IFN-γ-induced PD-L1 expression on human melanocytes is impaired in vitiligo

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Abstract
Mounting evidence shows that the PD-1/PD-L1 axis is involved in tumor immune evasion. This is demonstrated by anti-PD-1 antibodies that can reverse tumor-associated PD-L1 to functionally suppress anti-tumor T-cell responses. Since type I and II interferons are key regulators of PD-L1 expression in melanoma cells and IFN-γ-producing CD8+ T cells and IFN-α-producing dendritic cells are abundant in vitiligo skin, we aimed to study the role of PD-1/PD-L1 signalling in melanocyte destruction in vitiligo. Moreover, impaired PD-1/PD-L1 function is observed in a variety of autoimmune diseases. It is, therefore, hypothesized that manipulating PD-1/PD-L1 signalling might have therapeutic potential in vitiligo. The PD-1+ T cells were abundantly present in situ in perilesional vitiligo skin, but expression of PD-L1 was limited and confined exclusively to dermal T cells. More specifically, neither melanocytes nor other epidermal skin cells expressed PD-L1. Exposure to IFN-γ, but also type I interferons, increased PD-L1 expression in primary melanocytes and fibroblasts, derived from healthy donors. Primary human keratinocytes only showed increased PD-L1 expression upon stimulation with IFN-γ. More interestingly, melanocytes derived from non-lesional vitiligo skin showed no PD-L1 upregulation upon IFN-γ exposure, while other skin cells displayed significant PD-L1 expression after exposure. In a vitiligo skin explant model, incubation of non-lesional vitiligo skin with activated (IFN-γ-producing) T cells from vitiligo lesions was previously described to induce melanocyte apoptosis. Although PD-L1 expression was induced in epidermal cells in these explants, this induction was completely absent in melanocytes. The lack of PD-L1 upregulation by melanocytes in the presence of IFN-γ-producing T cells shows that melanocytes lack protection against T-cell attack during vitiligo pathogenesis. Manipulating PD-1/PD-L1 signalling may, therefore, be a therapeutic option for vitiligo patients.

KEYWORDS
autoimmunity, B7-H1 antigen, immune tolerance, programmed cell death 1 receptor, vitiligo
1 | INTRODUCTION

Vitiligo is a cutaneous pigment disorder characterized by white skin patches due to loss of melanocytes, the pigment-producing cells of the skin. A research study has clarified immune-mediated melanocyte destruction in vitiligo-affected skin, caused by autoreactive melanocyte-specific CD8+ T cells. Moreover, antibody responses against melanocyte antigens, such as tyrosinase and TRP-2, can be found in the sera of patients with vitiligo. In addition, an imbalance of CD4+/CD8+ T-cell ratio and regulatory T cells might contribute to T-cell-mediated pathogenesis of vitiligo. Finally, T cells with a resident phenotype (tissue-resident memory T (T_RM) cells) contribute to disease development and flare-up in human vitiligo.

Immune checkpoints, for example programmed cell death 1 (PD-1), modulate immune responses by regulating peripheral tolerance, thereby preventing unwanted inflammatory responses and autoimmunity. Immunotherapy for melanoma shows that anti-PD-1 antibodies can break peripheral tolerance and thereby reactivate anti-tumor responses in patients. Moreover, 2–43% of patients with melanoma treated with immunotherapy, vitiligo-like depigmentation occurs, which indicates the presence of immune reactivity against both melanoma cells and melanocytes. Vitiligo development in patients with melanoma has been associated with a favourable prognosis. As vitiligo can also develop after treatment with anti-PD-1 antibodies, this suggests the involvement of PD-1 and its ligand programmed cell death ligand 1 (PD-L1) in the regulation of tolerance at the level of melanocytes.

Therapeutically targeting PD-1 or PD-L1 seems a promising treatment strategy for human vitiligo, impaired PD-1/PD-L1 function is involved in a variety of autoimmune diseases, for example type I diabetes and rheumatoid arthritis, indicating the rationale to test the therapeutic potential of increasing PD-1/PD-L1 signalling in autoimmune. Considering that IFN-γ and type I interferons play a crucial role in vitiligo pathogenesis and both are known to induce immune evasion by PD-L1 expression in melanoma, the question arises to what extent PD-L1 upregulation and resulting immune regulation occur in vitiligo.

To our knowledge, in situ PD-1 expression and (cytokine-induced) PD-L1 expression in vitiligo remain incompletely studied. This study, therefore, aimed to study in situ PD-1/PD-L1 expression in human vitiligo specimens. Second, we aimed to investigate the role of PD-1/PD-L1 checkpoint signalling in melanocyte destruction in vitiligo and how this is influenced by interferons. Our results show lack of (IFN-γ-induced) PD-L1 expression by melanocytes from vitiligo patients, in contrast to healthy donor melanocytes, and point to an insufficient ability of melanocytes of vitiligo patients to protect themselves against autoreactive T cells.

2 | METHODS

2.1 | Patient samples

This study was conducted in accordance with the Declaration of Helsinki. All patients were informed and signed written informed consent approved by the Institutional Medical Ethics Review Committee (NL 64983.018.18). Biopsies were obtained from patients with non-segmental vitiligo (n = 22) aged ≤18 years old of the Netherlands Institute for Pigment Disorders at the Amsterdam University Medical Centers. Two or four millimetre punch biopsies were collected from non-lesional and perilesional vitiligo skin. Perilesional skin biopsies were taken from skin flanking the depigmented macule. Within this area, the inflammation of vitiligo is visible. Vitiligo activity was scored according to the vitiligo disease activity (VIDA) score. According to this score, we defined active vitiligo as progression or depigmentation within 6 months prior to inclusion (score +4 to +2). Vitiligo patients that score a VIDA score of 0 or −1 were considered stable. Skin biopsies from active vitiligo patients were used for immunohistochemistry and skin explant assays, whereas samples from stable vitiligo patients were used for cytokine exposure and FACS analyses. Healthy donor skin (n = 8) was obtained as discarded tissue after plastic surgery of the breast or abdomen. The Institutional Medical Ethics Review Committee granted a waiver for the anonymous use of human leftover material.

The demographic characteristics of patients with non-segmental vitiligo are represented in Table 1.

| Characteristics | Vitiligo |
|-----------------|---------|
| Subjects        | 22      |
| Gender          |         |
| Male            | 9       |
| Female          | 13      |
| Age (mean ±SEM) | 43.1 ± 2.9 |
| Disease activity|         |
| Active          | 16      |
| Stable          | 6       |

Abbreviation: SEM, standard error of mean.
PD-L1 (clone MIH1, eBioscience) and anti-human Melan-A (clone A19-P, Biorbyt Ltd). Antigen-antibody binding was visualized using PermaRed/AP (Diagnostic Biosystems), Vector® NovaRED, or Vector® Blue (both Vector Laboratories, Inc.) chromogen. Sections were counterstained with haematoxylin (Klinipath) and mounted for review. Sections of tonsil specimens with confirmed high expression of the target molecules served as positive control.

2.3 | Quantification of immunohistochemical staining analyses of tissue sections

Assessing the presence and multitude of marker-expressing cells was performed by two observers independently (M. Willemsen and G. Krebbers) using high-power field microscopy and comprised the analysis of the entire skin biopsy per section. Subsequently, the number of positive cells per mm² was determined for each staining. Images (magnification x400) were acquired on a Leica DM microscope using Leica software (Leica Biosystems).

2.4 | Tissue preparation

Skin biopsies of discarded breast and abdomen were dissociated into single-cell suspensions by enzymatic degradation using the Whole Skin Dissociation Kit and mechanical dissociation using the gentleMACS™ Dissociator (both from Miltenyi Biotec). Thereafter, cells were collected by filtering through a 70 µm cell strainer. Alternatively, skin biopsies were incubated in 50 µL dispase (Sigma-Aldrich) at 4°C overnight. The next day, epidermis and dermis were separated with forceps. Epidermal skin was fragmented and incubated in 0.05% trypsin/EDTA (Thermo Fisher Scientific) for 5 min at 37°C. Trypsin was neutralized by FCS, and cells were collected by filtering through a 70 µm cell strainer.

2.5 | Cell culture

Primary melanocytes were cultured in Medium 254 supplemented with 1% human melanocyte growth supplement, 50 U/mL penicillin and 50 µg/mL streptomycin (all from Thermo Fisher Scientific). Primary fibroblast cultures were cultured in DMEM supplemented with 2.5% FCS (both Thermo Fisher Scientific), 50 U/mL penicillin and 50 µg/mL streptomycin. The human keratinocyte cell line HaCat was cultured in IMDM (Thermo Fisher Scientific) supplemented with 8% FCS, 2 mM L-glutamine (Thermo Fisher Scientific), 50 U/mL penicillin and 50 µg/mL streptomycin. The human keratinocyte cell line HaCat was cultured in IMDM (Thermo Fisher Scientific) supplemented with 8% FCS, 2 mM L-glutamine (Thermo Fisher Scientific), 50 U/mL penicillin and 50 µg/mL streptomycin. Epidermal cell suspensions were plated in keratinocyte SFM medium (Thermo Fisher Scientific) supplemented with bovine pituitary extract (Thermo Fisher Scientific), 50 U/mL penicillin and 50 µg/mL streptomycin to obtain primary keratinocyte cultures. Additionally, primary healthy keratinocytes were kindly provided by the S. Gibbs Lab.

2.6 | Cytokine exposure

One day prior to exposure, cells were seeded in a 6-well plate at a density of 2 × 10⁵ cells per well in a total volume of 2 mL medium with supplements and culture conditions as stated above. After 24 h, cells were left unexposed or exposed for 48 h with 500 U/mL IFN-γ (R&D Systems), IFN-α1b, IFN-α2a, IFN-α2b or IFN-β1a (all 1000 U/mL, and all from ImmunoTools). After 48 h, cells were harvested for quantitative PCR and flow cytometry analysis.

2.7 | RNA isolation, cDNA synthesis and quantitative PCR

Cells were detached by scraping in cold PBS on ice. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following the protocol of the manufacturer. RNA was transcribed into cDNA according to the instructions of the manufacturer using the Promega AMV reverse transcriptase kit (Promega). An additional on-column DNase treatment was performed using the RNase-Free DNase (Qiagen). Gene expression levels of PD-L1 were measured by using quantitative PCR (qPCR) performed on a Bio-Rad CFX Connect (Bio-Rad Laboratories, Inc.). The following oligonucleotide sequences were used: β-actin forward 5'-GATCGGCGGCCTCACTCTG, reverse 5'-GACTCGTCACTCCTGCTTGC, HSRPS18 forward 5'-AGTTCCAGCATATTGCGAG, reverse 5'-CTCTTGGTGAAGT CAATGTC and PD-L1 forward 5'-GAAGTACGATACGAGGCTG, reverse 5'- TACCACAGACTTGATGG (all from Biologio Lab Equipment B.V.). Expression levels were calculated according to the 2ΔΔCT method and normalized to the reference genes (β-actin and HSRPS18).

2.8 | Flow cytometry

To detect the effect of cytokine exposure, cells were stained for flow cytometry analysis. The following antibodies were used: APC-conjugated mouse anti-human PD-L1 (clone MIH1, Thermo Fisher Scientific), PE-conjugated mouse anti-human CD119 (clone GIR-208, BioLegend) and FITC-conjugated mouse anti-human Melan-A (clone A103). Cell surface staining was performed in FACS buffer (PBS supplemented with 1% bovine serum albumin and 0.05% NaN₃). Subsequently, cells were fixed in Fixation Buffer and stained intracellularly in Perm/Wash Buffer (both from BioLegend), according to the instructions of the manufacturer. Antibody binding was analysed on a FACS Canto II, and data were analysed using FlowJo software (both from BD Biosciences).

2.9 | Skin explant assay

Cryosections of the previously described skin explant assays were used. In short, perilesional skin biopsies, flanking the vitiligo...
lesion, were cultured in 24-well plates in 1 mL IMDM supplemented with 10% heat-inactivated human AB serum (Cambrex), 20 U/mL interleukin-2 (EuroCetus), 5 ng/mL interleukin-15 (Strathmann Biotec GmbH & CO., KG), 15 μg/mL gentamycin (Duchefa), 2 mM L-glutamine, 50 U/mL penicillin and 50 μg/mL streptomycin and 50 mM 2-mercaptoethanol (Sigma-Aldrich) in a humidified atmosphere at 37°C and 5% CO₂. To promote T-cell outgrowth, 1.25 μL/mL anti-CD3/CD28 mAb-coated T-cell expander beads (Thermo Fisher Scientific) were added to the culture. CD8⁺ T-cell-enriched perilesional T-cell populations were prepared using anti-human CD8 mAb microbeads and magnetic cell separation columns (both from Miltenyi Biotec), according to the instructions of the manufacturer. Next, non-lesional skin biopsies were co-cultured in a 96-well round-bottom plate in the absence or presence of 3–5 × 10⁵ autologous perilesional CD8⁺ T cells for 48 h in 200 μL per well IMDM with supplements and culture conditions as stated above. Subsequently, explants were washed three times in PBS and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe) for further immunohistochemical analysis on cryosections of the explants.

2.10 Gene expression analysis

The R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) was used for analysis of gene expression profiles of lesional and non-lesional skin biopsies from stable non-segmental vitiligo patients from Singh et al. (2017)(GSE75819).²²

2.11 Statistical analysis

Statistical analysis was performed using Graphpad Prism software (Graphpad Software Inc.). Comparisons were made with ANOVA analysis or Student’s t-test. Correlations were performed using linear regression analysis. p-values < 0.05 were considered statistically significant; *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

3 RESULTS

3.1 Significant PD-1⁺ T cells but limited PD-L1⁺ cells in perilesional vitiligo skin biopsies

To explore the role of PD-1/PD-L1 in human vitiligo, we studied in situ PD-1 and PD-L1 expression in perilesional human vitiligo skin (see patient characteristics in Table 1). CD3⁺ cells were observed in all perilesional vitiligo skin biopsies, both dermal and epidermal (Table 2, Figure 1A). Similarly, PD-1⁺ cells were present in 13/13 (100%) perilesional vitiligo skin biopsies and the vast majority (75%) of PD-1⁺ cells co-localized with CD3⁺ cells, indicating the presence of PD-1-expressing T cells (Table 2, Figure 1B–C). Although PD-1⁺ cells were abundantly present in vitiligo skin, expression of the ligand PD-L1 was largely absent in perilesional vitiligo skin (Table 2, Figure 1D). Epidermal cells were PD-L1⁻ in all vitiligo skin specimens, whereas PD-L1 was only expressed on dermal cells in 46% (6/13) of the patients (Table 2, Figure 1D). If PD-L1⁺ cells were observed, even though very few cells did, 78% of PD-L1-expressing cells co-localized with T-cell infiltrates (Table 2, Figure 1E). Most importantly, PD-L1 expression could not be detected on epidermal melanocytes in perilesional skin biopsies (Table 2, Figure 1D), indicating that neither in situ melanocytes nor other epidermal skin cells of vitiligo patients expressed PD-L1, while PD-1-expressing T cells were present.

| TABLE 2 | PD-1 and PD-L1 expression in perilesional vitiligo skin |
|-----------------|-----------------|-----------------|
| Number of patients/total (%) | + | - |
| CD3 | Epidermis | 12/14 (86) | 2/14 (14) |
| | Dermis | 14/14 (100) | 0/14 (0) |
| PD-1 | Epidermis | 12/13 (92) | 1/13 (8) |
| | Dermis | 13/13 (100) | 0/13 (0) |
| PD-1 + CD3 | Epidermis | 11/11 (100) | 0/11 (0) |
| | Dermis | 12/12 (100) | 0/12 (0) |
| PD-L1 | Epidermis | 0/13 (0) | 13/13 (100) |
| | Dermis | 6/13 (46) | 7/13 (54) |
| PD-L1 + CD3 | Epidermis | 0/5 (0) | 5/5 (100) |
| | Dermis | 5/5 (100) | 0/5 (0) |

Figure 1D). Since in situ PD-L1 expression was absent in perilesional vitiligo skin and PD-1⁺ T cells were present, we investigated to what extent PD-L1 expression on human skin cells can be influenced by cytokines present in the skin micro-environment. It is known that CD8⁺ T cells in (peri)lesional vitiligo skin produce high levels of IFN-γ⁰²¹ and that IFN-γ can induce PD-L1 expression in melanoma.⁰¹ Similarly, IFN-α-producing plasmacytoid dendritic cells infiltrate active vitiligo skin,¹⁶,¹⁷ and although PD-L1 expression is primarily upregulated by IFN-γ in melanoma, stimulation with type I interferons also led to increased PD-L1 expression.⁰⁵ Exposure to IFN-γ, but also type I interferons (including IFN-α1b, IFN-α2a/b and IFN-β1a), increased constitutive PD-L1 mRNA expression on primary human melanocytes, derived from healthy donors (Figure 2A). Concomitantly, PD-L1 protein expression was significantly induced upon cytokine exposure (Figure 2A). Similarly, in vitro cytokine exposure led to PD-L1 induction on primary human fibroblasts on both mRNA and protein levels (Figure 2B). The human keratinocyte cell line, HaCat, and primary human keratinocytes showed increased PD-L1 protein expression only after stimulation with IFN-γ but not after stimulation with type 1 interferons (Figure 2C–D).

Likewise, PD-L1 expression was induced upon exposure to type I and II interferons in melanoma cell lines, and blood- or skin-derived dendritic cells (Figure S1–S2). These data show that pro-inflammatory
**FIGURE 1** Significant PD-1⁺ T cells but limited PD-L1⁺ cells in perilesional vitiligo skin biopsies. (A–E) Immunohistochemistry and positive cells/mm² in whole skin, epidermal and dermal skin of CD3 (A), PD-1 (B) and PD-L1 (D) in perilesional biopsies of vitiligo patients. Immunohistochemistry of PD-1 (orange) and CD3 (blue) (C) and PD-L1 (orange) and CD3 (blue) (E) in perilesional biopsies of vitiligo patients. The percentage of CD3⁺ cells per PD-1⁺ (C) and PD-L1⁺ cells are indicated in the right graphs. Evident double-positive cells are indicated by the arrowheads (C, E). Representative immunohistochemical staining is shown. Bars equal 100 µm
IFN-γ and type I interferons can induce PD-L1 upregulation in healthy donor (skin) cells.

3.3 IFN-γ exposure does not induce PD-L1 expression on primary vitiligo melanocytes

As shown, PD-L1 can be upregulated in various cell types in healthy skin, including melanocytes, upon exposure to inflammatory IFN-γ (Figure 2). We explored IFNG mRNA levels in a RNAseq dataset of Singh et al. (2017). This dataset contains bulk RNAseq data of lesional and non-lesional skin biopsies from 15 stable non-segmental vitiligo patients. IFNG levels were significantly increased in depigmented, lesional, non-segmental vitiligo skin compared to non-lesional, pigmented skin (Figure 3A). Also, IFNG positively correlates with PD-L1 expression in lesional vitiligo skin (Figure 3A), and thus, PD-L1 upregulation would be expected. IFNA levels were not significantly different between lesional and non-lesional skin in this dataset (data not shown). We, therefore, studied whether IFN-γ could influence the expression of PD-L1 on melanocytes from vitiligo patients. As it is time-consuming and hard to culture primary melanocytes for in vitro experiments, PIG3V cells, a cell line that originates from non-lesional melanocytes of a patient with non-segmental vitiligo, are often used to study vitiligo melanocytes. Unfortunately, PIG3V cells and PIG1 cells, which originate from healthy melanocytes, were not suitable for use, because of HPV E7-induced PD-L1 expression. Therefore, we chose primary cell suspensions as they most closely resemble melanocytes in vivo. Exposure of skin cell suspensions from healthy donors to IFN-γ led to increased PD-L1 expression on both melanocytes (Melan-A+) and the Melan-A−, other skin cells in the suspension (Figure 3B). In vitiligo, PD-L1 expression was induced on Melan-A+ other skin cells, similar to skin cells derived from healthy donors (Figure 3B). Most interestingly, exposure to IFN-γ did not induce expression of PD-L1 on melanocytes from non-lesional skin of vitiligo patients (Figure 3B), despite comparable CD119 (IFN-γ receptor) expression in comparison with healthy melanocytes (Figure 3C), indicating that specifically vitiligo melanocytes, but not other skin cells, do not upregulate PD-L1 upon IFN-γ exposure.

As IFN-γ-producing CD8+ T cells are abundantly present in vitiligo-affected skin, in situ cytokine-induced PD-L1 expression was analysed in an autologous skin explant model. In this model, non-lesional skin biopsies were co-cultured with medium alone or with autologous perilesional-derived CD8+ T cells. Our previous research study has shown that CD8+ T cells, obtained from vitiligo perilesional skin, infiltrate autologous and non-lesional skin biopsies, mediate melanocyte destruction. This model is, therefore, suitable for in vivo IFN-γ production and melanocyte killing. In non-lesional skin biopsies cultured in medium only, few cells expressed PD-L1, mostly in dermal infiltrates (Figure 4A). In contrast, PD-L1 was massively upregulated in the epidermis of non-lesional skin biopsies cultured in the presence of perilesional CD8+ cells (Figure 4A). Expression was not restricted to lymphocytic infiltrates but seen on the majority of epidermal cells, except for melanocytes (Figure 4B). These ex vivo in situ data show that during T-cell-mediated attack of melanocytes in non-lesional vitiligo skin, no upregulation of PD-L1 occurs in melanocytes, in contrast to the other skin cells. Melanocytes from vitiligo patients thereby differ from healthy donors in their susceptibility to melanocyte-reactive T-cell immunity.

4 DISCUSSION

This study reveals important insights into PD-1/PD-L1 signalling in human vitiligo. We show that while PD-1+ T cells were abundantly present in vitiligo skin, neither melanocytes nor other epidermal skin cells expressed its ligand PD-L1. Moreover, in contrast to healthy melanocytes, fibroblasts, keratinocytes and dendritic cells, vitiligo melanocytes did not upregulate PD-L1 expression upon exposure to IFN-γ or in the presence of IFN-γ-producing activated T cells. Vitiligo melanocytes thereby seem incapable of inhibiting attack by autoreactive T cells through the PD-1/PD-L1 pathway. These data indicate that inherent differences in PD-L1 regulation in melanocytes from vitiligo patients as compared to healthy donors explain their selective susceptibility to melanocyte-reactive T-cell attack.

Previous studies have shown the presence of PD-1-expressing cells in blood and skin of patients with vitiligo. Both (regulatory) CD4+ and CD8+ T cells expressing PD-1 were more abundant in blood of vitiligo patients than in healthy donors, whereas the percentage of regulatory T cells was significantly decreased in vitiligo patients. Likewise, PD-1+ cells were significantly increased in lesional and perilesional skin compared to normal, pigmented skin of patients with vitiligo and this correlated positively with CTLA-4-expressing cells. A recent study demonstrated an impaired regulatory T-cell-suppressive function in blood of patients with vitiligo, and therefore, it can be postulated that perilesional PD-1-expressing cells are mainly CD4+ T cells, which have lost their suppressive function. Nevertheless, considering the reduction in regulatory T cells and abundance of melanocyte-specific CD8+ resident memory T (T RM) cells in depigmented vitiligo skin, which have been shown to express PD-1, it can be postulated that PD-1+ cells in vitiligo skin are mainly melanocyte-reactive CD8+ T RM cells. Although
FIGURE 3  PD-L1 expression by vitiligo melanocytes remains unchanged after IFN-γ exposure. (A) Bar plot showing 2log of IFNG mRNA expression in lesional and non-lesional skin biopsies (left) and correlation analyses of 2log of IFNG mRNA expression and 2log of PD-L1 mRNA expression in lesional skin biopsies (right) from stable non-segmental vitiligo patients (n = 15). (B) Fold induction of PD-L1 expression and histograms of Melan-A⁺ (left) and Melan-A⁻ (right) cells of healthy control (n = 8) and non-lesional vitiligo skin (n = 6) after no exposure or exposure to IFN-γ (500 U/mL). (C) Mean MFI CD119 expression of Melan-A⁺ cells of healthy control (n = 8) and non-lesional vitiligo skin (n = 6). Error bars: mean ±SEM. Student’s t-test shows significance as indicated: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001. Correlations were performed using linear regression analysis.
PD-1 signalling may involve low cytotoxic potential of skin T RM cells, others have demonstrated that PD-1-expressing T RM cells remained reactive and capable of providing immune protection or mediating immune pathology. Consistent with this, treatment with PD-L1 fusion protein repressed the numbers of melanocyte-reactive T cells and increased regulatory T-cell numbers in a Pmel-1 T-cell receptor transgenic vitiligo mouse model. PD-L1 fusion protein treatment specifically repressed the activation of Vp12-expressing T cells, as measured by IFN-γ production, and reversed depigmentation in Pmel-1 mice. Although the main PD-1-expressing T-cell subset in vitiligo remains indistinct, targeting PD-1/PD-L1 signalling will either increase regulatory CD4+ T-cell numbers and function or repress cytotoxic CD8+ T-cell activity, both positively affecting peripheral tolerance.

This study demonstrates the absence of PD-L1 expression in perilesional vitiligo skin and impaired induction of PD-L1 expression upon IFN-γ exposure in primary melanocytes derived from vitiligo patients. To date, gene expression profiling has not revealed differentially expressed genes between vitiligo patients and healthy individuals that are specifically involved in IFN-γ receptor signalling pathways regulating PD-L1 expression. Also, there are no scRNA-seq data on vitiligo melanocytes and other skin cells available yet. Since PD-L1 is upregulated upon IFN-γ exposure in vitiligo skin cells, this impaired response seems to affect melanocytes only and will, therefore, probably not be detected in skin transcriptome analysis of whole skin biopsies but in scRNAseq analyses of melanocytes only. Together, this suggests a role for PD-L1 in the lack of peripheral tolerance in vitiligo, providing a rationale to target this axis in vitiligo treatment.

In conclusion, this study shows the absence of (IFN-γ-induced) PD-L1 expression by melanocytes of vitiligo patients and thereby reveals a potential intrinsic melanocyte defect in vitiligo. This study also highlights the presence of PD-1-expressing T cells in vitiligo skin, which strengthen the notion of manipulating PD-1/PD-L1 signalling to induce peripheral tolerance to melanocytes in vitiligo.

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CONFLICT OF INTEREST
CJMM was employed by ISA Pharmaceuticals. The authors declare that the research study was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTION
MW, GK, EPMT, KJW, AL and VAL conducted all experiments, immunohistochemical analysis and data analysis. MW prepared figures and drafted and prepared the manuscript. NFP and VSN provided patients for this study. MW, WJB, CJMM, MB and RML designed experiments and participated in manuscript revision. All authors have read and approved the final manuscript. RML supervised the project.
REFERENCES

1. Bergqvist C, Ezzedine K. Vitiligo: a review. Dermatology. 2020;236(6):571-592. doi:10.1159/0005060103

2. Frisoli ML, Essien K, Harris JE. Vitiligo: mechanisms of pathogenesis and treatment. Annu Rev Immunol. 2020;38:621-648. doi:10.1146/annurev-immunol-100919-023531

3. Cheuk S, Schlums H, Gallais Serezal I, et al. CD49a expression defines tissue-resident CD8(+) T cells poised for cytotoxic function in human skin. Immunity. 2017;46(2):287-300. doi:10.1016/j.immuni.2017.01.009

4. Dwivedi M, Laddha NC, Arora P, Marfatia YS, Begum R. Decreased regulatory T-cells and CD4(+)/CD8(+) ratio correlate with disease onset and progression in patients with generalized vitiligo. Pigment Cell Melanoma Res. 2013;26(4):586-591. doi:10.1111/jcm.12105

5. Cheuk S, Schlums H, Gallais Serezal I, et al. CD49a expression defines tissue-resident CD8(+) T cells poised for cytotoxic function in human skin. Immunity. 2017;46(2):287-300. doi:10.1016/j.immuni.2017.01.009

6. Boniface K, Jacquemin C, Darrigade AS, et al. Vitiligo skin is imprinted with resident memory CD8 T cells expressing CXCR3. J Invest Dermatol. 2018;138(2):355-364. doi:10.1016/j.jid.2017.08.038

7. Okazaki T, Honjo T. The PD-1/PD-L pathway in immunological tolerance. Trends Immunol. 2006;27(4):195-201. doi:10.1016/j.it.2006.02.001

8. Luke JJ, Flaherty KT, Ribas A, Long GV. Targeted agents and immunotherapies: optimizing outcomes in melanoma. Nat Rev Clin Oncol. 2017;14(8):463-482. doi:10.1038/nrclinonc.2017.43

9. Teulings HE, Limpens J, Jansen SN, et al. Vitiligo-like depigmentation in human skin. Autoimmun Rev. 2007;6(3):138-142. doi:10.1016/j.autrev.2006.09.010

10. Dwivedi M, Laddha NC, Arora P, Marfatia YS, Begum R. Decreased regulatory T-cells and CD4(+)/CD8(+) ratio correlate with disease onset and progression in patients with generalized vitiligo. Pigment Cell Melanoma Res. 2013;26(4):586-591. doi:10.1111/jcm.12105

11. Boniface K, Jacquemin C, Darrigade AS, et al. Vitiligo skin is imprinted with resident memory CD8 T cells expressing CXCR3. J Invest Dermatol. 2018;138(2):355-364. doi:10.1016/j.jid.2017.08.038

12. Okazaki T, Honjo T. The PD-1/PD-L pathway in immunological tolerance. Trends Immunol. 2006;27(4):195-201. doi:10.1016/j.it.2006.02.001

13. Luke JJ, Flaherty KT, Ribas A, Long GV. Targeted agents and immunotherapies: optimizing outcomes in melanoma. Nat Rev Clin Oncol. 2017;14(8):463-482. doi:10.1038/nrclinonc.2017.43

14. Teulings HE, Limpens J, Jansen SN, et al. Vitiligo-like depigmentation in patients with stage III-IV melanoma receiving immunotherapy and its association with survival: a systematic review and meta-analysis. J Clin Oncol. 2015;33(7):773-781. doi:10.1200/JCO.2014.574756

15. Quaglini P, Marenco F, Osella-Abate S, et al. Vitiligo is an independent favourable prognostic factor in stage III and IV metastatic melanoma patients: results from a single-institution hospital-based observational cohort study. Ann Oncol. 2010;21(2):409-414. doi:10.1093/annonc/mdn325

16. Boasberg PD, Hoon DS, Piro LD, et al. Enhanced survival associated with vitiligo expression during maintenance biotherapy for metastatic melanoma. J Invest Dermatol. 2006;126(12):2658-2663. doi:10.1038/sj.jid.5507045

17. Hua C, Boussemart L, Mateus C, et al. Association of vitiligo with tumor response in patients with metastatic melanoma treated with pembrolizumab. JAMA Dermatol. 2016;152(1):45-51. doi:10.1001/jamadermatol.2015.2707

18. Phan QQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci USA. 2003;100(14):8372-8377. doi:10.1073/pnas.1533209100

19. WillemSEN M, Melief CJM, Bekkenk MW, Luiten RM. Targeting the PD-1/PD-L1 axis in human vitiligo. Front Immunol. 2020;11:579022. doi:10.3389/fimmu.2020.579022

20. Blank C, Kuball J, Voelkl S, et al. Blockade of PD-L1 (B7–H1) augments human tumor-specific T cell responses in vitro. Int J Cancer. 2006;119(2):317-327. doi:10.1002/ijc.21775

21. Garcia-Diaz A, Shin DS, Moreno BH, et al. Interferon receptor signaling pathways regulating PD-L1 and PD-L2 expression. Cell Rep. 2017;19(6):1189-1201. doi:10.1016/j.celrep.2017.04.031

22. Singh A, Gotherwal V, Junni P, et al. Mapping architectural and transcriptional alterations in non-lesional and lesional epidermis in vitiligo. Sci Rep. 2017;7(1):9860. doi:10.1038/s41598-017-10253-w

23. Liu C, Lu J, Tian H, et al. Increased expression of PD-L1 by the human papillomavirus 16 E7 oncoprotein inhibits antitumor immunity. Mol Med Rep. 2017;15(3):1063-1070. doi:10.3892/mmr.2016.6073

24. Tembhere MK, Parihar AS, Sharma VK, Sharma A, Chattopadhyay P, Gupta S. Alteration in regulatory T cells and programmed cell death 1-expressing regulatory T cells in active generalized vitiligo and their clinical correlation. Br J Dermatol. 2015;172(4):940-950. doi:10.1111/bjd.13511

25. Rahimi A, Hossein-Nataj H, Hajheydari Z, et al. Expression analysis of PD-1 and Tim-3 immune checkpoint receptors in patients with vitiligo: positive association with disease activity. Exp Dermatol. 2019;28(6):674-681. doi:10.1111/exd.13952

26. Awad SS, Touni AA, Gabril MY. Expression of immune checkpoint points in active nonsegmental vitiligo: a pilot study. Int J Dermatol. 2020;59(8):982-988. doi:10.1111/jid.14983

27. Giri PS, Dwivedi M, Begum R. Decreased suppression of CD8(+) and CD4(+) T cells by peripheral regulatory T cells in generalized vitiligo due to reduced NFATC1 and FOXP3 proteins. Exp Dermatol. 2020;29(8):759-775. doi:10.1111/exd.14157

28. Klarquist J, Denman CJ, Hernandez C, et al. Reduced skin homing by functional Treg in vitiligo. Pigment Cell Melanoma Res. 2010;23(2):276-286. doi:10.1111/j.1755-148X.2010.00688.x

29. Seidel JA, Vukmanovic-Stejic M, Muller-Durovic B, et al. Skin resident memory CD8(+) T cells are phenotypically and functionally distinct from circulating populations and lack immediate toxic function. Clin Exp Immunol. 2018;194(1):79-92. doi:10.1111/cei.13189

30. Park SL, Buzzai A, Rautela J, et al. Tissue-resident memory CD8(+) T cells promote melanoma-immune equilibrium in skin. Nature. 2019;565(7739):366-371. doi:10.1038/s41586-018-0812-9

31. Clarke J, Panwar B, Madrigal A, et al. Single-cell transcriptomic analysis of tissue-resident memory T cells in human lung cancer. J Exp Med. 2019;216(9):2128-2149. doi:10.1084/jem.20190249

32. Miao X, Xu R, Fan B, et al. PD-L1 reverses depigmentation in Pmel-1 vitiligo mice by increasing the abundance of Tregs in the skin. Sci Rep. 2018;8(1):1605. doi:10.1038/s41598-018-19407-w

33. Yu R, Broady R, Huang Y, et al. Transcriptome analysis reveals markers of aberrantly activated innate immunity in vitiligo lesional and
non-lesional skin. PLoS One. 2012;7(12):e51040. doi:10.1371/journal.pone.0051040

SUPPORTING INFORMATION
Additional supporting information may be found in the online version of the article at the publisher’s website.

App S1 Methods.
Fig S1 PD-L1 expression is upregulated by melanoma cell lines after cytokine stimulation

Fig S2 Cytokine stimulation upregulates the expression on PD-L1 on dendritic cells

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