

# Patho-immunological mechanisms of vitiligo: an integration of the immunogenetic milieu, with innate and adaptive immunities, as triggered by environmental stress factors

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## Abstract

Epidermal melanocyte loss in vitiligo, triggered by stresses ranging from trauma to emotional stress, chemical exposure or metabolite imbalance, to the unknown, can stimulate oxidative stress in pigment cells which secrete damage-associated molecular patterns that then initiate innate immune responses. Antigen presentation to melanocytes leads to stimulation of autoreactive T cell responses, with further targeting of pigment cell. Studies show a pathogenic basis for cellular stress, innate immune responses and adaptive immunity in vitiligo. Improved understanding of the aetiological mechanisms in vitiligo has already resulted in successful use of the Jak-1 inhibitors in vitiligo. In this review we outline the current understanding of the pathological mechanisms in vitiligo, and locate loci to which therapeutic attack might be directed.

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## Summary

Epidermal melanocyte loss in vitiligo, triggered by stresses ranging from trauma to emotional stress, chemical exposure or metabolite imbalance, to the unknown, can stimulate oxidative stress in pigment cells which secrete damage-associated molecular patterns that then initiate innate immune responses. Antigen presentation to melanocytes leads to stimulation of autoreactive T cell responses, with further targeting of pigment cell. Studies show a pathogenic basis for cellular stress, innate immune responses and adaptive immunity in vitiligo. Improved understanding of the aetiological mechanisms in vitiligo has already resulted in successful use of the Jak-1 inhibitors in vitiligo. In this review we outline the current understanding of the pathological mechanisms in vitiligo, and locate loci to which therapeutic attack might be directed.

**Key words:** Antibodies, Autoimmunity, Cytokines, Cytotoxic T Cells, Th1/Th2 Cells

## Introduction

Melanocyte damage and destruction is the underlying pathological event in vitiligo, a skin disease characterised by depigmented patches. Vitiligo has a worldwide prevalence of about one percent, and can be classified into non-segmental, segmental, mixed and unclassifiable/undetermined vitiligo [1, 2]. Differentiating subtypes may be important as they might have different aetiologies. Segmental vitiligo often maps to a blaschkoid or dermatomal distribution [3]. Vitiligo can affect any gender, race or geographic region with no significant difference [4].

Although non-life threatening, vitiligo can have a serious psychological impact on sufferers [5]. Vitiligo patients commonly experience feelings of stress, fear of spreading vitiliginous lesions, embarrassment, negative self-image or self-consciousness [6]. Moreover, patients with vitiligo often experience depression, anxiety and discrimination and stigmatisation from others resulting in low self-esteem and social isolation [7].

In some countries such as India, vitiligo is still confused with leprosy and patients are subjected to antagonism, insult and social stigma [8]. Vitiligo can also have a major negative impact on the marital status and sexuality of patients [9-11]. Furthermore, previous reports have demonstrated that vitiligo patients with decreased quality of life at treatment initiation face a lower response rate to a given therapy [12]. Therefore, the development of specific psychological intervention and quality of life measures may affect positively the outcome of vitiligo treatment and enhance the patient's self-esteem and confidence [13].

A variety of factors may trigger vitiligo, including emotional stress, physical trauma and chemical exposure to imbalances in endogenous neural factors, metabolites, cytokines or hormones, which can stimulate autoimmune responses, in individuals with the appropriate genetic susceptibilities that ultimately target melanocytes [2]. The melanocytes in vitiligo are highly vulnerable to damage and apoptosis under the action of triggering factors [14]. The treatment of vitiligo, including topical steroids, calcitonin-inhibitors, phototherapies, and surgical procedure, in the past has frequently failed to achieve satisfactory repigmentation, but recently, the Jak-1 inhibitors have shown promise [15].

## Histopathological Features of Vitiligo and Melanocyte Degeneration

The affected skin in vitiligo demonstrates melanin loss and absence of or reduced numbers of melanocytes in the epidermis [16]. This decrease varies according to the disease stage [13]. Melanocytes as revealed by using an appropriate monoclonal antibody technique or the Fontana Masson (FM) stain, remain present, however, they disappear from affected skin of vitiligo as the disease progress [17]. Immunohistochemical studies of

vitiligo lesions show the absence of melanocytes from the basal layer, but they may exist in decreased numbers and can demonstrate degenerative changes [18].

Even though it is clear that depigmentation in vitiligo results from melanocyte loss from the skin, it remains unclear whether this occurs through a degenerative or autoimmune process. Defective *in vitro* growth of melanocytes derived from individuals with vitiligo [19, 20] and increased susceptibility of vitiligo melanocytes to exogenous stimuli suggests that degeneration may elucidate melanocyte loss [21].

Melanocytes from vitiligo subjects were shown to poorly proliferate compared to healthy normal melanocytes [19] and also they show inadequate antioxidant activity with high levels of superoxide dismutase and low levels of catalase [22]. Under normal circumstances, superoxide dismutase catalyses the first step of dismutation by converting the superoxide anion into oxygen and hydrogen peroxide and then catalase enzyme transforms hydrogen peroxide into water and oxygen, protecting cells from reactive oxygen species (ROS). In fact, melanocytes synthesise high ROS levels as by-product of melanogenesis. Therefore, compensatory media supplements such as growth factors or catalase are required to culture melanocytes derived from vitiligo patients [20, 23]. Also, increased expression of hydrogen peroxide and elevated oxidative by-products within vitiligo patient skin has been reported [22, 24, 25]. In addition, melanocytes from vitiligo patients were more sensitive to *in vitro* oxidative therapies such as cumene hydroperoxide and UVB light [26, 27]. However, exogenous treatment of catalase (pseudocatalase), which was proposed to cure vitiligo by regulating ROS, was ineffective in treating vitiligo lesions [28]. Thus, dysregulated redox balance in vitiligo might be a consequence, but not a cause, of vitiligo. Melanocytes from vitiligo patients show morphological and physiological abnormalities. Those in peri-lesional borders are seen to be enlarged with longer dendritic ends than normal melanocytes [29]. However, rapid repigmentation of the skin following engrafting of human vitiligo lesional skin on nude mouse was achieved, indicating that intrinsic melanocyte defect was not the only cause of melanocyte destruction in vitiligo [30]. Histochemical and immunohistochemical examination shows infiltration of a large number of T lymphocytes at the edge of vitiligo lesions with a complete microscopic loss of melanin in lesional skin [31]. Therefore, it is certain that vitiligo melanocytes are abnormal compared to healthy melanocytes, but this abnormality does not seem to be sufficient for the disorder.

### Responses to Stress in Vitiligo

Melanocytes in the epidermis are regularly exposed to various environmental stressors e.g., ultraviolet (UV) radiation, pollution, microorganisms and oxidising chemicals, which can stimulate reactive oxygen species production [32].

Reactive oxygen species consist of a number of oxygen-based free radicals such as superoxide and hydrogen peroxide, formed during multiple physiological and pathological processes [33]. Such free radicals are constantly scavenged by antioxidants such as superoxide dismutase, catalase, vitamin C, and vitamin E. As mentioned above, in vitiligo patients, high levels of superoxide dismutase and low levels of catalase have been observed in their skin [34]. Hydrogen peroxide created from superoxide anion can easily cross melanocyte membranes causing cellular damage [33]. Even though melanin present in the skin protects melanocytes as well as adjacent keratinocytes through its ability to absorb UV radiation, its synthesis likewise results in higher amount of intracellular reactive oxygen species, causing to be melanocytes more vulnerable to oxidative stress [35, 36]. In addition, a decrease in the stability of tyrosinase-related protein-1 (TYRP1), which is required for melanin synthesis, has been observed in vitiligo melanocytes, allowing accumulation of melanin intermediates [37]. The build-up of intermediate products increases the risk of protein misfolding, hence activating the unfolded protein response. This in turn induces the restoration of endoplasmic reticulum homeostasis through the halting of protein translation, inducing misfolded protein degradation and promoting the synthesis of chaperons to facilitate protein folding, sustained activation of which leads to apoptosis [2].

Intrinsic defects may also render vitiligo melanocytes vulnerable to oxidative stress. Observed anomalies include a dilated endoplasmic reticulum, mitochondrial dysfunction, and an abnormal melanosome structure, all of which suggests that these pigment cells are less capable of dealing with such stressors than those from

healthy individuals [32]. ROS-mediated stress has been directly linked with generation of neoantigenic epitopes within beta islet cells [38], and likewise melanocyte stress may generate neoantigens. Elevated ROS in melanocytes of vitiligo subjects has been correlated with peroxidation, and thus it is likely that ROS generates melanocyte neoantigens via protein carbonylation and oxidation [39].

Cellular stress has been found to develop in healthy melanocytes exposed to phenolic derivatives such as 4-tertiary butylphenol and monobenzyl ether of hydroquinone[40]. Once melanocytes become stressed, they promote the secretion of inducible heat shock protein 70 (iHSP70), which has been detected in vitiligo melanocytes and seen to correlate with active disease [41]. Pathogens or damage-associated molecular patterns (DAMP), which can evoke inflammation via pattern recognition receptors (PRRs) including Toll-like receptors and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Indeed, NLRP1 has been linked with vitiligo in a linkage study [42]. Innate immunity is activated by the release of DAMPs from stressed cells. DAMPs are likely to be constantly released from stressed melanocytes resulting in skin inflammation in patients with vitiligo [43]. In agreement with this, uninvolved skin of vitiligo patients shows increased numbers of lymphocytes in comparison with healthy controls [44]. In addition, increased iHSP70 expression in the skin of vitiligo patients causes melanocytes loss [45]. Mice lacking expression of iHSP70 will not develop experimental depigmentation, suggesting a role for iHSP70 in vitiligo [46]. iHSP70 has been found to have potent adjuvant and chaperone properties [47]. Under conditions of oxidative stress, genetically compromised melanocytes secrete melanosomal peptides-chaperoned iHSP70 that can activate dendritic cells and release the inflammatory signal that initiates the immune response in vitiligo [46]. Secreted iHSP70 from stressed vitiligo melanocytes was reported to induce dendritic cells to elevate the expression of coactivation markers CD80 and CD86, stimulating immune responses to melanocytes [48, 49]. Therefore, it is likely that melanocyte stress can contribute to instigation of autoimmunity through both neoantigen generation and activation of innate immunity [39]. iHSP70 is thus a link between oxidative stress as a trigger and the onset of the autoimmune reaction in vitiligo.

### Innate Immunity

Innate immunity is based on the ability of PRRs to detect pathogen-associated molecular patterns (PAMPs) found in pathogens or DAMPs released by damaged cells [50]. Reactive oxygen species and iHSP70 produced by stressed melanocytes serve as DAMPs in vitiligo, and PRRs initiate the innate response [43]. Innate immune cells such as natural killer (NK) cells, macrophage and dendritic cells show aberrant activation in vitiligo skin and granzyme-B (GZMB)-expressing activated NK cells have been found [44, 46]. Vitiligo skin shows an increase in NK cells activating receptors (CD16+CD56+ and CD3+CD16+CD56+), an upregulation in CLEC2B, an activating ligand of NK cells, and a decrease in the inhibitory receptors (CD16+CD158a+). Vitiligo skin also demonstrates increased numbers of dendritic cells, which can destroy melanocytes when activated by iHSP70 [46, 51]. While chemicals can trigger vitiligo by inducing melanocyte stress, adding HSP70i alone aggravates vitiligo mouse model, probably via the activation of dendritic cells in the skin [45].

In addition, mutant HSP70i delivery, which interferes with the signaling pathway of endogenous HSP70i, could inhibit depigmentation in vitiligo mouse and swine models by interfering with dendritic cell activation [46, 52]. Thus, DAMPS, in particular HSP70i, can directly initiate vitiligo in animal models by activating dendritic cells. Activated, dendritic cells locally synthesise cytokines, inducing T cell activation and recruitment to the skin and, in local lymph nodes, recruit cytotoxic T cells, thus bridging the innate with the adaptive immunity [53]. Therefore, delivery of mutant HSP70i may offer a potential treatment for vitiligo by altering the innate immunity. The connection in vitiligo between cellular stress and cell-based immunity was illustrated when melanocytes, stressed by exposure to 4-tertiary butyl phenol, were noted to facilitate activation of dendritic cells thus rendering them melanocytotoxic *in vitro* [48]. Others have demonstrated that stressed melanocytes can activate melanocyte-specific CD8+ T cells, resulting in an autoimmune response and consequent pigment cell destruction [54]. Recently, perilesional keratinocytes from vitiligo skin, under oxidative stress *in vitro*, have been shown to exhibit increased expressions of NLR family pyrin domain containing 3 (NLRP3) and downstream cytokine IL-1 $\beta$ , an inflammasome regulator that may modulate innate

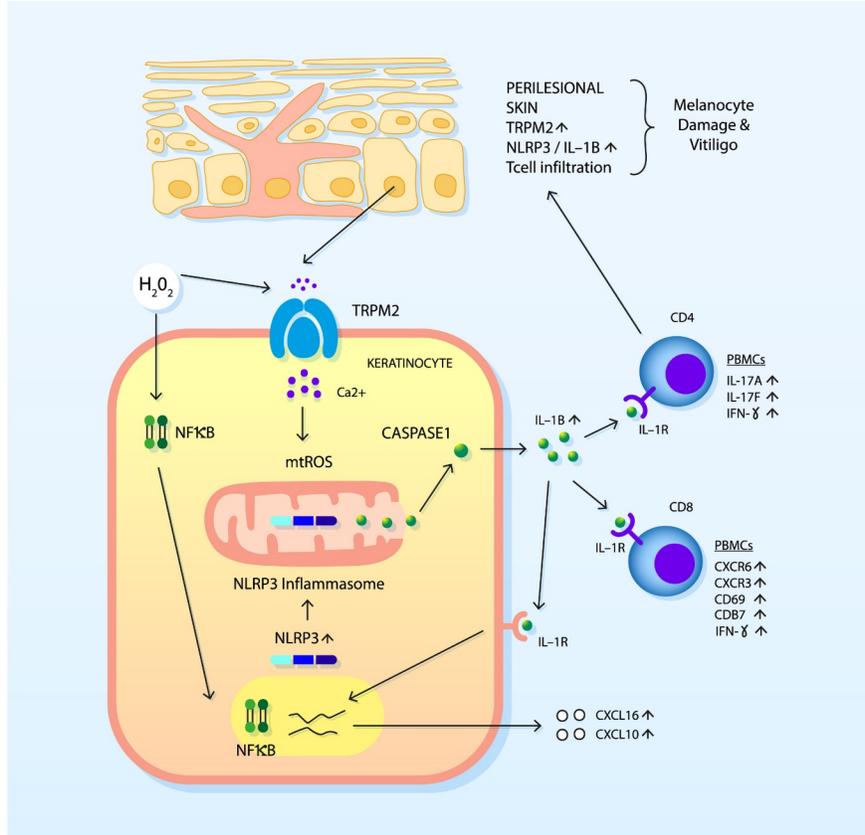
immune attack on melanocytes [55].

NLRP3 is a cytoplasmic NLR and is an essential constituent of the inflammasome in the innate immunity. The activation of NLRP3 inflammasome requires two signals. The first signal primes cells and induces NLRP3 expression by nuclear factor  $\kappa$ B (NF- $\kappa$ B)-mediated signaling [56], while the other signal requires mitochondrial reactive oxygen species (mtROS) and interaction of NLRP3 and apoptosis-associated speck-like protein containing a CARD (ASC) [57]. In addition, the NLRP3 inflammasome can also be activated through transient receptor potential cation channel subfamily M member 2 (TRPM2)-induced intracellular and mitochondrial calcium influx in  $H_2O_2$ -treated keratinocytes [55]. Once activated, NLRP3 inflammasome mediates caspase-1 cleavage which promotes synthesis of IL- $\beta$  [58, 59]. Subsequently, the function of CD8+ and CD4+ T cell is strengthened through IL1- $\beta$ /IL-1R signaling pathway [55]. IL1- $\beta$  elevated the expression of CXCR6 and CXCR3 in CD8+ T cells from vitiligo patients. Also, IL1- $\beta$  increased the synthesis of IL17A/F in CD4+ T cells and IFN- $\gamma$  in both CD8+ and CD4+ T cells [55]. IL1- $\beta$  in stressed keratinocytes also stimulated the expression of CXCL10 and CXCL16, ligands of CXCR3 and CXCR6 through NF- $\kappa$ B pathway, which promote the migration of cytotoxic T cells into vitiligo lesions [55] (**Figure 1**).

## Adaptive Immunity

### Infiltrating T Cells and their Role in Vitiligo

Following proinflammatory signalling in the skin, melanocyte antigens can be processed and presented by dendritic cells in the draining lymph node, resulting in the production of melanocyte-specific cytotoxic T lymphocytes and the generation of melanocyte-specific antibodies by B lymphocytes [60]. Histological studies of vitiligo lesions showed infiltration of lymphocytes at the border of depigmented lesions. These infiltrates consist mainly of CD8 + T cells, preferentially located in the dermo-epidermal borders neighbouring the melanocytes [61, 62]. CD8+ T cells are implicated in the destruction of melanocytes in vitiligo. Melanocytotoxic CD8+ T cells that express the skin homing marker, cutaneous lymphocyte-association antigen, infiltrate the lesional borders of vitiliginous skin, and their number correlates with disease extension and severity [63-65].



**Figure 1.** Activated NLRP3 inflammasome in keratinocytes promotes T cell responses in vitiligo. NLRP3 inflammasome in stressed keratinocytes is activated by NF-κB-mediated signaling as well as the interaction between mtROS and of NLRP3. NLRP3 inflammasome can also be activated through TRPM2-mediated intracellular and mitochondrial calcium influx. Once activated, NLRP3 inflammasome mediates caspase-1 cleavage which provoke synthesis of IL-β, resulting in the activation of D8+ and CD4+ T cells through IL1-β/IL-1R signaling pathway. IL1-β augments CXCR6 and CXCR3 expression in CD8+ T cells, IL17A/F synthesis in CD4+ T cells and IFN-γ production in both CD8+ and CD4+ T cells. IL1-β also promotes the expression of CXCL10 and CXCL16. TRPM2-facilitated NLRP3 inflammasome activation in keratinocytes stimulates T-cell function in vitiligo patients as demonstrated by upregulation of T cell activation markers CD69 and CD137 (Adapted from Li et al., 2020)[55].

Furthermore, perilesional infiltrating self-reactive CD8+ T cells in vitiligo patients recognise Melan-A/MART, tyrosinase (TYR) and melanocyte-specific protein, all of which are antigens involved in the melanogenic pathway [66].

Vitiligo patients showed elevated numbers of CD8+ T cells in their blood compared to healthy individuals [64].

Moreover, peri-lesional skin of vitiligo patients are highly infiltrated by melanocyte-specific CD8+ T cells [62], and these cells have the ability to kill melanocytes *in vitro* [62, 67, 68]. CD8+ T cells isolated from peri-lesional skin of vitiligo patients infiltrate explants of autologous healthy pigmented vitiligo skin and eliminate melanocytes in a way similar to the clinical pathology of vitiligo [68]. Importantly, isolated CD4+ T cells were incapable of inducing melanocyte apoptosis in autologous skin explants [68]. *In vitro* culture of CD8+ and CD4+ T cells derived from peri-lesional vitiligo skin secrete high levels interferon-γ (IFN-γ), a pro-inflammatory cytokine required for melanocyte-specific autoreactive CD8+ T cell recruitment [69].

These findings provide robust evidence that CD8+ T cells are equally essential and sufficient for destruction of melanocytes in vitiligo lesions.

## IFN- $\gamma$ Σιγναλινγ Πατηωαψ Αςτς ας α Κεψ Δριερ οφ $\Delta$ 8+ T έλλ Ρεςρυιτμεντ ανδ Φυςςτιον ιν Ϊτιλιγο

The mechanism by which CD8+ T cells trigger melanocytes apoptosis requires their secretion of proinflammatory cytokines IFN- $\gamma$  from CD8+ T cells [68]. Analysis of gene expression in human vitiligo lesional skin demonstrated increased expression of *IFRG* gene [70, 71], as well as genes induced by IFN- $\gamma$  and these include C-X-C chemokine receptor type 3 (*CXCR3*), a T cell chemokine receptor, and its ligands: *CXCL9*, *CXCL10*, and *CXCL11* [71]. In agreement with this finding, skin biopsies from vitiligo patient lesions also show prominent lymphocyte infiltrates that are primarily CXCR3+ [69, 71-73]. In addition, melanocyte-specific CD8+ T cells isolated from the skin and blood vitiligo subjects predominantly express CXCR3 receptor, and CXCL9 is a specific skin biomarker of active vitiligo [65]. Studies in mouse models of vitiligo established a critical role for pathway in pathogenesis of vitiligo, since INF- $\gamma$ , CXCR3, CXCL10 are all essential for vitiligo development [71, 74]. Neutralization of IFN- $\gamma$  with antibody treatment or the lack of *CXCR3* expression on T cells prevents the migration of autoreactive T cells into the skin and therefore do not cause depigmentation [71, 74]. Studies employing chemokine reporter mice showed that CXCL9 and CXCL10 are mostly produced by keratinocytes, and functional studies demonstrated that keratinocytes are primarily responsible for recruitment of autoreactive T cells [75].

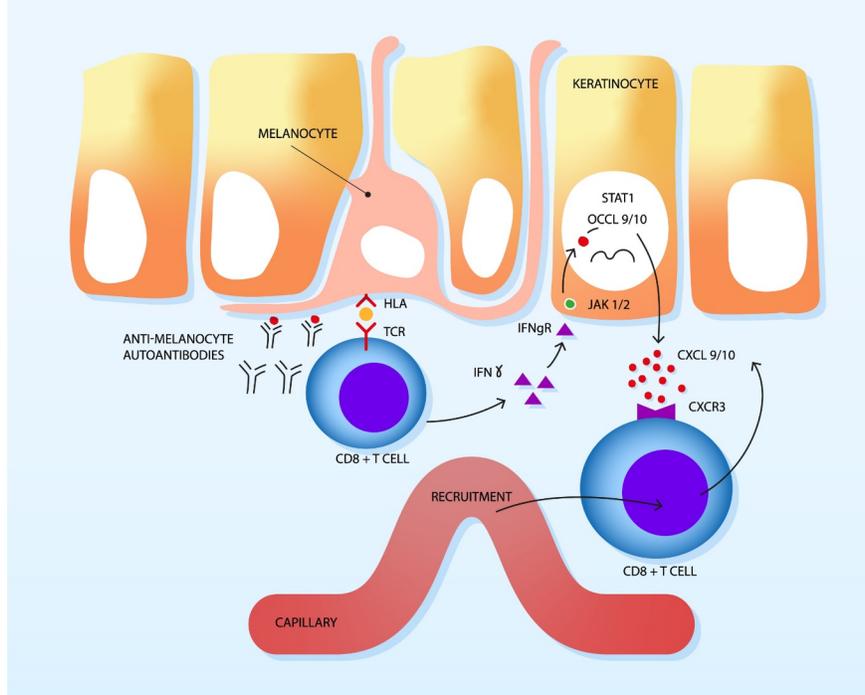
CXCL9 seems predominantly responsible for bulk recruitment of T cells to the skin, since when it is absent the number of melanocyte autoreactive T cells within lesional skin of vitiligo is decreased by tenfold [71]. However, in spite of reduced number of T cells, vitiligo severity remains unchanged, indicating that the over-recruitment of T cells occurs during vitiligo. In contrast, when CXCL10 is absent, the incidence and severity of vitiligo are decreased, yet bulk recruitment of T cells is unchanged [71].

Interestingly, in the absence of CXCL10, the quantity of T cells shown in the epidermis compared to the dermis in the skin is decreased, signifying that CXCL10 is responsible for T cell localisation within the skin and their effector function [71]. Thus, T cells produces IFN- $\gamma$ , which stimulates the production of CXCL9 and CXCL10 from keratinocytes to recruit more T cells and induce vitiligo progression [39]. As well as to vitiligo initiation and progression, the IFN- $\gamma$ -chemokine pathway is also needed for maintenance of established vitiligo lesions, as knocking out CXCR3 or blocking CXCL10 action prevents and reverses depigmentation in vitiligo [71] (**Figure 2**). Indeed, ruxolitinib, a janus kinase (JAK)-1,2 inhibitor, which interferes with IFN- $\gamma$  signalling pathway through preferential inhibition of JAK1 and JAK2, shows promise in vitiligo [76].

## Regulatory T Cells are Suppressors of Autoreactive Effectors and Inhibit Depigmentation

Regulatory T (Treg) cells and effector T cells are key factors in maintaining appropriate peripheral tolerance. Treg cells are a subgroup of CD4+ T cells that primarily represent a phenotype of CD4+, CD25+ and forkhead box P3 (Foxp3). They have a vital role in controlling autoimmunity by maintaining immunological unresponsiveness to self-antigens, playing a key role in preventing autoimmunity.

Patients who suffer from immuno- dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome lack Treg cells because of a mutation in the *FOXP3* gene and as a consequence suffer from other autoimmune diseases, including vitiligo [77]. Likewise, scurfy mutant mouse strain with defective FOXP3 lack Treg cells and shows widespread autoimmunity, underlining a critical role for Treg cells in maintenance of tolerance to self-antigens [78].



**Figure 2.** Progression of vitiligo requires continued recruitment of T cells which occurs via a positive-feedback loop. In the progressive phase of vitiligo, melanocyte-reactive CD8+T cells produce interferon-gamma on encountering melanocyte antigens. These induce keratinocytes to secrete CXCL 9 and CXCL 10, resulting in additional recruitment of lymphocytes to the site through the CXCR3 receptor (Adapted from Frisoli et al., 2020)[39].

In a mouse model of vitiligo, repigmentation was accompanied by an elevated infiltration of Tregs into the skin, possibly resulting in the prevention of immune responses against melanocytes [79]. In addition, studies reported an increase in vitiligo severity when Treg cells were depleted using either CD4 or CD25 antibodies [80, 81]. One study showed an increase in cutaneous Treg cell infiltration and a decrease in depigmentation when the expression of CCL22 was induced in the skin [82]. Another report revealed that adoptive transfer of exogenous Treg cells vitiligo prone mice at three weeks of age led to elevated number of cutaneous Treg cells and prevented vitiligo [81].

When vitiligo prone mice were injected with PD-L1P-Fc, Treg cell accumulation in the skin was enhanced and depigmentation was reversed [83]. These findings support the theory that the number of Treg cells in skin is critical for reducing depigmentation driven by T effectors and therefore helping to control vitiligo progression.

Several groups have reported disruption of Treg cells function, but there is no consensus as to where the disruption exactly lies: in density of Treg cells, suppressive effects, or immigration ability to the skin. Studies reported that peripheral Treg cells isolated from vitiligo patients showed a decreased ability to inhibit CD8+ T cells proliferation and activation *in vitro* [84]. However, another study showed normal Treg cell activity in vitiligo patients but decreased number in the skin, suggesting that reduced cutaneous localization of Treg cells to the skin, rather than diminished function of Treg cells, contributes to vitiligo pathogenesis [85]. Immunohistochemical studies revealed no significant reduction in Treg cell number in vitiligo lesional skin [86, 87], whereas another report showed a significant decrease in these cells [88]. It is therefore not clear how the function of Treg is disrupted in vitiligo patients, but effector T cell phenotype in human vitiligo also indicates the presence of defective Treg T cells. Naturally occurring Treg cells reduce self-reactive T cell

proliferation and activation, a phenomenon called anergy, and analysis of the phenotype of peripheral blood mononuclear cells indicates that melanocyte-autoreactive CD8+ T cells escape anergy in vitiligo patients [89]. Further studies are required to reliably determine how deficiency of Treg cells contributes to the development of vitiligo, and to examine the potential of improving Treg cell function as a novel treatment for vitiligo.

### **Immune-Dysregulation of T Helper Cells in Vitiligo**

The numbers of circulating Th1 and Th17 cells are increased in vitiligo compared to controls [90]. A mouse model of vitiligo shows a predominantly Th1-mediated pattern with a dominant role of CXCL10 [73]. Serum levels of interleukin 17 (IL-17), the effector molecule of Th17 cells, are raised in vitiligo patients, and IL-17 mRNA is elevated in their skin, implying a role for Th17 cells in driving the inflammatory response [91]. Recently, decreased frequencies of circulating Th1/Tc1, Th17/Tc17 and Th1/Th17-Tc1/Tc17 cells were shown in patients with vitiligo, suggesting possible migration of these cells into the skin [92]. Indeed, Th1/Th17, Tc1/Tc17 and Th17 cells have been touted as potential targets for therapy [93].

### **Resident Memory T Cells and their Function in Vitiligo**

The risk of repigmentation relapse in vitiligo lesions is common and occurs within the first year following treatment cessation [94]. To date, there is no treatment to prevent relapses. One study showed that vitiligo patients treated with narrow band-ultraviolet B (NB-UVB) lost their new pigment two years after cessation of treatment [95]. Notably, relapse occurs in exactly the same previously repigmented lesions, suggesting that autoimmune memory plays a role in the skin that is not cleared by current treatments [39]. Resident memory T (Trm) cells are a subset of long-lived T cells that persist within most nonlymphoid tissues after inflammation or infection driven by T cells [96, 97]. Trm cells of the skin are known of their long-term residence in the skin. They patrol their surroundings; the epidermis and papillary dermis, and rapidly respond when they encounter their cognate antigen [98]. They are defined by the cell surface expression of CD69, CD103, CD49a and CD44 [99]. As these cells are able to live in tissues for long time and rapidly evoke immunity reactions against viruses, they were strongly considered candidates for stimulating vitiligo lesional relapse [39]. Trm cells function has been the subject of debate. In some models, these cells sufficiently control viral titres during re-infection with no need for recirculating cells [100, 101]. In other contexts, they mainly synthesise cytokines for effector T cell recruitment from the circulation (Ariotti et al., 2014). However, CD8+ Trm cells are shown to have low cytotoxic potential.

In normal human skin CD8+ Trm cells demonstrated low levels of granzyme B and perforin [102]. Moreover, the effect of CD8+ Trm cells isolated from normal human skin on lysing allogenic target cells were significantly poorer than circulating effector memory T cells [103]. Trm cells mediate the immune response to melanoma via inhibiting tumour outgrowth instead of tumour elimination, indicating that Trm cells lack cytotoxic ability [104]. Based on their low cytotoxic activity and efficient ability of producing cytokines, Trm cells may be involved in recruitment of effector cells from circulation [39]. In vitiligo mouse model, it was found that blockade of T cell migration to the skin or depletion of recirculating memory T cells reversed vitiligo, although the number of Trm cells was not affected [105]. It was therefore concluded that Trm cells cannot independently maintain vitiligo in the absence of recruitment of further T cells [105]. In this vitiligo mouse model, autoreactive melanocyte-specific CD8+ Trm cells were shown to synthesise IFN- $\gamma$  and CXCL10, probably employing the IFN- $\gamma$ -chemokine pathway to maintain vitiligo lesions [105]. Thus, Trm cells are likely to mediate the long-term maintenance and relapse of the disease in patients via cytokine-mediated recruitment of circulating T cells [39]. Notably, vitiligo patients with stable disease are considered to have continuing immune response, as evidenced the existence of Trm cells in vitiligo perilesional skin [69, 106]. These may be involved during a flare-up, as previously reported in psoriasis [107].

### **Humoral Immunity in Vitiligo**

Besides cellular immunity, humoral (antibody-mediated) immunity adds supportive evidence for a pathological role of autoimmunity in vitiligo.

Different antibodies to melanocytes have been identified at significantly elevated levels in the sera of vitiligo

patients as opposed to healthy controls [108-111], and their levels are directly associated with the extent and activity of vitiligo [109, 112, 113], being present in 93% of patients with wider depigmentation (5-10% of skin area involved) and in only 50% of patients with minimal pigment loss (<2% of skin area involved) [114].

These anti-melanocyte antibodies belong to the immunoglobulin G (IgG) class [111], including subclasses IgG1, IgG2 and IgG3 [115], though IgA anti-melanocyte antibodies have also been detected [116].

Immunoprecipitation studies using melanocyte protein extracts have shown that antibodies in vitiligo patients are most frequently directed to antigens with molecular weights of 35, 40 to 45, 75, 90 and 150-kDa, these being found on the cell surface [117]. Some of these proteins, including those of 40-45, 75, and 150-kDa, appear to be common tissue antigens, whereas the 35 and 90- kDa proteins are preferentially expressed on melanocytes [118]. Additionally, other reports have identified vitiligo antibody targets of 45, 65, 70, 88, and 110-kDa, which are specifically expressed in melanocytes [119]. Various melanocyte-associated autoantigens have been reported. Antibodies against tyrosinase, a melanocyte-specific protein, have been extensively detected [120-122], as have antibodies against other proteins of the melanogenic pathway such as L-dopachrome tautomerase, TYRP1, PMEL, albeit at a low prevalence [123-125]. Considering this autoantibody response, rituximab, a monoclonal directed against CD20 protein expressed on the B cell surface, has shown promise in a small clinical trial in vitiligo patients [126]. A variety of circulating organ-specific antibodies against gastric parietal cells, pancreatic islet cells, thyroid and adrenal glands, are common in vitiligo patients' sera, though these are not recognised as major melanocyte antigens [127]. Phage display technology has identified other targets such as melanin-concentrating hormone receptor 1, tyrosine hydroxylase, heat-shock protein 90, osteopontin, ubiquitin-conjugating enzyme, translation-initiation factor 2, GTP-binding protein Rab38,  $\gamma$ -enolase,  $\alpha$ -enolase and lamin A [128-130], as well as four novel autoantigens glycoprotein non-metastatic melanoma protein b, melanocortin 1 receptor, OCA2-encoded P protein and GTP-binding protein Rab27A (unpublished data). Vitiligo-associated antibodies are capable of melanocytotoxicity *in vitro* and *in vivo* by complement-mediated cytotoxicity and by antibody-dependent cellular cytotoxicity [131, 132]. Melanin-concentrating hormone receptor 1 block the function of the receptor, though it is not known if this affects melanocyte function [133]. Melanocyte expressed of HLA-DR and intercellular adhesion molecule-1 (ICAM-1) induced by anti-melanocyte antibodies may make them a target for cytotoxic T cells [134]. Presently, it unclear whether melanocytes are a primary or secondary target of the humoral immunity in vitiligo. Autoantibodies might arise from a genetic susceptibility to immune dysregulation at the T or B lymphocyte level, with lack of tolerance to pigment cell antigens, or from an immune response against melanocytes damaged by other mechanisms, such as T cell destruction [135]. Repigmentation observed in vitiligo patients receiving immunosuppressive treatments supports the notion of an immune-mediated process in vitiligo [13]. Tacrolimus, a reagent that suppresses T cells by blocking cytokine gene-activating cofactor calcineurin works in vitiligo [136, 137], as do topical corticosteroids, which suppress T lymphocyte activity and B cell antibody responses [138]. Phototherapy, reduces the number of Langerhans cell in the skin and down regulates expression of vitiligo-associated melanocyte antigens [139].

### Pathogenic Mechanisms of Autoimmunity in Vitiligo

The ability of vitiligo-associated antibodies to destroy melanocytes has been demonstrated *in vitro* by both complement-mediated cytotoxicity and antibody-dependent cellular cytotoxicity [131, 133, 140].

*In vivo*, the administration of IgG from vitiligo patients into human skin grafted onto nude mice has been shown to induce melanocyte destruction [132]. In a reconstructed epidermis model, sera from 9/13 (69%) vitiligo patients induced the detachment of melanocytes, although this was not related to disease extent or activity [141]. Vitiligo patient antibodies against MCHR1 were demonstrated to block the function of the receptor [133]. However, it is not known if or how this activity could affect the melanocyte function [133]. Moreover, IgG anti-melanocyte antibodies are able to induce the expression of HLA-DR and ICAM-1 on melanocytes and the release of IL-8 from melanocytes [134]. By enhancing the antigen-presenting activity of melanocytes in this way, they become targets for cytotoxic T cells. Presently, it has not been determined if melanocytes are a primary or secondary target of the humoral immune response in vitiligo. They might

arise from a genetic susceptibility to immune dysregulation at the T or B lymphocyte level, leading to lack of tolerance to pigment cell antigens [142]. Alternatively, vitiligo antibodies might originate from an immune response against melanocytes damaged by some other mechanism [142].

Interestingly, the normally intracellular melanocyte antigens TYRP1 and PMEL can be expressed on the cell surface and so can be accessible by antibodies [143, 144]. In addition, pigment cell antibodies in vitiligo might be secondary to destruction of the melanocyte from another immune cause such as T-cells, but that once triggered the antibodies are themselves destructive to melanocytes. While the potential for cytotoxic CD8+ T cells to eliminate melanocytes in both vitiligo and melanoma immunotherapy is clear, the exact mechanism that these cells use is not fully understood. Several cytotoxic effector proteins such as perforin, granzyme, Fas ligand and cytokines can be used to destroy target cells [145]. It is believed that cytotoxic T cells mainly use perforin and granzyme as fast-acting method of destroying cancer cells or virus-infected cells, whereas Fas ligand-driven killing mechanism may act as slower-acting alternative process [146]. In fact, several intracellular signaling pathways promote cytotoxic T cell killing via perforin, granzyme and Fas ligand, and therefore it is currently unclear how these pathways are selectively used by T cells and how they communicate [145]. Additionally, T cell-mediated killing mechanism in autoimmune vitiligo may differ from that of cancer or viral-infected cells. Therefore, how melanocyte in vitiligo are eliminated is not yet clear, and this requires further studies in order to unveil the exact mechanism involved in details. Melanocytes from perilesional skin of vitiligo patients demonstrates physiological and histological abnormalities which may induce autoimmune reaction against melanocytes. Genes that are implicated in antigen presentation confer a significant risk for the development of vitiligo [147]. One hypothesis suggests that modified proteins called neo-antigen can be extremely immunogenic stimulating an immune response against them, since thymic epithelial cells responsible for T cell education do not synthesise such proteins [148]. This leads to the formation of highly self-reactive T cells with high-affinity receptors that target neo-antigens [148]. Melanoma is immunogenic owing to somatic DNA mutation that result in neo-antigen generation [149]. However, self-reactive T cells are unlikely to target mutated proteins in vitiligo, since untransformed melanocytes do not have the ability to mutate their DNA [39]. Several biochemical processes have been involved in neo-antigen formation as well such as deamidation, carbonylation, citrullination, oxidation and alternative mRNA splicing [38]. These processes are implicated in generating neo-antigen in untransformed beta islet cells [38].

### **Cytokines Imbalance and their Role in Vitiligo Pathogenesis**

Cytokine imbalance has been shown in vitiligo skin [150]. Elevated serum levels of soluble IL-2 receptor, which correlated with disease activity in vitiligo patients, have been reported, as have increased synthesis of IL-6, a cytokine induces ICAM-1 expression on melanocytes which could facilitate leukocyte-melanocyte interaction, as well as elevated levels of IL-8, neutrophils, T lymphocytes, and basophils recruiter [151, 152]. Recently this has been added to by the revelation of elevation of T helper (Th) 1 (IL-2, interferon (IFN)- $\gamma$ , TNF- $\beta$ ), Th2 (IL-4, IL-5, IL-10, IL-13) and Th17 (IL-17, IL-23)-innate cytokines in the serum of all 44 vitiligo patients examined, with a higher ratio of Th1/Th2 cytokines [153]. Expression of the pro-inflammatory cytokine TNF- $\alpha$  is significantly elevated in lesional and peri-lesional vitiligo patient skin, whereas a variety of melanogenic mediators such as endothelin-1, stem cell factor, basic fibroblastic growth factor and granulocyte monocyte-colony stimulating factor are expressed at lower levels in vitiliginous lesions [154]. Recently it has been shown that melanocyte adhesion is disrupted in vitiligo skin through increased levels of MMP-9, produced by keratinocytes in response to IFN- $\gamma$  and TNF- $\alpha$ , and disturbing E-cadherin on the pigment cells [155]. Both human patients and a mouse model of vitiligo, show high levels of IFN- $\gamma$ , the cytokine required for the cutaneous recruitment of melanocyte-specific autoreactive CD8+ T lymphocytes, and of IFN- $\gamma$ -induced cytokine CXCL10, and its receptor CXCR3, found on autoreactive CD8+ T cells [71]. Knocking out CXCR3 or blocking CXCL10 action prevents and reverses depigmentation in vitiligo [76].

### **Malignant Melanoma-Associated Vitiligo**

Malignant melanoma is a type of skin cancer resulted from uncontrolled growth of melanocytes. Although the exact mechanism implicated in the pathogenesis of malignant melanoma-associated vitiligo is still unclear, the immune reactivity against malignant melanoma cells, especially CD8+ T cells, is thought to play a critical role

in vitiligo development [156, 157]. Malignant melanoma-associated vitiligo may arise from immune response directed against malignant melanoma-associated antigens expressed by both melanocytes and malignant melanoma cells. Indeed, antibodies reactive to tyrosinase [158], TYRP1, dopachrome tautomerase and Pmel17 [159] have been found in some malignant melanoma patient sera. Malignant melanoma studies showed that these melanocyte specific-antigens were recognised by self-reactive CD8+ T cells [160]. Following immunotherapy for metastatic malignant melanoma, enhanced efficacy is associated with CD8+ T cells. Immunotherapy for malignant melanoma involves blocking T-cell checkpoint inhibitors, which interfere with T cell tolerance in tissues, and adoptive cell therapy, which expands T cells that infiltrate autologous tumor *ex vivo* for therapeutic reinjection into melanoma patients [39]. Importantly, tumour infiltration with CD8+ T cells is vital in the effectiveness of both strategies [161], and these cells are thought to regulate malignant melanoma via perforin-dependent cytotoxicity [145]. New-onset vitiligo is commonly triggered by malignant melanoma therapy, and this occurs in about 4% such patients who are treated with immunotherapy [162]. Notably, vitiligo patches initiated by malignant melanoma immunotherapy are packed with CD8+ T cells that are specific to melanocytes, similar to idiopathic vitiligo patches [163]. Thus, CD8+ T cells are crucial to both the eradication of malignant melanoma and the pathogenesis of vitiligo. Therefore, the immune response in malignant melanoma patients which causes melanocyte damage is suggested to be cell-mediated, driven by CD8+ T cells and not by a humoral response. Thus, CD8+ T cells are crucial to both the eradication of malignant melanoma and the pathogenesis of vitiligo. Melanocyte-specific antibodies in malignant melanoma patients are likely to arise as a secondary immune response after melanocyte destruction via cell-mediated effects. Furthermore, the serum titres of malignant melanoma-related antibodies are low and their levels do not differ in patients with and without malignant melanoma-associated vitiligo. Vitiligo-like lesions in malignant melanoma patients receiving immunotherapy are considered as a good prognostic factor and have been correlated with a longer survival in these patients [164]. Development of vitiligo during treatment of malignant melanoma can be used as a clinical indication to predict and maintain the response [164].

## Genetic Factors

Genetic involvement in vitiligo is obvious from a simple examination of family histories: 15-20% of vitiligo patients have at least one affected first-grade relative [165]. However, vitiligo does not show a Mendelian mechanism of inheritance, but is the polygenic with multiple susceptibility loci [147]. The concordance rate in monozygotic twins is 23%, suggesting involvement of non-genetic factors [166]. Genome-wide association studies have revealed that approximately half of vitiligo susceptibility genes encode immune-regulatory proteins, while the remainder encode melanocyte-specific proteins [147]. Several studies have shown that multiple loci contribute to vitiligo susceptibility (**Table 1**). An observed association between HLA types and vitiligo, and other autoimmune disorders, can be elucidated by several pathways that ultimately result in disruption in self-antigen recognition (Jin et al., 2012a). This can lead to autoreactive T cell development and/or failure to produce effective Tregs population [60]. Genome-wide association studies have also reported a subset of other immune regulatory genes that are also key mediators of adaptive immunity such as *CD80* (activation of T cells), cytotoxic T lymphocyte antigen-4; *CTLA4* (inhibition of T cells), *GZMB*(cytotoxicity of T cells), forkhead box protein P3; *FOXP3*(development and function of regulatory T cells), lymphoid protein tyrosine phosphatase non-receptor type 22; *PTPN22*(down-regulation of T cell activation) and arginine–glutamic acid dipeptide repeats protein; *RERE* (regulation of cell apoptosis) [167]. An association of vitiligo with genes that play a role in innate immunity, such as NACHT leucine-rich-repeat protein 1 (*NLRP1*), interferon-induced helicase C domain 1 (*IFIH1*) and caspase-7 (*CASP7*), has also been found in genome-wide association studies [168]. In addition to immune regulatory genes, several genes that are only expressed in melanocytes and involved in melanocyte function have been identified as vitiligo susceptibility loci. These include *TYR*, *PMEL*, melanocortin 1 receptor (*MC1R*), *OCA2* [169]. Such genes encode for enzymes or proteins recognised as autoantigens in vitiligo, facilitating an anti-melanocyte autoimmune response [122, 124].

**Table 1: A summary of genes associated with susceptibility to vitiligo**

Type	Gene	Protein	Function
HLA	<i>HLA-A</i>	HLA class I histocompatibility antigen, A	Peptide transport
	<i>HLA-DRB1 and DQA1</i>	HLA class II histocompatibility antigen, DRB1 and DQA1	Peptide transport
	<i>HLA class III</i>	HLA class III histocompatibility antigen	Involvement in immune response
Immune- regulatory	<i>AIRE</i>	Autoimmune regulator	Mainly in thymus
	<i>BACH2</i>	Transcription regulator protein BACH2	B cell development
	<i>C1QTNF6</i>	Complement C1q tumour necrosis factor-related protein 6	Induction of immune response
	<i>CASP7</i>	Caspase-7	Execution of apoptosis
	<i>CCR6</i>	Chemokine-cytokine receptor 6	Regulation of cell migration
	<i>CD44</i>	CD44 antigen	Involvement in cell adhesion
	<i>CD80</i>	T-lymphocyte activation antigen CD80	Co-stimulatory molecule
	<i>CLNK</i>	Cytokine-dependent hematopoietic cell linker	Positive regulation of hematopoiesis
	<i>CTLA4</i>	Cytotoxic T-lymphocyte protein 4	Inhibition of T cell activation
	<i>CXCR5</i>	C-X-C chemokine receptor type 5	Involvement in chemotaxis
	<i>FASLG</i>	FAS ligand	Regulation of apoptosis
	<i>FOXP1</i>	Forkhead box protein P1	Regulation of immune response
	<i>FOXP3</i>	Forkhead box protein P3	Regulation of immune response
	<i>GZMB</i>	Granzyme B	Mediation of cytotoxicity
	<i>IFIH1</i>	Interferon-induced helicase C domain 1	Regulation of antiviral response
	<i>IKZF4</i>	Zinc finger protein Eos	Regulation of gene expression
	<i>IL2RA</i>	Interleukin-2 receptor subunit alpha	Regulation of cell growth
	<i>NLRP1</i>	NACHT leucine-rich-repeat protein 1	Regulation of inflammation
	<i>PTPN22</i>	Lymphoid protein tyrosine phosphatase non-receptor type 22	Negative regulation of signaling
	<i>SH2B3</i>	SH2B adapter protein 3	Development of immune system
	<i>SLA</i>	Src-like-adaptor	Regulation of cell signaling
	<i>TOB2</i>	Protein TOB2	Involvement in cell cycle
	<i>TSLP</i>	Thymic stromal lymphopoietin	Cytokine
<i>UBASH3A</i>	Ubiquitin-associated and SH3 domain-containing A protein	Support of cell signaling	
Melanocyte function	<i>XBP1</i>	X-box-binding protein 1	Regulation of gene expression
	<i>ASP</i>	Agouti signalling protein	Melanin production
	<i>FOXD3</i>	Forkhead box D3	Regulation of cell differentiation
	<i>MC1R</i>	Melanocortin 1 receptor	Regulation of melanin production
	<i>OCA2</i>	OCA2 gene	Melanin production
	<i>PMEL</i>	Melanocyte-specific protein PMEL	Structural component of melanosomes
	<i>TYR</i>	Tyrosinase	Regulation of melanin production
Metabolism-related genes	<i>ZMIZ1</i>	Zinc finger protein MIZ type 1	Regulation of gene expression
	<i>ACE</i>	Angiotensin-converting enzyme	Regulation of blood pressure
	<i>CAT</i>	Catalase	Protein degradation
	<i>EDN-1</i>	Endothelin-1	Regulation of blood pressure
	<i>LPP</i>	Lipoma-preferred partner	Potential role in cell signaling
	<i>RERE</i>	Arginine-glutamic acid dipeptide repeats protein	Regulation of gene expression
	<i>RNASET2</i>	Ribonuclease T2	Oxidative stress response

## Neuronal Theory

Neuronal elements were originally thought to have a role in vitiligo through catecholamine released from epidermal nerve endings which might be cytotoxic to melanocytes or by autonomic dysfunction producing pigment cell destruction [203, 204]. Clinical pointers towards neural involvement included the distribution along blaschkoid lines in segmental vitiligo, and the occasional observation of a true dermatomal appearance, e.g. in the trigeminal areas [167, 204].

Ultrastructurally vitiligo skin shows degeneration of fine cutaneous nerves in 42% of cases, with Schwann cells showing thickened basement membrane in 75% of instances and axonal damage in a half [203, 205]. Changes in neuropeptides, notably neuropeptide Y, are evident at the margins of vitiligo patches [205]. This led to the postulation that neuropeptides, potentially neuropeptide Y, which has effects on the immune system through receptors located on several immunologically active cells including T cells, NK cells, dendritic cells, granulocytes, and macrophages, released from nerve ending release next to melanocytes, could provoke a local immune reaction and melanocyte destruction [206, 207]. Treg cells, seen as central to melanocyte destruction in vitiligo, can be induced by vasoactive intestinal polypeptide, and can alter the Th1/cytotoxic T cell (Tc)1 balance that is skewed in vitiligo [208]. Hence, the neural and neuro-endocrine systems, neuropeptides and neurotransmitters, interacting with the immune systems, need to be taken into account in the

causation of vitiligo [209].

### Convergence Theory

The convergence theory attempts to link together the potential causal mechanisms of vitiligo [210]. It suggests multiple sequential stages in pathogenesis beginning with an elicitation stage perhaps due to mechanical friction and skin trauma, emotional stresses, chemical exposure or imbalances in endogenous neural factors, metabolites, cytokines or hormones [5, 206, 211-214]. Such factors, in an individual whose genetic make-up predisposes to immune activation and melanocyte destruction, it is proposed result in oxidative stress within melanocytes which subsequently express HSP70 and chaperoned melanocyte antigens, presented by dendritic cells to T cells in regional lymph nodes, resulting in proliferation of melanocyte-specific cytotoxic T cells and melanocyte destruction — the stage of immune activation [54, 135, 166].

Absent or reduced fully-functional skin-infiltrating Tregs contribute to the on-going immune response and disease spread [86]. Antibodies against melanocyte-specific proteins such as tyrosinase, generated in response to melanocyte destruction, damage pigment cells by activating complement or by antibody-dependent cellular cytotoxicity [122, 133].

### Conclusion and Prospects

Sufficient is now understood about the pathogenesis of vitiligo to permit targeted pharmacological intervention at the appropriate immunological steps. However, the exact modus by which the genetic interacts with the environment, and with the immune system still requires considerable elucidation. What can be said is that both environmental factors and cell-intrinsic defects instigate stress responses in melanocytes, resulting in the synthesis of DAMPs that elicit innate immune cells, which in turn activate adaptive immunity that ultimately targets melanocytes. A genetic predisposition to autoimmunity may underlie inappropriate immune responses in vitiligo, but immune responses may occur secondarily as a result of melanocyte damage by other factors, as when autoantibodies have been observed directed against intracellular pigment cell proteins — exposure of cryptic epitopes and protein modification occurring during apoptosis [215]. Following processing by dendritic cells, antigenic proteins can be presented to either autoreactive T cells, which evaded clonal deletion, or to naïve T cells, which have not been tolerised against cryptic epitopes [216]. In consequence, antibodies can be secreted following autoreactive B cell stimulation by activated autoreactive CD4+ T lymphocytes [216], which may then act to further aggravate vitiligo. However, it is possible that antibodies play no part in the pathogenesis of vitiligo, but might indicate the existence of autoreactive anti-melanocyte T cells are capable of destroying melanocytes, a scenario that merits further investigation.

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