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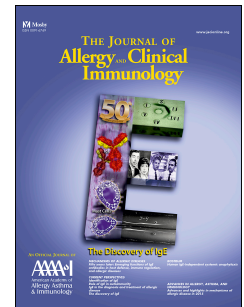
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Vitiligo: Mechanistic Insights Lead to Novel Treatments

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Abbreviations/Acronyms: Alpha-melanocyte stimulating hormone (α -MSH), damage-associated molecular patterns (DAMPs), glycogen synthase kinase- β (GSK- β), Genome wide association studies (GWAS), Herpes Simplex Virus (HSV), Janus Kinase (JAK), narrow band UVB (nbUVB), psoralen plus UVA (PUVA), reactive oxidative species (ROS), single-nucleotide polymorphisms (SNPs), Signal Transducer and Activator of Transcription (STAT), T regulatory cell (Treg), resident-memory T cells (Trm), Wingless-related integration site (WNT), vitiligo activity scoring index (VASI), vitiligo extent score (VES), vitiligo European task force measurement (VETF).

Key words:

vitiligo, treatment, T cell, IFN-g, CXCL9, CXCL10, CXCR3, resident memory, regulation, stress, innate immunity, inflammasome, NLRP1, NLRP3, melanocyte, regeneration, clinical trials, repigmentation, hair.

Abstract

Vitiligo is an autoimmune disease of the skin characterized by patchy depigmentation. Current treatments are moderately effective at reversing disease by suppressing autoimmune inflammation in the skin and promoting the regeneration of melanocytes. Recent basic and translational research studies have significantly improved our understanding of disease pathogenesis, which is now leading to emerging treatment strategies based on targeted therapy. Here we discuss important clinical characteristics of vitiligo, current therapies, their limitations, advances in understanding disease pathogenesis, emerging targeted treatments, and strategies to optimize clinical trials to efficiently and effectively test these new treatments.

Introduction

Vitiligo is an autoimmune disease of the skin that results in disfiguring white spots (**Figure 1a**), which often negatively affect patients' self-esteem and quality of life^{1,2}. Like many autoimmune diseases, complex interactions among genetic, environmental, and stochastic factors contribute to disease pathogenesis in vitiligo³. However unlike many other autoimmune diseases, current treatments are capable of reversing vitiligo by suppressing the immune system and stimulating regrowth from a natural reservoir of melanocyte stem cells. These stem cells can survive autoimmune attack due to their location within hair follicles, which are immune privileged sites similar to other organs like the brain and eye. As a result, successful repigmentation of vitiligo patient skin initially presents in a perifollicular pattern (**Figure 1b,c**). Certain anatomical sites that lack hair follicles are difficult to treat, including the hands (particularly the knuckles and fingertips), feet, other bony prominences, lips, and genitals (**Figure 1d**). In addition, lesions in which the hair is depigmented have a poor prognosis. Thus, patient expectations must be managed with this information in mind.

Existing treatments for vitiligo (**Table 1**) include topical steroids and calcineurin inhibitors, which suppress immune responses in the superficial skin where the immune infiltrate is located. These are most useful for treating disease that is localized, comprising less than 5% of the body surface area (BSA)^{4,5}. Phototherapy, initially administered as psoralen plus UVA (PUVA) but now primarily as narrow band UVB (nbUVB), is effective for more widespread disease, or disease that is active and expanding with the appearance of new lesions. Excimer laser, which emits a single wavelength of light in the nbUVB range, is also effective when treating localized disease that is unresponsive to topical therapies, or when a more rapid response is desired⁶. When highly active, vitiligo can be stabilized through oral pulse steroid therapy, which is continued until another

therapy (usually nbUVB) is therapeutic⁷. While moderately effective, current treatments for vitiligo are both financially and practically burdensome.

Over the past two decades, basic and translational research studies have identified several mechanistic pathways that promote vitiligo, which offer promising opportunities to develop new, targeted treatments. The discovery that CD8⁺ T cells are both necessary and sufficient to mediate melanocyte destruction in humans provided a foundation for ongoing investigation of T cells in vitiligo disease⁸. Likewise, early findings that melanocytes from vitiligo patients grew poorly *ex vivo* and were more susceptible to oxidative stress provided insight that melanocyte abnormalities contribute to vitiligo pathogenesis⁹⁻¹¹. Current evidence supports involvement by both CD8⁺ T cells and melanocyte stress, and thus understanding of both aspects of disease presents opportunities for therapeutic development.

IFN- γ Pathway and T Cell Recruitment in Vitiligo

CD8⁺ T cells are key effectors that drive melanocyte destruction in vitiligo, and several studies have identified critical pathways involved in the recruitment of these pathogenic cells to skin tissue. Gene expression analysis of human lesional skin revealed upregulation of IFN- γ and IFN- γ -dependent genes, including the T cell chemokine receptor CXCR3 and its multiple ligands CXCL9, CXCL10, and CXCL11¹². CXCR3⁺ cells are present within vitiligo skin biopsies, and antigen-specific T cells in vitiligo patient blood are enriched for the CXCR3 receptor¹²⁻¹⁵. Functional studies in a mouse model of vitiligo were consistent with the human observations, and then revealed that vitiligo functionally requires the IFN- γ -chemokine signaling axis. Blockade of this pathway disrupts T cell recruitment to the skin, as well as subsequent melanocyte destruction and pigment loss. Furthermore, this approach not only prevented vitiligo in mice, but was able to reverse disease as evident by perifollicular repigmentation, similar to the response seen in humans^{12,16,17}. A more

recent study found that keratinocytes are the major source of chemokine production during vitiligo, and that disrupting IFN- γ signaling only in keratinocytes mitigated disease in mice¹⁸. This suggests that targeting IFN- γ signaling in keratinocytes, which can easily be done topically, may also be an effective treatment strategy.

Similar to the mouse model, blockade of the IFN- γ pathway appears to successfully treat vitiligo in humans. IFN- γ activates its associated receptor IFNGR and a downstream intracellular JAK-STAT signaling pathway. Two case reports found that vitiligo patients receiving either of the two JAK inhibitors tofacitinib or ruxolitinib rapidly repigmented. The patient treated with ruxolitinib was found to have a stable, elevated level of CXCL10 in his serum, which rapidly decreased after treatment with the JAK inhibitor. This observation was consistent with the hypothesis that the mechanism of improvement was through inhibition of IFN- γ signaling and resulting loss of chemokine production¹⁹⁻²¹. Consistent with the key role of keratinocytes in promoting vitiligo progression in mice, a case series recently reported that topical ruxolitinib was effective in repigmenting a small number of vitiligo patients, particularly on the face²².

Based on these studies, a phase 2 clinical trial has been initiated to test the effectiveness of topical ruxolitinib for vitiligo treatment (NCT02809976). In addition to JAK inhibitors, multiple other drugs have been developed that target the IFN- γ pathway, many having passed Phase I trials with a good safety profile, and these could be tested as vitiligo treatments (**Figure 2**). We have found in our mouse model that blocking any component of the IFN- γ signaling pathway is effective at mitigating disease, and thus multiple options exist for developing novel therapeutics that target autoimmunity in vitiligo^{12,17,18,23}.

Memory T Cells in Vitiligo

As mentioned above, vitiligo lesions are reversible with treatment. However, these lesions frequently return when treatment is discontinued, with relapse occurring in 40% of patients within the first year after stopping treatment. This relapse can be significantly decreased using periodic treatment with topical calcineurin inhibitors, which strongly suggests that a memory component of autoimmunity persists long-term within lesional skin²⁴.

CD8⁺ resident-memory T cells (Trm) remain within the skin and mucosae for very long periods after viral infections to prevent reinfection, and are thought to be responsible for controlling herpes virus recurrences, including HSV-induced cold sores²⁵⁻²⁹. Trm have also been associated with inflammatory skin diseases including mycosis fungoides, psoriasis, and vitiligo^{30,31}; however, their functional contributions to maintain disease are still unclear. Cheuk et al. reported that Trm are highly enriched within vitiligo patient skin and possesses cytotoxic effector function when exposed to inflammatory cytokines, and thus may play a role in the persistence of vitiligo lesions³¹. Another group found that Trm are present in vitiligo lesions in a mouse model of melanoma and vitiligo, and may be partly responsible for mediating protection against recurrence of melanoma³². Future studies may determine that targeting Trm in vitiligo could be an effective treatment strategy, and could potentially be durable, even after discontinuing the treatment.

Lymphocyte Regulation in Vitiligo

Abnormal regulation of lymphocytes is an important component of vitiligo pathogenesis. Genome wide association studies (GWAS) have identified risk alleles related to T cell and B cell regulation and tolerance: *CTLA4*, *FASLG*, *PTPN22*, *UBASH3A*, and others^{3,33}. Polymorphisms in *FOXP3*, *BACH2*, and *IL2RA*, all important drivers of T regulatory cell (Treg) development and function, are associated with vitiligo risk, which implicates Tregs as important factors in determining vitiligo

initiation and/or progression³⁴. The incidence of vitiligo is also increased in patients with X-linked syndrome (IPEX), who lack Tregs³⁵. Autoreactive effector T cells in vitiligo patients have a dysregulated phenotype compared to healthy controls, suggesting ineffective Treg function³⁶. However, it is unclear exactly how Tregs may be dysfunctional in vitiligo patients. Studies are conflicting whether Tregs are present in normal or decreased numbers, whether there is a defect in their skin-homing potential, and whether they possess abnormal regulatory function³⁷⁻⁴¹.

Melanocyte Stress in Vitiligo

While CD8⁺ T cells mediate autoimmunity in vitiligo, the question remains why melanocytes are selectively targeted for autoimmune attack. In melanoma, T cells target tumor cells due to the immunogenicity of neoantigens produced by somatic mutations, yet no current evidence of protein modification has been found to explain T cell autoimmune attack in vitiligo⁴². However, melanocyte stress does appear to initiate autoimmune inflammation in vitiligo. Translational and clinical research studies suggest that intrinsic and/or extrinsic factors induce the cellular stress response in melanocytes, which then activates innate immunity within the skin to trigger the initial inflammation that leads to autoimmunity⁴³⁻⁴⁵. Melanocytes from non-lesional skin biopsies of vitiligo patients demonstrate a dilated endoplasmic reticulum, which represents structural evidence of cell stress⁴⁶. Both lesional and non-lesional skin of vitiligo patients also show elevated reactive oxidative species (ROS), indicating that melanocyte stress and redox aberrations pre-exist within the skin prior to autoimmune onset⁴⁷.

Intrinsic factors capable of promoting melanocyte stress include genetic risk alleles, which have been implicated through genome wide association studies (GWAS). Several non-HLA single-nucleotide polymorphisms (SNPs) associated with vitiligo lie within the melanocyte-specific enzyme *tyrosinase (TYR)*³³. While these polymorphisms have been suggested to primarily

promote presentation of autoantigen for autoimmune targeting, they may also induce stress through protein misfolding or mark abnormal enzymatic activity during melanogenesis, which has been strongly associated with promoting cellular stress through production of ROS^{48,49}. Another SNP associated with an elevated risk for developing vitiligo is *X-box binding protein 1 (XBP1)*, which may augment the cell stress response in vitiligo^{34,50}.

Several experimental vitiligo treatments have sought to reduce melanocyte stress. Aimed at compensating for diminished expression of redox enzymes within vitiligo patient skin, a topical metal ion formulation with anti-oxidative properties, termed pseudocatalase, was tested for its ability to boost the effectiveness of narrow-band UVB (NB-UVB) phototherapy^{47,51}. However, this study was unblinded and uncontrolled, and controlled studies have since failed to show a benefit of pseudocatalase treatment⁵²⁻⁵⁴. Some antioxidant dietary supplements reportedly provide a mild enhancement of NB-UVB phototherapy effectiveness^{55,56}. Pooled antioxidant supplementation with α -lipoic acid, vitamin C, vitamin E, and polyunsaturated fatty acids was found to improve the response to NB-UVB phototherapy⁵⁷. Similarly, *polypodium leucotomos* extract may quench oxidative free radicals, and has been reported to enhance the NB-UVB response⁵⁸. *Ginkgo biloba* possesses both antioxidant properties as well anti-inflammatory effects⁵⁹. This may explain its mild efficacy reported in two small clinical studies^{60,61}. Therefore, the use of antioxidant therapies has demonstrated limited efficacy, yet future studies may identify more effective therapies as the pathways that contribute to cellular stress in melanocytes are better defined.

While genetic factors clearly play a role in vitiligo risk, they do not account for all of this risk. In fact, identical twins are concordant for vitiligo only 23% of the time, strongly implicating other factors as well, such as environmental exposures that can act as extrinsic factors to influence vitiligo onset⁶². Exposure to a number of chemicals has been reported to induce and exacerbate vitiligo, including monobenzyl ether of hydroquinone, 4-tert-butylphenol, 4-tert-butylcatechol, and others⁶³. These

chemicals are similar in that they are all phenols, and likely act as analogs of the melanogenesis substrate tyrosine, also a phenol. Toxicity to these vitiligo-associated chemicals also depends on interaction with tyrosinase and other melanogenic enzymes, which increases cellular stress in the melanocyte and initiates inflammation through activation of innate immunity^{63,64}. A number of common household products have additionally been implicated in vitiligo induction, including permanent hair dyes, detergents, and others^{63,65}. Exposure to a cosmetic skin lightening cream led to the development of vitiligo in over 18,000 users in Japan in 2013, and the chemical culprit in the cream was likely to be the tyrosine analogue rhododendrol^{63,66}. Therefore, management of vitiligo includes counseling patients about these risks so they can consider avoidance of these products, and future studies to identify key chemicals in these products could lead to safer formulations.

Innate Immune Response in Vitiligo

Inflammation responsible for initiating autoimmunity in vitiligo appears to result from immune activation directly by stressed melanocytes. These cells produce damage-associated molecular patterns (DAMPs) whose secretion leads to activation of nearby dendritic cells, and their production of pro-inflammatory cytokines⁶⁷⁻⁶⁹. The DAMP most associated with vitiligo, HSP70i, is induced within perilesional and lesional vitiligo skin, and functionally worsens disease in a mouse model of vitiligo by inducing and/or recruiting activated, inflammatory dendritic cells^{68,70-72}. Furthermore, a mutant HSP70i protein with altered C-terminal domain was able to prevent disease by reducing dendritic cell activation and subsequent T cell responses. Inhibiting melanocyte-induced innate immune responses, therefore, represents another potential new therapeutic strategy⁷².

A recent study found that skin inflammation due to chemically-induced melanocyte stress in a mouse model required the innate immune receptor NLR Family Pyrin Domain Containing 3

(NLRP3), suggesting that this receptor, its associated proinflammatory inflammasome complex, or its downstream effector cytokine IL-1 β , may be possible therapeutic targets to prevent the initiation of disease or its spread⁷³. In addition to activation of NLRP3 by chemical-induced melanocyte stress, the inflammasome receptor *NLRP1* has been associated with vitiligo in genetic studies⁷⁴. Vitiligo-associated NLRP1 increases inflammasome processing of pro-IL-1 β to functional IL-1 β , which further implicates inflammasome activation and innate inflammation with vitiligo pathogenesis⁷⁵. Perilesional vitiligo skin, where disease is most active, also has elevated IL-1 β expression, suggesting that the IL-1 β pathway may contribute to vitiligo progression^{76,77}. To our knowledge anti-IL-1 β targeted therapies have not been tested in vitiligo patients.

IL-17 Pathway Cytokines in Vitiligo

TNF- α and IL-17, which are key targets for immunotherapy in psoriasis, are also mildly elevated in vitiligo lesions and have been proposed to be potential treatment targets for vitiligo⁷⁸. However it is currently unclear whether they play any functional role in vitiligo pathogenesis. Diseases mediated by IL-17 and TNF- α like psoriasis, the prototypic Th17 skin disease, typically have epidermal acanthosis, prominent neutrophil recruitment, and symptomatic inflammation with erythema and scale, features that are absent in vitiligo⁷⁹. In addition, targeted immunotherapies that disrupt TNF- α , IL-17, and other cytokines in this pathway are so far reportedly ineffective for vitiligo, and some have even initiated new-onset vitiligo in patients receiving treatment for other diseases⁸⁰⁻⁸³. Further, we found limited production of IL-17 and related cytokines in vitiligo lesions compared to IFN- γ -induced cytokines, and Trm found in psoriasis lesions produced large amounts of IL-17, while Trm in vitiligo lesions produced IFN- γ without any elevated IL-17^{12,31}. These observations suggest that TNF- α and IL-17 are not a major drivers of vitiligo pathogenesis.

Promoting Melanocyte Regeneration in Vitiligo

While inhibition of autoimmunity alone is an effective treatment strategy, promoting melanocyte regeneration may further improve treatment responses. The best example of this is the efficacy of surgical procedures that transplant melanocytes from an unaffected site to depigmented lesions in stable vitiligo. This includes older approaches like split-thickness skin grafting or punch minigrafts, as well as transfer of blister roofs from nonlesional to lesional skin sites. A newer approach is to create a single cell suspension from nonlesional donor epidermis and transfer the combined melanocyte and keratinocyte cellular mixture to lesional skin prepared through dermabrasion or laser ablation. However because active autoimmunity will quickly destroy newly transplanted melanocytes, procedural approaches are only effective when the disease is stable, typically characterized by the lack of spread of existing lesions or the appearance of new lesions within the previous 1-2 years. Segmental vitiligo, a particularly stable subtype of vitiligo characterized by unilateral depigmentation that is limited by the midline (**Figure 4**), is uniquely responsive to surgical approaches, with excellent success rates of between 58% and 84%⁸⁴⁻⁸⁶.

Alpha-melanocyte stimulating hormone (α -MSH) activates melanocyte proliferation, migration, differentiation, and function. A randomized clinical trial demonstrated that a synthetic analogue of α -MSH, afamelanotide, increased the efficacy of NB-UVB phototherapy⁸⁷. However, treatment with afamelanotide induced hyperpigmentation of non-lesional skin, causing some patients to withdraw from the study due to the enhanced contrast of vitiligo lesions. Aimed more specifically at promoting differentiation of melanocyte stem cells without causing hyperpigmentation, Regazzetti et al. reported that oxidative stress impairs melanoblast differentiation by mitigating WNT- β -catenin signaling⁸⁸. Treatment of vitiligo lesional skin explants with a synthetic WNT agonist stimulated melanocyte differentiation⁸⁸. Inhibition of glycogen synthase kinase- β (GSK- β), a negative regulator of WNT- β -catenin signaling, showed identical effects as the WNT agonist, demonstrating that

multiple targets within the WNT- β -catenin pathway could represent viable options for an adjunct vitiligo treatment⁸⁸.

Planning for Efficient and Informative Clinical Trials

Clinical trials to test some of the new, targeted therapies described above are being planned. Careful selection of patient populations will be important for designing successful trials that are able to accurately measure treatment responses. For example, subjects with vitiligo located primarily in hairless skin and those with lesions containing white hair do not have a strong capacity for repigmentation (**Figure 5**), and thus may need to be excluded from these studies, at least initially. Duration of disease has been previously reported to negatively affect prognosis to treatment⁸⁹; however, this may be indirectly through increased presence of white hairs with long-term disease. In our experience, patients with long-duration disease have repigmented well as long as their lesions contain hair with normal pigment. Subjects with the segmental variant of vitiligo are frequently excluded from clinical trials because this variant is less responsive to medical treatments, probably because of its early development of hair depigmentation. However as mentioned above, these patients are ideal for surgical treatments. Thus patients with active inflammation may represent the best population to test immunomodulators, while those with stable disease may be best for therapies that promote melanocyte regeneration.

Vitiligo is slowly responsive to treatment, with early signs of repigmentation visible clinically within 2-3 months of initiating treatment and statistically measurable improvement taking up to 6 months to observe. Outcome measures must be carefully selected, and options include the vitiligo activity scoring index (VASI), vitiligo European task force measurement (VETF), the newly developed vitiligo extent score (VES), and the developing photography-based objective scoring methods^{90,91}.

Biomarkers of disease activity can help to identify subjects with active inflammation, and may

serve as excellent early indicators of treatment responses^{12,14,92-95}. These include both serum biomarkers as well as biomarkers that can be directly measured within lesional skin, which may have better sensitivity and specificity. We recently described a minimally invasive skin sampling technique through the induction of blisters in lesional and nonlesional skin, which provides blister fluid for analysis of infiltrating immune cells and inflammatory cytokines that reflect both disease activity as well as early treatment responses¹⁵.

Conclusion

Vitiligo is a unique autoimmune disease due to the reservoir of melanocyte stem cells that provides ability for disease reversal. Although the U.S. Food and Drug Administration has not yet approved any medical treatments for repigmentation of vitiligo, existing off-label therapies are moderately effective, while basic and translational research have provided insights into vitiligo pathogenesis that offer promising targets for new drug development. Vitiligo appears to be initiated by the melanocytes themselves, which exhibit cellular stress that can be influenced by both intrinsic and extrinsic factors. Genetic risk alleles may increase endogenous stress or decrease the threshold that can be tolerated by the cells, while environmental factors like chemicals and household products may exacerbate stress pathways to initiate disease in predisposed individuals. Cytotoxic T cells are necessary and sufficient for melanocyte killing in vitiligo, and targeting their recruitment appears to be an effective therapeutic strategy (**Table 1**). Innate immune activation may provide the connection between melanocyte stress and adaptive immune responses, which could potentially be targeted as well to mitigate initial activation of autoimmunity. Clinical trials to test these emerging therapies are on the horizon, and must be carefully planned for efficient and informative outcomes. We are witnessing a revolution in our understanding of vitiligo pathogenesis, and this revolution is sure to lead to better treatments for our patients in the near future.

Figure Legends:

Figure 1. White patches of depigmentation characteristic of vitiligo (A), perifollicular repigmentation following successful treatment of disease (B,C), and difficulty repigmenting bony prominences of the knuckles relative to the dorsum of hands (D).

Figure 2. IFN- γ signals to keratinocytes and Langerhans cells, stimulating production of CXCL9 and CXCL10. CXCR3 recognizes these chemokines, leading to recruitment of CXCR3⁺ CD8⁺ T cells. Autoreactive T cells kill melanocytes and establish long-lived resident memory within the skin. Drugs that target aspects of this pathway are shown in red.

Figure 3. UV light, melanogenesis, and vitiligo-associated genetic polymorphisms (shown in red) may contribute to intrinsic melanocyte stress. Several environmental phenols also interact with melanogenic enzymes to induce stress, which then leads to secretion of damage-associated molecular patterns (DAMPs) that stimulate nearby innate immune cells through pattern recognition receptors (PRRs). Shown in red, vitiligo-associated genetic polymorphisms may impact both melanocyte stress and innate immune response. Hypothesized pathways are denoted with a "?", and significant stress from exogenous insult is denoted by thick arrows.

Figure 4. Segmental vitiligo is characterized by unilateral depigmentation that does not cross the midline.

Figure 5. Repigmentation is impaired at anatomic sites where there is little hair growth. Lack of repigmentation on the volar wrists in a patient whose entire forearms were otherwise successfully treated (A). White hairs do not support perifollicular repigmentation relative to pigmented hairs (B).

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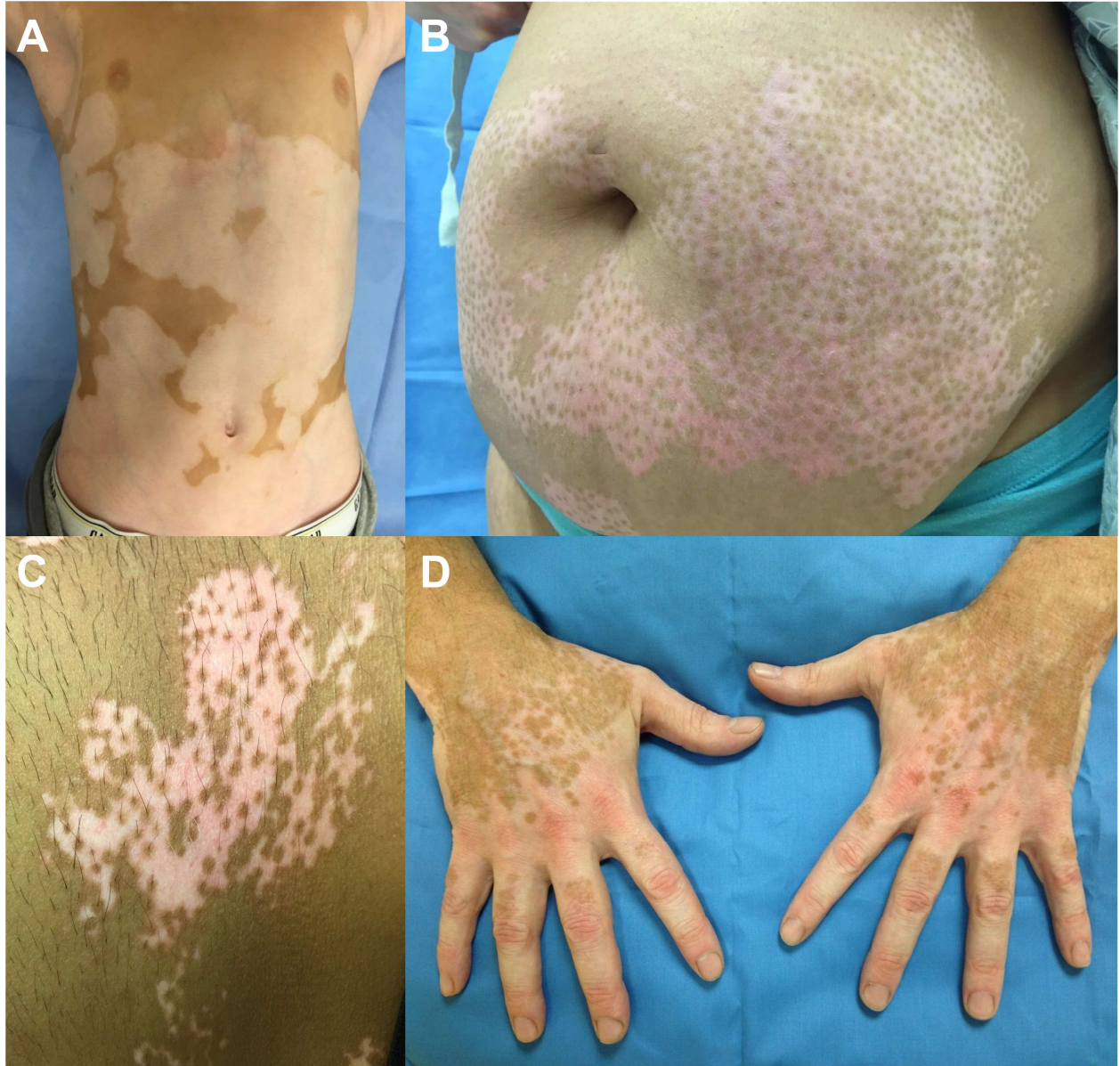
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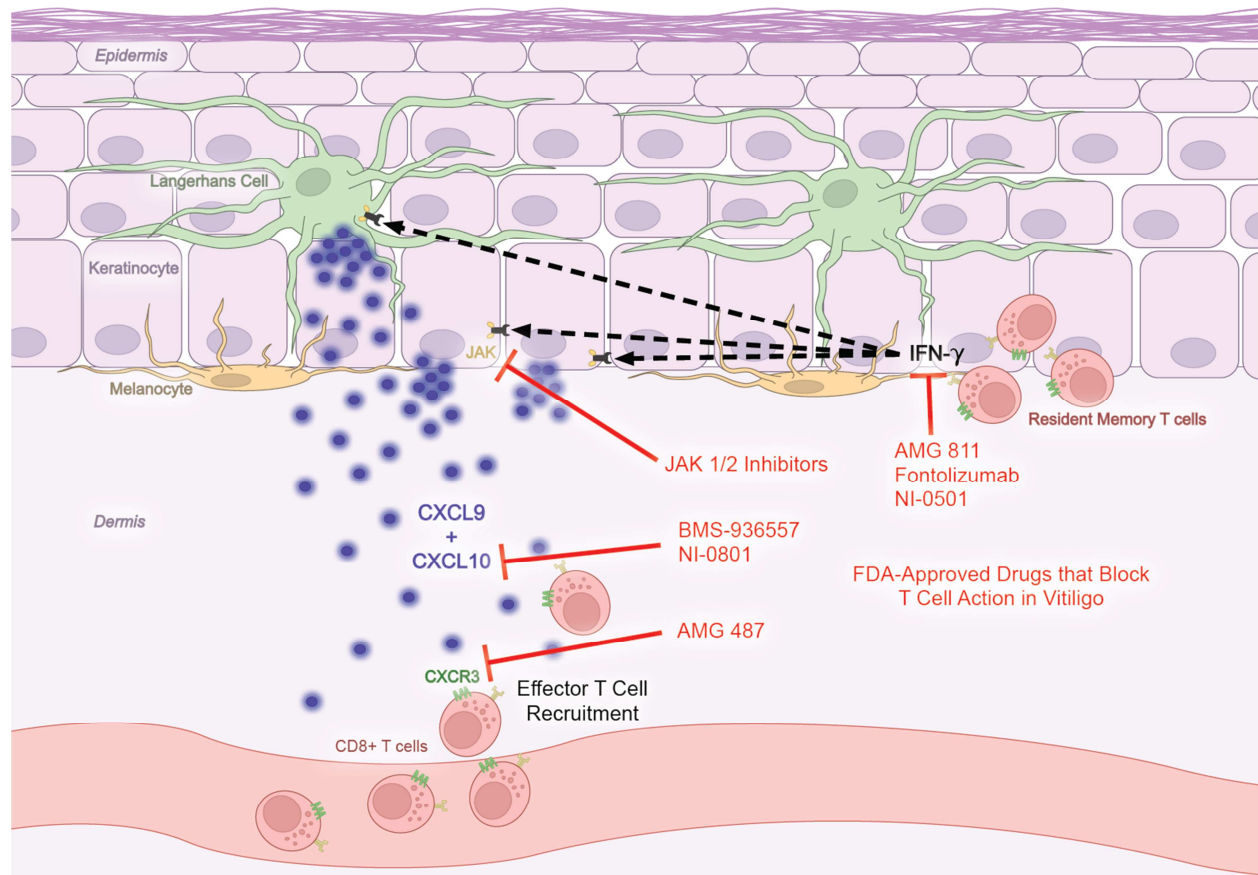
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Table1. Current and Emerging Vitiligo Treatments

<i>Current:</i>
<u>Topical Immunosuppression</u> Topical Steroids Topical Calcineurin Inhibitors
<u>Phototherapy</u> Narrow-Band UVB (nbUVB) Excimer Laser
<u>Oral Immunosuppression</u> Oral Pulse Steroids
<u>Surgical</u> Melanocyte Transplant Procedures
<i>Emerging:</i>
<u>Targeted Immunosuppression</u> IFN- γ Pathway Inhibitors
<u>Melanocyte Growth Promoters</u> Afamelanotide WNT- β -catenin Signaling Enhancers





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