon 57 (GGA→GAA; designated C allele), and codon 52 (CGT→TGT; designated D allele) (Sumiya *et al.,* 1991; Lipscombe *et al.,* 1992; Hansen and Holmskov, 1998). These variant alleles in the collagen like domain cause structural changes in MBL and will lead to its low plasma levels and thus results in plasma concentrations varying by more than 1000-fold within the population (Garred *et al.,* 2003; Thiel, 2007). The promoter region of the *MBL2* gene contains a number of regulatory elements, which affect transcription of the gene and hence Polymorphisms in the promoter region, thus affecting the plasma MBL concentration directly. Besides these exon 1 variant alleles, SNPs at promoters -550 (G/C; allele L) and -221 (G/C; allele X) have also been associated with low plasma levels of MBL (Madsen *et al.,* 1995). An association between *MBL2* gene codon 54 (allele B) polymorphism and susceptibility to vitiligo has been described in Turkish population (Onay *et al.,* 2007).

1.9.5 *NALP1* :

The *NALP1* gene, mapped to chromosome 17p13, has been linked to vitiligo and associated autoimmunity (Jin Y *et al.,* 2007). The *NALP1* gene encodes NALP1, a leucine-rich repeat protein 1, a member of the nucleotide oligomerization domains-like receptors (NLRs) family. The NLRs are a group of cytoplasmic pattern recognition receptors, which nonspecifically recognize microbial products, such as lipopolysaccharide, thereby stimulating innate immunity.

The *NALP1* gene encodes NLRP1, a leucine-rich repeat protein 1, a member of the nucleotide oligomerization domains-like receptors (NLRs) family. The NLRs are a group of cytoplasmic pattern recognition receptors, which nonspecifically recognize microbial products, such as lipopolysaccharide, thereby stimulating innate immunity. NALP1 contains five domains, a protein-protein interaction N-terminal pyrin domain followed by centrally located NACHT (NAIP CIIA HET-E and TP1 family proteins) domain responsible for oligomerization and activation of the NLRs, a domain comprised of five tandem leucine-rich repeat (LRR) domains that is speculated to bind microbial ligands, a FIIND domain, and a finally a C-terminal caspase recruitment (CARD) domain (Martinon and Tschopp, 2005).

The primary function of human NALP1 is caspase activation. Upon activation by appropriate PAMPs, resulting in its oligomerization, human NALP1 binds adapter protein ASC via its N-terminal PYD. ASC, in turn, binds pro-caspase-1, forming a multiprotein complex termed an 'inflammasome' (Martinon *et al.,* 2002). The C-terminal CARD of NALP1 can also bind both pro-caspase-1 and caspase-5 (Tschopp *et al.,* 2003) (Figure 14). These members of the caspase family activate IL-1b and IL-18, as well as causing apoptosis, as highlighted previously. NALP1 may have other functions besides activation of caspase-1 in response to bacterial PAMP, MDP. In this regard, the CARD of NALP1 binds the CARD of Apaf-1, a caspase-9 activating protein that becomes activated by cytochrome c when released from mitochondria (Bruey *et al.,* 2007; Chu *et al.,* 2001) (Figure 15). Thus, it is possible that NALP1 contributes to apoptosis via caspase-9 activation in some contexts. Also, it is possible that Apaf1 and components of patched receptor complex are intrinsic activators of NALP1. Cytochrome c-mediated oligomerization of Apaf-1 conceivably could create

a platform for recruitment of NALP1, resulting in caspase- 1 activation via a mechanism whereby the Nterminal PYD domain of NLRP 1 binds ASC, which in turn binds pro-caspase-1, while the C-terminal CARD of NRLP1 binds the CARD of Apaf1. Activated patched receptors also could theoretically create an oligomerized protein complex at the plasma membrane for NRLP1 recruitment, thus promoting caspase activation.

1.9.5.1 *NALP1* Polymorphisms:

Given its location in the NALP1 protein, the missense mutation at L155H potentially could impact oligomerization of the protein, although other explanations are also possible. Polymorphisms in the promoter of the human *NALP1* gene presumably affect expression, but whether the result is increased versus decreased levels of NALP1 protein is unknown.

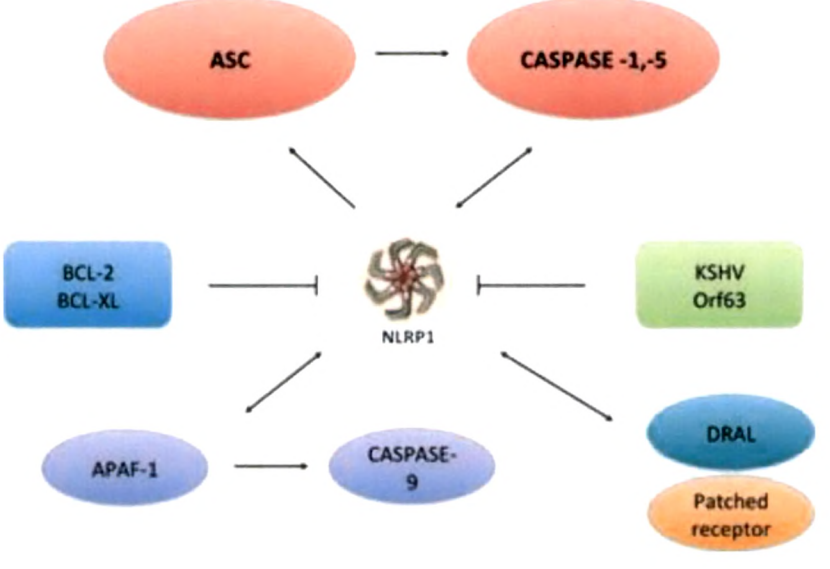

Figure 14. Protein interactions involving NALP1. Proteins known to interact with NALP1 are indicated, linking them to inflammation, apoptosis and other cellular functions.

Variations in the *NALP1* gene have been reported to confer risk for vitiligo and extended autoimmune disorders in Caucasian patients from the United Kingdom, the United States, and Romania (Jin Y *et al.,* 2007, Jin Y *et al.,* 2007). A recent report demonstrated that in a Norwegian population, Polymorphisms in *NALP1* are

associated with the organ-specific autoimmune conditions, Addison's disease, and type 1diabetes.

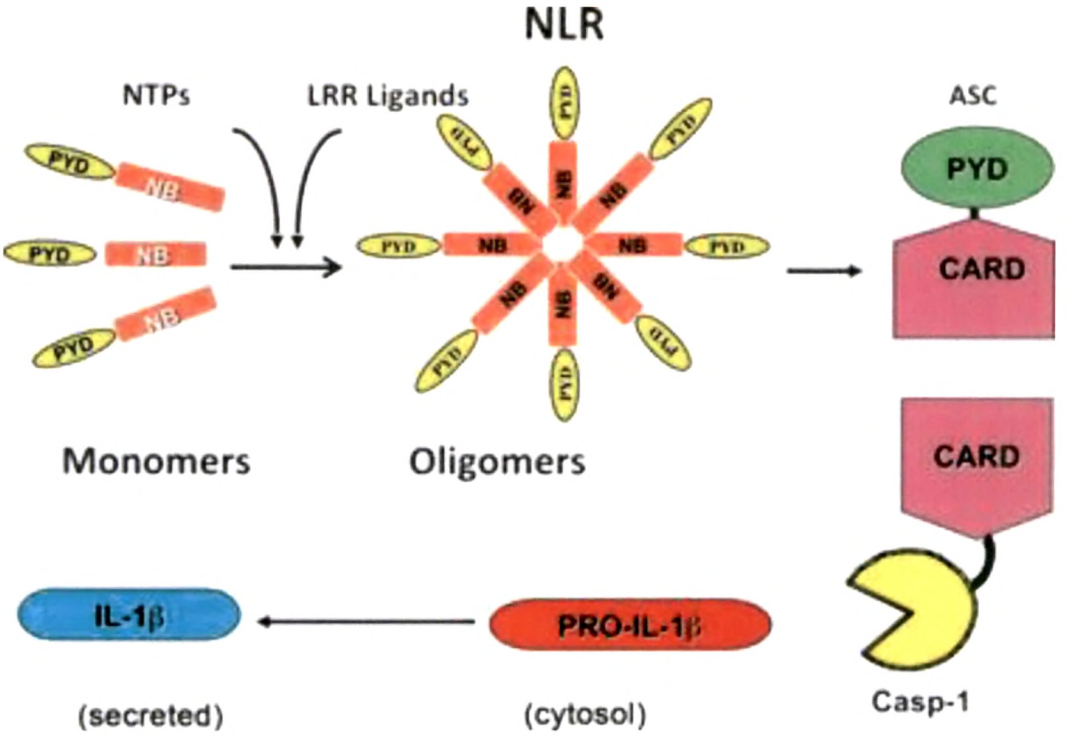

Figure 15. Inflammasome assembly. The steps involved in assembling the caspase-1-activating inflammasomes are depicted.

1.9.6 *MYG*1 (Melanocyte proliferating gene):

MYG1, C12orf10 in human is a ubiquitous nucleo-mitochondrial protein involved in early developmental processes (Philips *et al.,* 2009); however *MYG*1 expression in normal adult tissues is stable and seems to be changed mainly as a response to stress/illness conditions (Kingo *et al.,* 2006, Hawse *et al.,* 2003, Kõks *et al.,* 2004). MYG1 is predicted to possess metal-dependent protein hydrolase activity. MYG1 protein is highly conserved and present in all eukaryotes from yeast to humans (Nepomuceno-Silva *et al.,* 2004).

According to global expression analysis, *MYG1* tends to be up-regulated in undifferentiated and pluripotent cells. Kingo *et al.,* 2006 have suggested the involvement of *MYG*1 in vitiligo pathogenesis by showing elevated expression of *MYG1* mRNA in both uninvolved and involved skin of vitiligo patients. In addition,

MYG1 has been found to be consistently up-regulated also in skin biopsies from patients with atopic eczema (Sääf *et al.*, 2008).

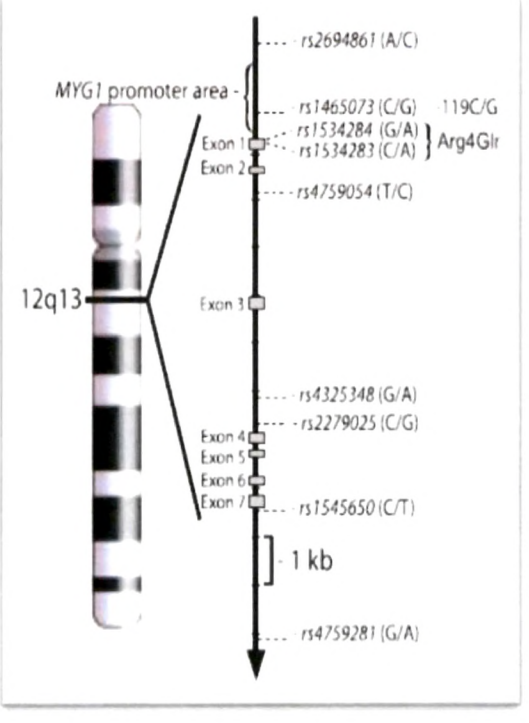

Figure 16: Relative positions of selective SNPs in *MYG* are represented by their ID numbers from NCBI's dbSNP database.

1.9.6.1 *MYG* Polymorphisms:

Philips *et al.*, (2010) have proposed that *MYG1* gene may be involved in vitiligo pathogenesis. This study demonstrated that both *MYG1* promoter polymorphism -119C/G and Arg4Gln polymorphism (Figure 17) in the mitochondrial signal of *MYG1* have a functional impact on the regulation of the *MYG1* gene and promoter polymorphism (-119C/G) is related with susceptibility for actively progressing vitiligo.

Philips *et al.*, (2010) showed that *MYG1* mRNA levels in skin samples of healthy controls correlate with *MYG1* promoter polymorphism -119C/G and subjects with homozygous -119G allele have significantly higher *MYG1* transcript levels than subjects with homozygous -119C allele. The study confirmed higher activity of -119G promoter by using *in vitro* luciferase reporter assay and showed alteration of p300

(E1A-associated 300 kDa protein) binding site is a potential reason why *MYG1* -119G promoter allele is more active. The -119G promoter was on average more than 2.5 fold more active regardless of the length of the promoter fragment (Philips *et al.*, 2010).

1.9.7 *IFNG* (Interferon γ):

Interferons are a small group of cytokines that include alpha, beta and gamma interferons (IFN-α,β,γ). Interferonγ, also known as type II interferon, is a single copy gene located on human chromosome 12q24 spans approximately 5.4 kb consisting of four exons and three introns (Gray *et al.*, 1982; Naylor *et al.*, 1983). It encodes a polypeptide of 166 amino acids with a 20 amino acid signal peptide and is an acid-labile protein which has antiviral, immune-regulatory and anti-tumour properties (Schroder *et al.*, 2004).

It alters transcription of up to 30 genes involved in a variety of physiological and cellular responses. IFNγ binding to IFNGR1 & IFNGR2 (interferon gamma receptors) activates the JAK-STAT pathway by phosphorylation of Jak and thereby activating STAT transcription factor (Horvath *et al.*, 2004). JAK-STAT signalling pathway is the best-characterised and commonly accepted IFN signalling pathway (Gough *et al.*, 2008). In addition, IFNγ activates antigen presenting cells (APCs) and promotes Th1 differentiation by up regulating the transcription factor and inhibits the development of Th2 cells (Oriss *et al.*, 1997). IFNγ is a pleiotropic cytokine that is a key regulator of development and functions of the immune system.

Apart from functions in host defense, IFNγ may also contribute to autoimmune pathology. Although IFNγ production was shown to be disease-limiting in autoimmune models such as murine experimental allergic encephalomyelitis (EAE) (Espejo *et al.*, 2001), it may contribute to autoimmune nephritis (Heremans *et al.*, 1978). In humans, IFNγ is implicated in pathology of diseases such as systemic lupus erythematosus (Lee *et al.*, 2001), multiple sclerosis (Panitch *et al.*, 1987), and insulin-dependent diabetes mellitus (Wang *et al.*, 1997).

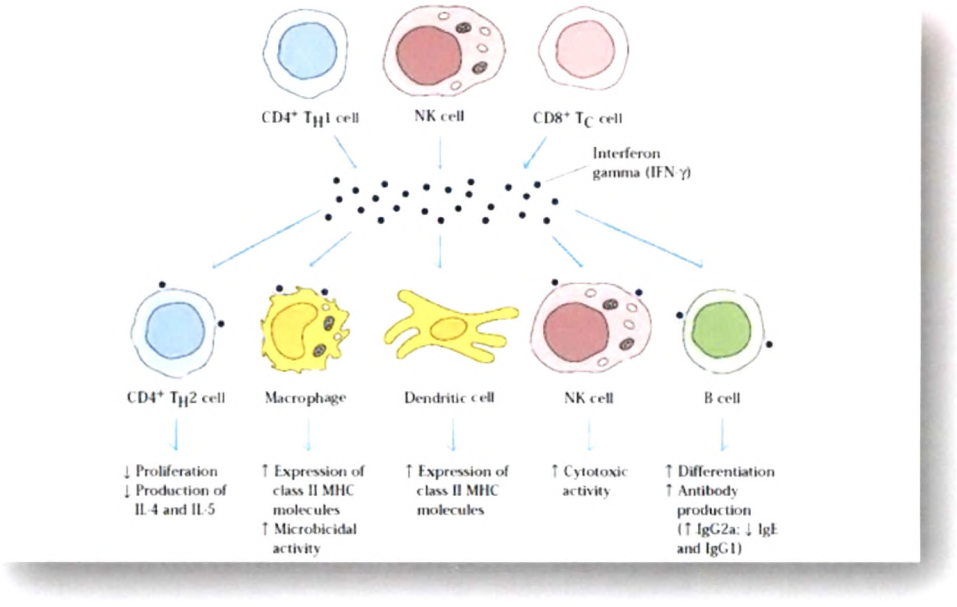

Figure 17: IFN-γ: Mode of Action (Kuby, *Immunology*, 6 th *Ed.*, 2007)

It has been suggested that melanocyte death is mediated by apoptosis in the context of autoimmunity and cytokines such as IFNγ and TNFα can initiate apoptosis (Huang *et al.,* 2002). Additionally, IFNγ and TNFα induce the expression of intercellular adhesion molecule-1 (ICAM-1) on the cell-surface of melanocytes (Yohn *et al.,* 1990). Increased expression of this adhesion molecule on the melanocytes enhances T cell/melanocyte attachment in the skin and may play a role in the destruction of melanocytes in vitiligo (Al Badri *et al.,* 1993).

The *IFNγ* coding region is invariant, with no reported polymorphisms (Hayden et al., 1997). There are two well-known single-nucleotide polymorphisms in the *IFNγ* gene. A CA repeat microsatellite sequence in the noncoding region of the first intron is polymorphic. Allele 2, with 12 CA repeats is associated with constitutively high IFNγ production in vitro. In addition, a single nucleotide A/T polymorphism at the 5' end of the CA repeat region in the first intron of the *IFNγ* gene (+874 A/T polymorphism; rs2430561) has been correlated with the presence or absence of the microsatellite allele 2 (Pravica *et al.,* 1999). Also, the presence of *IFNγ* (+874 Thi/Alo) polymorphism creates a putative NF-κB binding site in intron1 and shows preferential

binding to the T allele and correlates with high IFN-γ producer phenotype (Pravica *et al.*, 2000).

1.10 TREATMENT

Vitiligo is a difficult disease to treat. Although the treatment of vitiligo has improved during the last decade, it is still not satisfactory. Several treatment modalities are currently in use; however these methods usually induce incomplete pigmentation. Vitiligo treatment can be classified into two broad categories i.e. non-surgical therapies and surgical therapies (Nordlund *et al* 1993; Van Geel, 2001).

1.10.1 Non-surgical therapies:

1.10.1.1 Psoralen photochemotherapy:

Psoralenes are furocoumarin tricyclic hydrocarbon compounds. Psoralen photochemotherapy consists of photosensitizing psoralen with ultraviolet A in the 320-400 nm range (PUVA). PUVA and UVB therapies are widely used in the treatment of many skin disorders including vitiligo. The rationale of PUVA is to induce remissions of skin diseases by repeated controlled phototoxic reactions (Matsumura and Ananthaswamy, 2004). These reactions occur only when psoralenes are photoactivated by UVA. In systemic treatment, 8 methoxypsoralen or 4,5,8 trimethoxypsoralen is administrated before radiation exposure. The UV dosage is gradually increased until minimal erythema of vitiligo lesions occurs. How PUVA therapy stimulates the inactive melanocytes is unknown (Kovacs, 1998). The mechanism underlying the therapeutic effects of the combination of psoralen plus UVA is generally assumed that UVA induced DNA psoralen photoadducts impair the cell replication (Honig *et al* 1994). Inhibition of cell proliferation is observed at psoralen concentration and UVA doses which do not affect the cell viability (Luftl *et al.*, 1998); on the other hand higher doses cause irreversible DNA damage, resulting in both apoptosis and necrosis (Johnson *et al.*, 1996). It has been confirmed that the repigmentation is derived from the melanocyte reservoir in the hair follicles (Cui *et al.*, 1991). It has been demonstrated that PUVA irradiation of normal melanocytes *in vitro* inhibits the DNA and protein synthesis and affects EGF receptor and vitiligo associated melanocyte antigen expression. It is difficult to explain the PUVA induced

repigmentation of vitiligo on the basis of these different mechanisms. It has been proposed that PUVA could stimulate the production of melanocyte growth factor or may deplete antigens on vitiligo melanocytes, thus blocking the binding of specific autoantibodies, (Kao and Yu 1992). PUVA is immunosuppressive and this action of PUVA on T lymphocytes could be the reason for its therapeutic effect on vitiligo (Akyol *et al.,* 2002). It was proposed that PUVA inhibits gene transcription, which ultimately results in the shut down of cytokine release. Neuner *et al* showed the effect of PUVA on the release of the pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α from human peripheral blood mononuclear cells resulting in a significant reduction in these cytokines, thus causing the anti-inflammatory activity of PUVA (Neuner *et al* 1994).

1.10.1.2 Water bath PUVA:

The most recent model in phototherapy is water bath PUVA, in which the patient lies in a bath tub containing psoralen water for 15 min so that the drug gets absorbed on the skin and then goes for light therapy. This kind of therapy is especially beneficial in children for whom oral medicines are not safe (Aragane *et al.*, 2001). Another method of psoralen treatment, used rarely for pediatric patients with small, scattered vitiligo patches, involves the application of a very dilute solution of the drug directly to the affected skin area. This is then exposed to sunlight. Such topical treatment makes a person very liable to severe burn and blisters following too much sun exposure whereas water bath PUVA has the advantage of being done at home, and does not damage the entire skin surface.

1.10.1.3 Broadband UVB:

This phototherapy uses an emission spectrum of 290-320 nm (Koster and Wiskemann 1990).

1.10.1.4 Narrowband UVB:

In this phototherapy an emission spectrum of 310-315 nm is used (Westerhof and Korbotova 1997). Narrow band UVB therapy or TL-01 therapy is the latest in phototherapy for the treatment of vitiligo. In this therapy there is no need to take oral psoralen or apply psoralen. The therapy is very safe and can be safely administered

even to children. Narrow band UVB is much safer than full spectrum UVB. If exposure to natural sunlight is equal to 100% UV radiation , using a narrow band UV light is roughly 1% UV radiation . The advantage of UVB therapy over PUVA regimen is reflected by shorter duration of treatment (Van Geel *et al.,* 2001).

1.10.1.5 Topical immunomodulators:

Topical immunomodulatory agents such as tacrolimus and pimecrolimus offer several advantages in the treatment of vitiligo. These agents are well tolerated in children and adults and they can be used for long duration without evidence of atrophy or telangiectasias, the common complications associated with long term steroid use (Grimes, 2005). Tacrolimus is a topical immunomodulatory agent that affects T cell and mast cell functions by binding to cytoplasmic immunophilins and by inactivating calcineurin. Tacrolimus inhibits the synthesis and release of pro inflammatory cytokines and vaso active mediators from basophils and mast cells (Tharp, 2002). Pimecrolimus, which has a mechanism of action similar to tacrolimus, also can induce repigmentation in vitiliginous lesions (Mayoral *et al* 2003). As tacrolimus, pimecrolimus induces maximal repigmentation on sun-exposed areas.

1.10.1.6 Calcipotriol:

It is a synthetic analogue of vitamin D3. Vitamin D3 binds to vitamin D receptors in the skin, affecting melanocyte and keratinocyte growth and differentiation. It also inhibits T cell activation (Dusso and Brown 1998). Melanocytes express 1, 25-dihydroxyvitamin D3 receptors, which may stimulate melanogenesis.

1.10.1.7 Pseudocatalase:

The discovery of low epidermal catalase levels in involved and uninvolved skin of patients with vitiligo suggested a major stress arising from increased epidermal H_2O_2 generation (Schallreuter *et al* 1991). However, catalase mRNA levels in melanocytes and keratinocytes from patients is normal compared to healthy controls (Maresca *et al.*, 1997). One consequence of H_2O_2 accumulation is the oxidative degradation of the porphyrin active site of the catalase leading to its deactivation (Aronoff *et al.,* 1965).

Pseudocatalase is a bis (Mn) bicarbonate complex for the removal of H_2O_2 in the epidermis of vitiligo patients (Schallreuter *et al.,* 1995). Pseudocatalase functions as a pro-drug requiring UV light for the full activation of the complex (Schallreuter *et al.,* 1999a). Successful removal of the high levels of epidermal H_2O_2 in vitiligo was shown with a topical application of pseudocatalase in several studies (Schallreuter *et al.,* 1995). It has been demonstrated that *in vitro* and *in vivo* use of pseudocatalase leads to the recovery of $6BH_4$ whose recycling process is perturbed in vitiligo and thus leads to repigmentation (Schallreuter *et al.,* 2001).

1.10.1.8 Khellin and UVA:

Khellin is a furanochrome and combined with UVA, it is as effective as PUVA therapy in the treatment of vitiligo without having the phototoxicity associated with psoralens (Nordlund *et al.*, 1993).

1.10.1.9 Fake tanning products:

Cover creams or self tanning products are special drug cosmetics that can be used to match most skin patches when medical treatment is not successful. Patients with vitiligo are required to protect their depigmented skin against excessive sun exposure by wearing protective clothing. Tattooing is rarely recommended. It works best for the lip area, particularly in people with dark skin. However, it is difficult to perfectly match the skin, and tends to look worse over time. Cosmetics can be used to improve the appearance of the white areas not covered by clothing. Sunscreens give coolness to the affected areas and also prevent the normal skin around the patches from becoming darker. Bleaching or depigmentation of the normal skin and autologous transplantation of skin are an option for those who are severely affected (Samantha *et al.*, 2008).

1.10.2 Surgical therapies:

Several treatment modalities such as PUVA, -UVB and local corticosteroids are currently used in the treatment of vitiligo. However, these treatments usually induce incomplete repigmentation. Surgical methods intended to repigment vitiligo are an interesting therapeutic option if patients have stable disease (Ongenae *et al.,* 2001).

All surgical techniques have the same basic principle: to transplant autologous melanocytes from a pigmented donor skin to regions without melanocytes (Ongeneae *et al.,* 2001). Basically there are two types of surgical techniques, tissue grafts and cellular grafts. Tissue grafts are full thickness punch grafts and, split thickness grafts and suction blister grafts. With tissue grafts, only a limited surface area can be treated but with good results in the majority of cases.

1.10.2.1 Full thickness punch grafts:

In this method punch grafts from normally pigmented skin are implanted in the affected area. Repigmentation is based on the 'pigment spread phenomenon' by grafted piece of normal skin. The grafts are implanted into perforations previously made at the recipient site using a biopsy punch under local anesthesia (Ongenae *et al.*, 2001). The success rate of full thickness punch grafts is in between 68-82% (Malakar and Dhar 1999; Boersma *et al.,* 1995; Falabella *et al.,* 1988). Punch grafting is easy to perform and does not require special equipment or a laboratory set up. Difficult areas such as lips could be treated successfully; however it is not suitable for body folds (Malakar and Dhar 1999).

1.10.2.2 Split thickness grafts:

This technique has a high success rate of 78–91% (Olsson and Juhlin 1998; Kahn and Cohen 1998). After obtaining a split thickness skin graft using a dermatome it can be applied directly to the derma braded recipient area. Temporary small epithelial milia like cysts can be observed in the recipient area during the first months, especially on the face and neck. Scar or keloid formation at the donor site is reported in 12% of the patients treated with split thickness grafts. As donor tissue is limited more than one split skin grafting session can be necessary (Ozdemir *et al.*, 2002).

1.10.2.3 Suction blister grafts:

Grafts are carefully removed with sharp scissors and forceps after harvesting the graft. This epidermal sheet is then grafted onto the denuded recipient site. The success rate is 73–88%. Pigment spread after epidermal blister grafting can be enhanced by pre operative radiation therapy of the donor site using PUVA. Temporary hyper

pigmentation can be seen in the grafted sites in 2–65% (Ozdemir *et al.*, 2002). The eyelids, lips and bony prominences can be treated using this method.

1.10.2.4 Cultured epidermal grafts:

A shave biopsy of normally pigmented skin is the source of epidermal cell culture. The cultured sheet is released by treatment with dispase and attached to petroleum gauze as support. Subsequently the gauze to which the epithelium adheres will be applied onto the dermabraded recipient site and covered with occlusive dressing (Kumagai and Uchikoshi 1997). Success rate of this method is in between 33-54%.

1.10.2.5 Non-cultured keratinocytes and melanocytes:

Transplantation technique with a suspension of non cultured keratinocytes and melanocytes in the treatment of depigmented lesions is effective. Donor skin is obtained from the occipital area and immersed for 18 h in 0.25% trypsin solution. The following day the epidermis of the donor skin can be separated from the dermis *in vitro* using fine forceps. After several procedures a cellular suspension is obtained (Mysore and Salim, 2009). Liquid nitrogen is used to induce blisters in the recipient area. The cellular suspension from the donor site is injected into each blister at the recipient area after aspiration of the viscous blister fluid. The intact blister top is a natural dressing that holds the transplanted cells in place. It is important not to separate keratinocytes from melanocytes before grafting because factors furnished by keratinocytes sustain melanocyte growth (Ozdemir *et al.*, 2002). The success rate of this therapy is more than 70% (Gauthier and Surleve-Bazeille 1992).

1.10.2.6 Cultured melanocytes:

Lerner *et al.*, 1987 first described the use of cultured pure autologous human melanocytes. They cultured melanocytes of a shave biopsy from normally pigmented skin *in vitro* with the addition of several growth factors and chemical media. The success rates vary between 22-72%.

1.10.2.7 Depigmentation:

Depigmentation or the removal of remaining pigmentation is normally done in patients who have greater than 50% of their bodies affected and who have demonstrated recalcitrance to repigmentation. Depigmentation is permanent and irreversible. Monobenzylether of hydroquinone is used as a depigmenting agent (Nordlund *et al* 1993).

1.10.3 Herbal products

1.10.3.1 Anti-vitiligo® (True Herbals, Lahore, Pakistan):

Anti-vitiligo® (True Herbals, Lahore, Pakistan) is a traditional herbal formulation which was available internationally since November 2003. It is effective both in disease of recent onset as well as long standing established cases. Formulation contains the following ingredients.

1.10.3.2 Psoralea corylifolia:

It is a rich source of naturally occurring psoralen. It sensitizes human skin to the tanning effect of UV and sun light. *P. corylifolia* has been traditionally used both orally as well as in the form of topical preparations. Oxidative stress is widely believed to be one of the likely causative factors in the initiation of white skin patches of vitiligo. Hence, the protective, anti-oxidative and anti stress properties of *P. corylifolia* may contribute to the improvement in the hypo-pigmented white skin patches of vitiligo.

1.10.3.3 Black cumin:

Seeds of Nigella sativa have also been having an immunomodulatory as well as anti cancer effect, which is due to augmentation of T cell and natural killer cell mediated immune responses.

1.10.3.4 Barberry root:

Barberry root or the root of *Berberis vulgaris* contains numerous chemicals and bioactive compounds of medical significance. It contains for example the alkaloids like berbamine, berberine, and oxyacanthine. Other compounds include tannins,

chelidonic acid and resins. It is also quite rich in B-vitamin thiamine, lutein, vitamin C, beta-carotene, zeaxanthin, zinc, chromium, and cobalt. This herb has also been shown in scientific studies to possess antioxidant and cytoprotective properties.

1.10.3.5 Kalawalla® (American Life Style, New York, USA):

Kalawalla® (American Life Style, New York, USA) is a herbal product that works as a natural immunomodulator with proven immunomodulating effect. The product contains *Polypodium leucotomos* standardized extracts. *P. leucotomos* is a fern plant extract that has been used in Europe to treat vitiligo for over 10 years with encouraging results. The extract can help to regulate the immune system bringing it to its healthiest, strongest and balanced levels. Repigmentation results can be seen within the first month of taking the product. *P. leucotomos* standardized extract has been known to increase the lymphocyte levels. It is also known to regulate the CD4/CD8 ratios to their normal values.

1.10.3.6 Piperine:

The synthetic derivatives of piperine can stimulate pigmentation in the skin especially when combined with UVR treatment. The studies have compared the effects of piperine and its analogues tetrahydropiperine (THP), cyclohexyl analogue of piperine (CHP) and reduced CHP (rCHP) when applied to the skin of mice, either alone or followed by UV treatment. CHP did not show significant results while piperine, THP and rCHP did induce pigmentation in the skin. When used alone, the compounds stimulated pigmentation to an even, light brown color within six weeks. However, by accompanying the use of piperine or THP with UV, the skin became significantly darker, and within only seven weeks as compared to other treatments which take a year.

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