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Viewpoint

Vitiligo: interplay between oxidative stress and immune system

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Abstract: Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis, linked with both genetic and non-genetic factors. The precise *modus operandi* for vitiligo pathogenesis has remained elusive. Theories regarding loss of melanocytes are based on autoimmune, cytotoxic, oxidant–antioxidant and neural mechanisms. Reactive oxygen species (ROS) in excess have been documented in active vitiligo skin. Numerous proteins in addition to tyrosinase are affected. It is possible that oxidative stress is one among the main principal causes of vitiligo. However, there also exists ample evidence for altered immunological processes in vitiligo, particularly in chronic and progressive conditions. Both innate and adaptive arms of the immune system appear to be involved as a primary event or as a secondary promotive consequence. There is speculation on the interplay, if any, between ROS and the immune system in the pathogenesis of vitiligo. The article focuses on the scientific evidences linking oxidative stress and immune system to vitiligo pathogenesis giving credence to a convergent terminal pathway of oxidative stress–autoimmunity-mediated melanocyte loss.

Key words: autoimmunity – melanocyte – oxidative stress – vitiligo

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Introduction

Vitiligo is a common dermatological disorder of the epidermis and hair follicles, manifesting clinically as expanding hypopigmented lesions of the skin. It affects 0.5–1% of the world population, and its incidence ranges from 0.1 to 8.8% in India (1,2). Absence of melanocytes in the skin lesion due to their destruction has been suggested to be the key event in the pathogenesis of vitiligo (3). The aetiology of vitiligo remains obscure despite being in focused debate for the last six decades (3–6), and hence, it is important to unravel the underlying pathomechanisms of vitiligo.

A single dominant pathway appears unlikely to account for all cases of melanocyte loss in vitiligo, and apparently, a complex interaction between genetic, environmental, biochemical and immunological events is likely to generate a permissive milieu (Fig. 1). Loss of melanocytes in vitiligo appears to occur through a combination of several mechanisms that act in concert. Here, we discuss the possible interconnections of oxidative stress and immune system that are involved in melanocyte loss. There might be alteration in melanocyte-specific proteins by the action of reactive oxygen species (ROS), which results in the generation of neoantigens, autoimmunity and melanocytorrhagy leading to defective apoptosis.

Oxidative stress and vitiligo

Oxidative stress is considered to be one of the possible pathogenic events in melanocyte loss (7,8). Defective recycling of tetrahydrobiopterin in whole epidermis of patients with vitiligo is related to the intracellular production of H_2O_2 (9,10). In addition, an increased intracellular production of ROS due to mitochondrial impairment (11) and a compromised antioxidant status (8,12,13) supports the concept of a possible systemic oxidative stress in vitiligo. This accumulated oxidative stress causes DNA damage, lipid and protein peroxidation (14,15) (Fig. S1). Many proteins are altered and show partial or complete loss of functionality due to H_2O_2-mediated oxidation. H_2O_2 can also function as an inhibitor of tyrosinase, or in the presence of H_2O_2, DOPA (dihydroxyphenylalanine) substrate can generate a secondary complex that can bind and inhibit tyrosinase (16).

Elevated extracellular calcium levels and inhibition of thioredoxin reductase also contribute to the generation of oxidative stress in the vitiligo epidermis (17,18). Several sources have been documented for the unusual production/accumulation of epidermal H_2O_2 [Table 1: (8–12), (19–29)]. Our studies also showed systemic oxidative stress in patients with vitiligo due to an imbalance in enzymatic and non-enzymatic antioxidant systems (20,25) and significant decrease in acetylcholine esterase (AChE) activity (30), which could be due to H_2O_2-mediated oxidation of AchE (31), thus emphasizing the role of oxidative stress in precipitation of vitiligo. Moreover, our recent study suggests oxidative stress as the initial triggering factor in precipitating vitiligo. Patients with early onset (<3 months) of vitiligo showed significant decrease ($P = 0.005$) in the levels of antimelanocyte antibodies compared to patients with long duration (>3 months), and moreover, erythrocyte lipid peroxidation levels were significantly increased ($P = 0.0085$) in patients with early-onset vitiligo compared to patients with long-standing vitiligo.

Further, increased levels of ROS in melanocytes may cause defective apoptosis resulting in release of aberrated proteins, which can serve as autoantigens leading to autoimmunity (32). The intracellular levels of H_2O_2 and other ROS also increase in response to cytokines such as TNFα (tumor necrosis factor α) and TGFβ1 (transforming growth factor β1), which are potent inhibitors of melanogenesis (33–36). High ROS also increase the levels

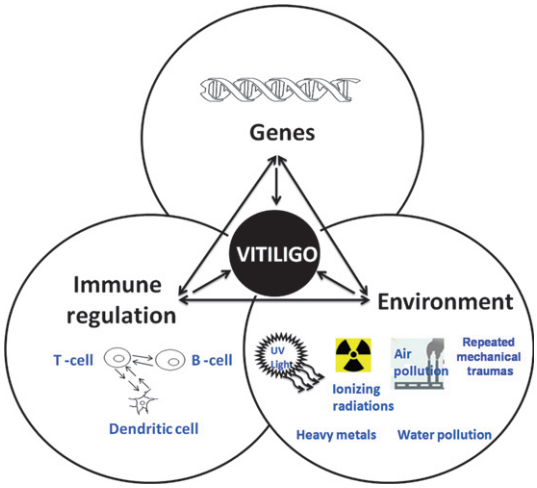

Figure 1. Interplay of genes, environment and immune system in precipitation of vitiligo: interaction of genes with environment and immune system leads to vitiligo. Susceptible genes under the effect of environmental trigger like: generation of ROS by various environmental sources (UV and ionizing radiations, air and water pollution, heavy metals etc.) and repeated mechanical traumas can result into aberrant immunological responses (i.e. cellular and humoral immune response) resulting into autoimmunity.

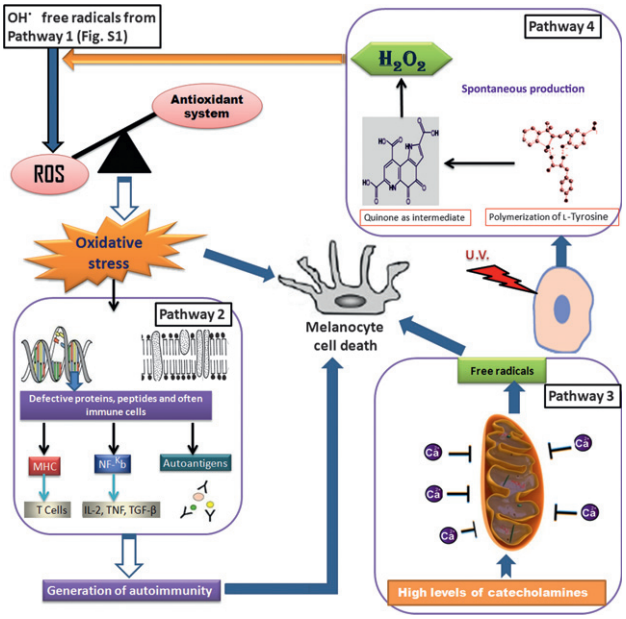

Figure 2. Different pathways for melanocyte loss: (i) Generation of ROS by various metabolic processes. (ii) Imbalance in ROS generation and antioxidant system leads to accumulation of free radicals resulting in oxidative stress. This accumulation causes DNA damage, synthesis of defective proteins and membrane disintegration which provokes immune system resulting in autoimmunity. (iii) Increased catecholamines inhibits mitochondrial calcium uptake which results in generation of free radicals. (iv) Exposure to UV radiation leads to spontaneous production of quinones in melanocytes which in turn results into ROS generation.

of cytokines, including IL-2 (interleukin-2), which upregulate the expression of anti-apoptotic protein, Bcl-2 (B-cell lymphoma-2), thereby making T cells resistant to apoptosis (Fig. 2; pathway 2) (37). Moreover, transepidermal loss of melanocytes under stress conditions (adrenaline and H_2O_2) supports the hypothesis that non-segmental vitiligo (NSV) melanocytes have an intrinsic defect, which limits their adhesion in a reconstructed epidermis (38), thus leading to melanocytorrhagy (39–41).

Autoimmunity and vitiligo

Vitiligo lesions are characterized by an infiltration of inflammatory cells, particularly cytotoxic, helper T cells and macrophages. This infiltration is most prominent in the perilesional skin just prior to clinical appearance of vitiligo. Only early-stage lesions show non-specific infiltrate of lymphocytes in the epidermis and the dermis suggesting involvement of T cells in active vitiligo lesions (42). Elevated antibody levels against melanocyte antigens in 2624 patients showed increased frequency of autoimmune disorders such as hypothyroidism, pernicious anaemia, Addison's disease, systemic lupus erythematosus and inflammatory bowel disease in vitiligo probands and their first-degree relatives suggesting a common genetic aetiological link between vitiligo and other autoimmune diseases (43,44). Further, Michelsen (45) has proposed antibody-based and T-cell-based dominant mechanisms in generalized and localized vitiligo, respectively, as the contributory factors for autoimmune vitiligo. Thus, humoral and cell-mediated immune mechanisms are likely to be involved in the melanocyte destruction.

Humoral immune response in vitiligo

Antibodies against melanocyte antigens are detected in the sera of patients with vitiligo, and a correlation exists between melanocyte antibody levels and disease activity (46–49). Tyrosinase is the principal antigen recognized by these antibodies (49,50). Our recent study has also suggested that 75% of patients with vitiligo had antimelanocyte antibodies in their circulation. Kemp et al. (51) found that 23% of the patients with non-segmental vitiligo were positive for tyrosine hydroxylase antibodies.

The other melanocyte antigens recognized by autoantibodies are gp100/Pmel 17 (a melanosomal matrix glycoprotein) and tyrosinase-related proteins 1 and 2 (TRP 1 and TRP 2) (52,53) (Table S1). These cell differentiation antigens are localized primarily to melanosomes (54). A summary of the autoantigens implicated in vitiligo is given in Table S1 (41,52,53,55–61). *In vitro* studies showed that vitiligo antibodies are able to destroy melanocytes by complement-mediated damage and antibody-dependent cellular cytotoxicity (62). The selective loss of melanocytes might result from antibody reactivity directed to the antigens preferentially expressed on pigment cells, which might result from a genetic predisposition to immune dysregulation at the T-cell level (50,63). Moreover, B-cell infiltration in juxtaposition to depigmented zones supports the idea that the autoimmune phenomenon is mediated by a humoral mediator or is local to some areas of skin (64).

Cell-mediated immunity

The high frequencies of melanocyte-reactive cytotoxic T cells in the peripheral blood of patients with vitiligo, perilesional T-cell infiltration and melanocyte loss *in situ* suggest the involvement of cellular autoimmunity in vitiligo pathogenesis (65–69). In particular, active cases of vitiligo were demonstrated to have higher levels of cytotoxic T cells (70). Histopathological and immunohistochemical studies have confirmed the presence of infiltrating $CD8^+$ T cells in generalized vitiligo (71–76). *In vitro* studies demonstrated an increased production of pro-inflammatory cytokines IL-6 and IL-8 by monocytes of active patients with vitiligo, which will affect effector cell migration, effector target attachment and

Table 1. Sources for epidermal/systemic H_2O_2 generation/accumulation in vitiligo

Source	References	H_2O_2 generation/accumulation	Increase/decrease
Monoamine oxidase A	Schallreuter et al. (19)	Epidermal	Increase
Superoxide dismutase	Agrawal et al. (20); Hazneci et al. (21)	Blood	Increase
Glucose 6 phosphate dehydrogenase	Agrawal et al. (20)	Blood	Decrease
NADPH oxidase	Schallreuter et al. (10)	Epidermal	Increase
Photooxidation of pterins	Rokos et al. (22)	Epidermal	Increase
Nitric oxide synthases	Gibson and Liley (23)	Epidermal	Increase
Short circuit in 6BH4 recycling	Schallreuter et al. (9); Kaufman et al. (24)	Epidermal	Increase
Catalase	Dell'Anna et al. (11); Schallreuter et al. (12); Maresca et al. (8); Shajil and Begum (25)	Blood and epidermal	Decrease
Glutathione peroxidase/reduced glutathione	Beazley et al. (26); Dell'Anna et al. (11); Agrawal et al. (20); Yildirim et al. (27)	Blood	Decrease
Tyrosinase-related protein 1	Jimbow et al. (28)	Epidermal	Decrease
Xanthine oxidase	Koca et al. (29)	Blood	Increase

also cause B-cell activation (77). In most patients with vitiligo, the balance of cytotoxic/suppressor and helper/inducer T cells in peripheral blood is disturbed (64,78). Moreover, in progressive disease, the $CD4^+/CD8^+$ ratio is decreased among skin-infiltrating T cells (79).

Recent studies have demonstrated that the number and suppressive effects of peripheral T regulatory cells in progressive generalized patients with vitiligo were significantly reduced, suggesting an impairment in their ability to inhibit the proliferation (76,80,81). Nevertheless, Abdallah and Saad (82) also showed a dysfunction of Tregs by the elevation of Tregs and Teffs in generalized patients with vitiligo suggesting that Tregs were unable to control the immunological attack and destruction of melanocytes by cytotoxic T cells. In addition, our findings demonstrated decreased levels of both *sCTLA4* and *flCTLA4* transcripts in patients, suggesting the disturbance in the suppressive capacity of Tregs and thus emphasize the role of cellular immunity in vitiligo (83).

Recently, the role of Th17 cells has gained more attention in vitiligo, as immunohistochemical analysis showed Th17 cell infiltration in vitiligo skin samples in addition to $CD8^+$ T cells (84,85). Moreover, the studies provide evidence for the influence of a Th17 cell-related cytokine environment (IL-17A, IL-1β, IL-6 and TNFα) in local depigmentation in autoimmune vitiligo (84,85). IL-17 has also been reported to be involved in augmented production of ROS (86), thereby implicating its role in oxidative stress-mediated cell damage. In addition, studies have also found increased levels of IL-17 in serum, lesional skin (87) and in neutrophils of patients with vitiligo (88), thus suggesting an important role of Th17 cytokine in the pathogenesis of vitiligo.

Genetics of vitiligo

Vitiligo is characterized by multiple susceptibility loci, incomplete penetrance and genetic heterogeneity and may involve genes associated with the biosynthesis of melanin, antioxidant system and regulation of autoimmunity (89,90). Recent studies suggest that genetic factors may play a major role in the pathogenesis of vitiligo. Our study also suggests that 21.93% of Gujarat patients with vitiligo exhibited positive family history and 13.68% patients had at least one affected first-degree relative (91). Because vitiligo is a polygenic disease, several candidate genes including *MHC*, *ACE*, *CAT*, *CTLA4*, *COMT*, *ESR*, *MBL2*, *PTPN22, HLA*, *NALP1, XBP1, FOXP1* and *IL-2RA* that are involved in regulation of immunity have been tested for genetic association with generalized vitiligo (89,92,93).

Recently, we have shown positive association between HLA-A*33:01, HLA-B*44:03 and HLA-DRB1*07:01 with patients with vitiligo from North India and Gujarat suggesting an autoimmune link of vitiligo in these cohorts (94). We have also shown that the three most significant class II region SNPs: rs3096691 (just upstream of NOTCH4), rs3129859 (just upstream of HLA-DRA) and rs482044 (between HLA-DRB1 and HLA-DQA1) are associated with generalized vitiligo (95). The genotype–phenotype correlation between *CTLA*-4, *IL*-4 and *TNFA* gene polymorphisms supported the autoimmune pathogenesis of vitiligo in Gujarat population (83,96,97), whereas our earlier studies on *CAT*, *GPX*, *MBL*-2, *ACE* and *PTPN*22 polymorphisms did not show significant association (98–101).

Cytokines and apoptosis

The exact pathway for loss of melanocytes is not yet known; however, apoptotic death has been suggested in vitiligo (102,103). Cytokines such as IL-1, IFNγ or TNFα are paracrine inhibitors of melanocytes and can initiate apoptosis (102). Our recent study has shown increased TNFα protein and transcript levels in patients with vitiligo, suggesting an early apoptosis of melanocytes (97). In addition, TNFα induces IL-1α, thereby promoting B-cell differentiation, immunoglobulin production and also cause maturation of dendritic cells, thus results in development of autoimmunity (65). Apoptosis of melanocytes in vitiligo may also be due to melanocyte-specific antibodies (73).

Kotobuki et al. (84) showed that IL-17A dramatically induced IL-1β, IL-6 and TNFα production in keratinocytes and fibroblasts, which can affect apoptosis of melanocytes. IL-6 and IL-13 secreting $CD8^+$ T cells from vitiligo perilesional margins may induce autologous melanocyte apoptosis (104). Also, an imbalance of keratinocyte-derived cytokines such as GM-CSF, bFGF, SCF, IL-6, IL-1α and TNFα in the lesional skin has been demonstrated, which could impair the normal life and function of melanocytes (105,106). Moreover, alteration in mRNA expression pattern of *IL-20RB*, *IL-22RA2*, *IL-28A*, *IL-28B*, *IL-28RA*, *IFNA1*, *IFNB1* and *IFNG* genes involved in regulation of survival/apoptosis of melanocytes has been observed in vitiligo skin and/or peripheral blood mononuclear cells (PBMC) (107).

Defective apoptosis and generation of autoimmunity

Melanocytes from patients with vitiligo demonstrate various abnormalities, including incompetent synthesis, processing of melanocytes, abnormal rough endoplasmic reticulum, homing-

receptor dysregulation and early apoptosis (5,103). Oxidative stress, which can induce apoptosis by cytochrome C–mediated pathway of caspase activation, may contribute to melanocyte loss in vitiligo lesions (108). 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. These modified autoantigens, which may also expose cryptic epitopes, may be processed by mature Langerhans cells and presented to T cells (109). Subsequently, the autoreactive $CD4^+$ T cells may stimulate autoreactive B cells to produce autoantibodies, whereas $CD8^+$ T cells may attack melanocytes directly (109). It is worth noting that efficient clearance of apoptotic cells is crucial for the avoidance of autoimmune responses to intracellular antigens.

Interplay of oxidative stress and immune system

The two major theories of vitiligo pathogenesis include autoimmune aetiology for the disease and 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. The synergistic interaction of oxidative stress with immune system may lead to either direct or indirect loss of melanocytes, as it has been previously suggested in melanocytorrhagic hypothesis (38). In addition, 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 loss of melanocytes (Fig. 2).

In autoimmune disorders, the immune system creates a chronic or relapsing inflammatory milieu in which ROS can accumulate with a toxic effect on surrounding cells. This can explain the pathogenesis of inflammatory vitiligo (110). The bottom line question that remains unanswered is what causes 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?

ROS are produced as by-products of melanogenesis in melanocytes and controlled by several redundant antioxidant enzymes. 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 ROS in the epidermis of patients with vitiligo (111). Moreover, there are several ways by which ROS, besides having a direct melanocytotoxicity, can induce an autoimmune attack against melanocytes. In fact, ROS are involved in specific early events in T-cell activation and antioxidants are involved in reducing T-cell proliferation, IL-2R expression and IL-2 production (112). The build-up of ROS along with possible immune system defects allows for the inappropriate autoimmune response against melanocytes (Fig. 2).

The melanogenic pathway involves the formation and polymerization of L-tyrosine, which is converted into L-dopaquinone with O-quinone as an intermediate product. Exposure to UV radiation for longer time causes the spontaneous production of O-quinone leading to the formation of H_2O_2 as a by-product (60) (Fig. 2, pathway 4). The structures of melanocytic macromolecules and small molecules, such as Melan-A and tyrosinase, may be changed by acute or chronic oxidative stress and can act as antigens (neoantigens). Neoantigens with sufficient homology or identity to host antigenic proteins induce autoreactivity. This phenomenon is referred to as 'molecular mimicry' (113). The presence of rheumatoid factors in the sera and lesions of patients with vitiligo can be explained by this mechanism (114). Over time, chronic oxidative stress could generate several adducted and⁄or non-adducted molecules that would essentially act as a neoantigens (115). More than one neoantigens/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 (113).

Further, increased phenols⁄catechols, in vitiliginous skin areas, may serve as surrogate substrates of tyrosinase, converting into reactive quinones (16). Such reactive quinones, whose production is enhanced by increased H_2O_2 in the vitiligo lesions, can covalently bind to tyrosinase (haptenation). This could give rise to a neoantigen, carried by Langerhans cells to the regional lymph nodes and stimulate the proliferation of cytotoxic T cells (116). Moreover, Kroll et al. (117) showed that 4-tertiary butyl phenol (4-TBP) exposure sensitizes human melanocytes to dendritic cell (DC)-mediated killing through release of HSP70 and DC effector functions. Recently, Elassiuty et al. (118) have demonstrated that stress-induced (UV, 4-TBP) melanocyte cell death is protected by haem oxygenase-1 (HO-1) overexpression, thereby contributing to beneficial effects of UV treatment for patients with vitiligo.

During chronic oxidative stress and other noxious processes, neoantigens potentially cause tissue damage and release a plethora of sequestered autoantigens. This process is referred to as the 'bystander effect'. Such an outburst of autoantigens from the target tissue would potentially amplify the effect of the neoantigens, leading to the breakdown of self-tolerance (113). These reports have yielded some interesting clues linking oxidative stress and immune system and provide an insight into the generation of autoimmunity due to oxidative stress. However, the conjunction of oxidative stress and autoimmune hypotheses is unable to explain the potential triggering factors and different depigmentation patterns observed in different types of vitiligo.

Major open questions

Based on the available data, melanocyte loss in vitiligo is still an enigma and the triggering factors are still being debated. Also, the proposed hypotheses have not been tried on animal models to support their validity. Moreover, the bilateral symmetrical distribution of vitiligo patches on skin demands more scrutiny. Further, in generalized vitiligo, the involvement of autoimmunity should vanquish all melanocytes in the skin but it is not so, why? However, it has been suggested that many external triggering factors (such as mechanical traumas) could play a crucial role in the final clinical expression of vitiligo and it is well known that vitiligo lesions are predominantly located on skin areas chronically submitted to repeated frictions and continuous pressures (119,120). Thus, the interconnections of the different hypotheses and their role in vitiligo pathogenesis are yet to be understood.

Conclusion

The pathogenesis of vitiligo, though, partially understood still remains complex and enigmatic to a greater extent. However, the presented scientific approaches in recent years have yielded some

interesting clues giving credence to both oxidative stress and autoimmune hypotheses with potential clinical relevance. Although the condition may be precipitated by multiple aetiologies, the interaction of oxidative stress with immune system clearly appears to be the key convergent pathway that initiates and/or amplifies the enigmatic loss of melanocytes. Better understanding of triggering factors for generation of autoimmunity in patients with vitiligo could pave the way towards the development of preventive/ameliorative therapies. Dissecting out this mode of skin depigmentation in vitiligo animal model/*in vitro* reconstructed epidermis [as previously reported; (38)] will be helpful in unravelling the vitiligo puzzle.

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Conflict of interests

The authors have declared no conflicting interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Intracellular ROS generation: In normal cellular process, O2 gets converted to O2.- (superoxide radicals) radicals by NAD(P) oxidase, lipoxygenase, xanthine oxidase, P450 monooxygenase.

Table S1. Antigens recognized by vitiligo autoantibodies.

