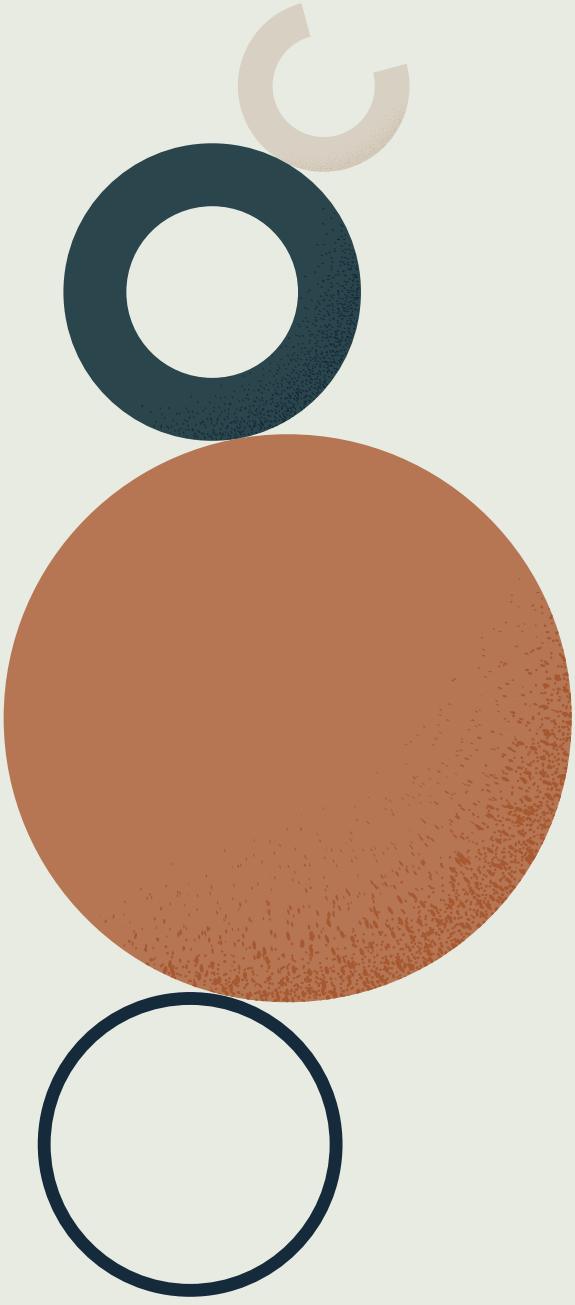
VITILIGO & MELANOMA

The fine balance between autoimmunity and immune escape



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Chapter 1

General introduction

Skin immune system

Skin immune cells

The human skin plays a critical role in immune defense against infection by the presence of various immune cells, including dendritic cells and T cells[1]. The skin consist of two main layers: the epidermis and the dermis (**Figure 1**). The outer layer of the skin, the epidermis, primarily constitutes keratinocytes. Keratinocytes are formed in the basal layer that proliferate, differentiate and move upward until they die and form the stratum corneum, which is critical for the barrier function of the epidermis[2]. Besides their structural character, keratinocytes activate and/or recruit dermal and circulating leukocytes by the production on cytokines and chemokines[3]. Aside from keratinocytes, Langerhans cells (type of dendritic cells), T cells, and melanin (pigment) producing cells, melanocytes, can be found in epidermal skin[1]. The dermis is mainly composed of fibroblasts, which are responsible for the production of extracellular matrix and collagen, but also contains blood vessels and lymphatic vessels, through which dendritic cells, T cells, B cells, and NK cells can migrate[1].

Initiation of a skin immune response

Upon skin infection, dendritic cells take up antigen and migrate to skin-draining lymph nodes to present it to pathogen-specific naïve $CD4^+$ or $CD8^+$ T cells. Naïve T cells that become activated, proliferate, differentiate into memory and effector T cells and migrate back to the infected site in order to eliminate pathogen-infected cells by the production of proinflammatory cytokines (e.g. IFN-γ and TNF-α)[4]. Upon clearance, the majority of effector T cells die, leaving behind a pool of memory T cells. Based on effector function and migration potential, circulating memory T cells can be defined into three subsets. Central memory T (T_{CM}) cells circulate and migrate to secondary lymphoid organs, while effector memory T (T_{EM}) cells have the capacity to migrate into peripheral tissues to clear the infection. Populations of memory T cells that permanently reside in peripheral tissues after an infection is cleared, are called resident memory T (T_{RM}) cells[5-7].

Concomitantly, dendritic cells in secondary-lymphoid organs can present and activate antigen-specific naïve B cells[8]. Following cognate

antigen encounter, B cells interact with T follicular helper (T_{FH}) cells and differentiate into short-lived immunoglobulin (Ig) M-secreting plasma cells or undergo additional proliferation, affinity maturation and class switching. The latter results in high-affinity and long-lived Ig-producing plasma cells and memory B cells. Upon reinfection, antigen-specific memory B cells can rapidly respond by the production of antibodies.

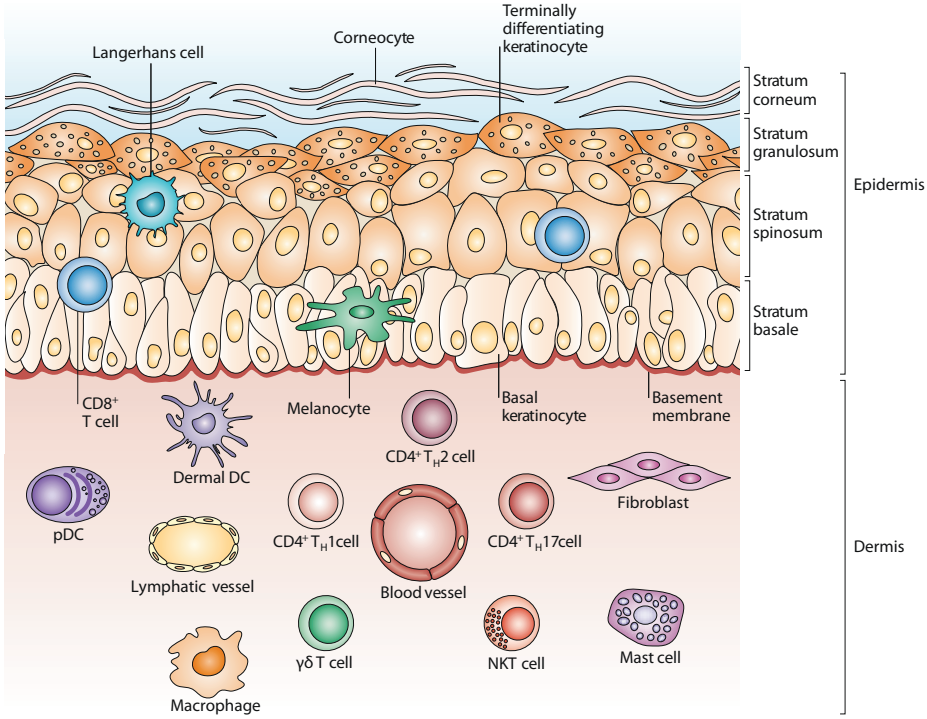

Figure 1. Skin anatomy and skin immune cells. The human skin consist of two layers: the epidermis and the dermis. The epidermis is mainly composed of keratinocytes, but also contains melanocytes, which produce pigment (melanin), Langerhans cells (type of dendritic cells) and T cells. The dermis constitutes fibroblasts that form collagen, elastic tissue and reticular fibers. Next to fibroblasts, it contains dendritic cells, different T cell subsets, natural killer T (NKT) cells, macrophages and mast cells. While absent in the epidermis, blood and lymphatic vessels are present throughout the dermis, enabling dendritic cells, T cells, B cells and NK cells to migrate. Reprint with permission from Springer Nature: Nature Reviews Immunology, Nestle *et al*., 2009[1].

Melanoma

Clinical features

Malignant melanoma is the most aggressive type of skin cancer and originates From melanocytes. Melanoma incidence has been increasing worldwide, with approximately 324.000 newly diagnosed patients in 2020[9]. Currently, melanoma represents the fifth most common form of cancer in the Netherlands, affecting 8300 patients annually[9]. In 2019, 751 patients died of melanoma in the Netherlands[10]. Melanoma affects a younger patient population than many malignancies, the median age at diagnosis is 53 years[11]. Melanoma has a high risk of invading lymphoid organs and spreading systematically and, thus, many patients develop metastasized disease. For melanoma has a high mortality once metastasized, accounting for roughly 57.000 deaths worldwide in 2020[9], it is important to diagnose melanoma at an early stage before metastasis occurs. Environmental and genetic factors may increase a person's risk of developing melanoma. Sunburn, intermittent sun exposure, fair skin, a high number of (atypical) nevi, eye color, hair color and genetic predisposition are known melanoma risk factors[12].

Immunogenicity

Generally, melanoma is considered a highly immunogenic, if not the most immunogenic, tumor[13]. Patients with melanoma occasionally experience spontaneous remission. More specifically, melanoma patients have melanoma-specific $CD8^+$ T cells that are capable of killing tumor cells. Additionally, anti-melanoma immunity can be found in patients with metastatic disease. Melanoma patients may, therefore, benefit from immunotherapy. Immunotherapy aims at inducing or increasing anti-tumor immune responses that eventually could lead to tumor regression. Immunotherapeutic strategies for melanoma include the use of (1) cytokines (e.g. interleukin-2 and interferons), (2) vaccines (dendritic cell-based or using tumor cells/tumor antigen-derived peptides), (3) adoptive cell therapy with tumor-infiltrating lymphocytes and (4) immune checkpoint inhibitors. Many of these trials were not taken to phase III trials because of lack of activity or failed to induce durable responses.

In the past decade, immunotherapy has become more successful, by the use of immune checkpoint inhibitors. Ipilimumab, a monoclonal antibody against cytotoxic T-lymphocyte antigen-4 (CTLA-4), was the first therapy to show an improvement in overall survival in patients with metastatic melanoma[14,15]. Long-term clinical responses were seen, but only in a minority of patients (10-20%). More recently, antibodies targeting programmed cell death 1 (PD-1) have been developed, showing higher response rates (40-52%)[16]. Antibodies blocking programmed cell death ligand 1 (PD-L1) have also been tested in metastatic melanoma patients, but despite their efficacy of 20-32% none of these currently available agents have been approved for the treatment of metastatic melanoma yet[17-19]. Concluding, immunotherapy for melanoma has shown clear objective clinical responses, supporting the hypothesis that overcoming tumor immune tolerance is needed to induce effective tumor clearance.

Vitiligo

Clinical features

Vitiligo is the most common skin depigmenting disorder, affecting approximately 1% of the general population, and is characterized by loss of melanocytes, resulting in white, depigmented skin patches[20]. Based on morphology and activity, vitiligo can be classified into segmental vitiligo (SV) and non-segmental vitiligo (NSV) (**Figure 2**). NSV, the commonest form, is characterized by symmetrical depigmentation of the body, whereas segmental vitiligo is less common (± 10%) and usually has a unilateral distribution[21]. Additionally, NSV shows an unpredictable disease course, while SV typically stabilizes a few months after onset. Finally, NSV is considered an autoimmune disorder and is closely associated with other autoimmune conditions, e.g. thyroid disease and alopecia areata, whereas systemic autoimmune comorbidities are less common in patients with SV[22,23].

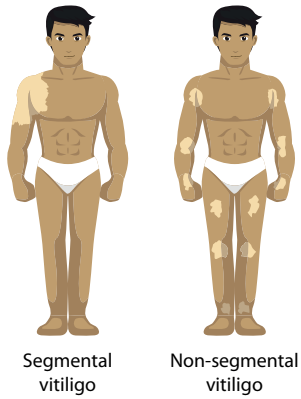

Figure 2. Types of vitiligo. Segmental vitiligo (left) is characterized by a unilateral distribution of the white patches, whereas in non-segmental vitiligo (right) depigmentation often appears symmetrical on both sides of the body.

Pathogenesis

Considering differences in clinical presentation and disease course, SV and NSV are believed to have distinct underlying pathogenic mechanisms. Koebner phenomenon is considered as an initial trigger in NSV patients[24]. Following this injury, damage-associated molecular patterns are released, oxidative stress is enhanced, and melanocytes can lose epidermal adhesion. Pro-inflammatory signaling by inflammasomes then leads to antigen processing by dendritic cells that migrate from the skin to the draining lymph nodes to present autoantigens to and activate autoreactive T cells. Melanocyte-specific, cytotoxic $CD8^+$ T cells that are capable of killing melanocytes are found to be increased in number both in blood and depigmented skin of NSV patients[25-27]. Moreover, infiltration of cytotoxic $CD8^+$ T cells positively correlates with disease severity[25]. Proinflammatory IFN-γ and CXCL10 signaling play a central role in driving this autoimmunity[28-31]. At the same time, antibody responses against melanocyte antigens, e.g. tyrosinase and TRP-2, have been found in the serum of patients with NSV[32], indicating that besides activation of $CD8^+$ T cells, a $CD4^+$ T cell and humoral response is initiated. Recently, T_{RM} cells were shown to have a prominent role in disease development and flare-up in human NSV. Autoreactive $CD8^+$ T_{RM} cells were increased specifically in vitiligo-affected skin compared to healthy unaffected donor skin[33,34], all together indicating immune disturbance in patients with NSV.

Contrary to NSV, SV is characterized by loss of melanocytes in a particular area. To date, a somatic mosaicism of melanocytes is the most plausible theory of the unique distribution pattern in SV[24]. Only recently, immune-mediated pathophysiology of SV has been recognized[23]. Increasing evidence has shown immune-based cytotoxic destruction of melanocytes in SV, with lesional IFN-γ-producing melanocyte-antigen reactive $CD8^+$ T cell infiltrates migrating to the basal layer[23]. However in contrast to NSV, this T cell-mediated melanocyte loss seems to be a local inflammatory response.

Treatment modalities

Because of its complex pathogenesis, treating vitiligo remains challenging. Current treatment modalities aim to suppress immune-mediated melanocyte destruction (in NSV) and/or induce repigmentation of the skin (in both SV and NSV)[35]. Topical corticosteroids and other immunosuppressants are usually given to NSV patients to reduce T cell activity and thus stabilize active vitiligo. To stimulate regeneration of melanocytes from the pigment cell reservoir, patients can undergo narrow-band ultraviolet B (NB-UVB) therapy. Phototherapy stimulates melanocyte proliferation and pigmentation, while inhibiting cutaneous immunity. Stable depigmented lesions that are unresponsive to local immune suppression or NB-UVB therapy can be treated with surgical melanocyte transplantation techniques, e.g. punch grafting of non-lesional skin[21]. Patients receiving melanocyte transplantation are often treated with phototherapy afterwards to restore skin pigmentation. Surgical treatments have proven to be successful (greater than 75% repigmentation of treatment sites) in patients with SV in 70 to 90% of treatments[36,37]. NSV patients, however, seem unresponsive to transplantation, with only 2 out of 17 patients showing repigmentation of greater than 75% of the treatment area[38].

Current treatment strategies combine NB-UVB therapy with topical corticosteroids and/or other immunosuppressants[39]. Clinical efficacy of current treatment modalities are not satisfactory, especially in NSV[40], as these are not effective in all patients, not all anatomic locations repigment and 40% of the patients relapse within a year after discontinuing treatment[27,41]. Therefore, new therapeutic strategies should be developed and evaluated for vitiligo patients.

Skin immune system in pigment cell disorders

Autoimmunity and tumor immunity

Similarities exist between autoimmunity and tumor immunity, exemplified by the association between vitiligo and melanoma. Adoptive transfer of gp100-specific CD8$^+$ T cells in mice bearing B16 melanoma cured the mice of the tumor, but also caused vitiligo[42]. Similarly, melanoma patients can develop vitiligo spontaneously or upon immunotherapy treatment. New-onset vitiligo occurs in 2-43% of melanoma patients treated with immunotherapy[43-46]. This depigmentation is caused by activated anti-melanoma immunity that targets not only malignant cells, but also healthy melanocytes[47]. Self-reactive CD8$^+$ T cells isolated from these melanoma patients were shown to recognize melanocyte differentiation antigens, e.g. Melan-A/MART-1 and gp100, expressed on both melanocytes and (over-)expressed on melanoma cells[48]. Presence of melanocyte-specific T cell responses in metastatic melanoma patients has been correlated with prolonged survival[43-45,49,50] and an objective response to pembrolizumab treatment was associated with a higher occurrence of vitiligo[50]. Conversely, vitiligo patients have 3-fold less risk of developing melanoma during life[51,52]. Considering this melanoma/vitiligo relationship, melanoma patients could benefit from anti-melanocyte immunity. On the other hand, vitiligo patients could benefit from increased tolerance to melanocytes.

Immune evasion by melanoma cells

Many melanoma patients develop metastasized disease, e.g. by an impairment of the host immune system. PD-1, expressed on activated T cells, is an immune checkpoint that engages to its ligand PD-L1[53]. PD-L1 is constitutively expressed by various immune cells and inducibly expressed on non-immune cells, including cancer cells[54]. Tumor-associated PD-L1 can functionally suppress T cell responses against melanoma and promote T cell apoptosis. Targeting these immune checkpoints has become one of the therapeutic challenges[55]. As mentioned earlier, monoclonal antibodies targeting PD-1 have been approved for clinical use and among first-line treatment options for advanced melanoma patients[56]. Nevertheless, responses to these therapies are still suboptimal, around 20-40% for PD-1 or

PD-L1-targeting monoclonal antibodies[53], e.g. because of dynamic changes in PD-L1 expression, indicating the need to study regulation of PD-L1 expression in the tumor-micro-environment.

Besides PD-1/PD-L1 signaling, tumor heterogeneity is commonly seen in melanoma patients. Heterogeneity involves the presence of cells with different phenotypic and molecular features within a tumor (intralesional) or between tumors (interlesional) in a patient. Intralesional heterogeneity is commonly explained by clonal evolution of a tumor, that arise e.g. from point mutations or phenotypic changes. Interlesional heterogeneity often results from intralesional heterogeneity of the primary tumor or, more precisely, heterogeneity of circulating tumor cells. As a result, metastatic lesions arise from different subpopulations within tumors.

These subpopulations of tumor cells harbor distinct phenotypic and molecular signatures, which results in differential levels of sensitivity to treatment[57]. Accordingly, tumor heterogeneity is thought to drive evolution of cancers and resistance to therapy, immunotherapy included[58]. In fact, during immunotherapy, many metastatic melanoma patients experience "mixed response", with some tumor lesions regressing and other ones progressing[59]. This might result from selection for antigen-negative tumor cells[60-62] or tumor cells with stemness properties that have a phenotype different from their differentiated counterpart[63,64]. Tumor cells with stemness features are less represented in most melanoma patients and are less immunogenic, being hardly targeted by specific immunity. This illustrates that melanoma cells can use tumor heterogeneity to evade immune destruction.

Moreover, immunotherapy itself can cause tumor heterogeneity, as it has been shown that tumor-specific cytotoxic T cells induce dedifferentiation of melanoma cells[65], which thereby acquire "stem cell-like" properties. Altogether, this indicates the need to study melanoma heterogeneity to overcome immune evasion. To reveal those subpopulations that are insufficiently targeted by current immunotherapies and/or to identify features of immunotherapy-induced subpopulations.

Immunomodulating factors in vitiligo

Immune checkpoint signaling plays a pivotal role in immune evasion by tumors. As shown in melanoma, targeting immune checkpoints is sufficient to break peripheral tolerance in a fraction of patients. While abundantly

studied in melanoma, immune regulation by PD-1/PD-L1 in vitiligo has received far less attention thus far. Impaired PD-1/PD-L1 function is involved in a variety of autoimmune diseases, among which type 1 diabetes and rheumatoid arthritis[66], indicating the rationale to test the therapeutic potential of increasing PD-1/PD-L1 signaling in autoimmunity. Concomitantly, PD-1/PD-L1 might be directly involved in vitiligo pathogenesis, indicating the need to study these molecules. If affected, manipulating PD-1/PD-L1 might influence peripheral tolerance to melanocytes in vitiligo, making it an interesting target in the treatment of vitiligo patients[67].

Aims and thesis overview

This thesis aims at 1) investigating the role of immune cells in two skin diseases, melanoma and vitiligo, with respect to resident and circulating immune cells, and 2) study the role of melanocytes and melanoma cells in evading the immune system.

Part 1 describes the involvement of the immune system in skin pigmenting disorders. Whereas segmental vitiligo stabilizes quickly, non-segmental vitiligo patients often experience active disease, with new lesions occurring during life. This suggests differences in pathogenic mechanisms involved. In **chapter 2**, we therefore investigate differences in circulating immune cells in human blood between patients with segmental and non-segmental vitiligo.

Besides systemic autoimmunity, local immune cells are also involved in skin depigmentation, as some lesions recur at the same skin sites as previous lesions, suggesting resident T cells are involved. **Chapter 3** reviews the literature on how skin-resident T_{RM} cells contribute to vitiligo and melanoma, as well as their potential as therapeutic targets in both diseases. Current literature suggest an important protective role for T_{RM} cells in melanoma. However, little is known about abundance of T_{RM} cells in non- and pre-malignant tissues. In **chapter 4**, we aimed to evaluate the expression patterns of markers expressed by skin-resident T cells in human skin specimens, representing the spectrum from healthy skin to metastatic melanoma.

Part 2 describes characteristics of melanocytes and melanoma cells that might contribute to immune evasion. To study melanocytes in more

detail, we first sought to optimize melanocyte isolation from human donor skin. In **chapter 5**, we describe a method to instantly isolate highly-purified human melanocytes from epidermal cell suspensions. **Chapter 6** is a critical review of the current literature on the PD-1/PD-L1 pathway in vitiligo as a new therapeutic target for vitiligo therapy. **Chapter 7** investigates the role of PD-1/PD-L1 checkpoint signaling in melanocyte destruction in vitiligo and how this is influenced by interferons.

Besides PD-1/PD-L1 signaling, melanoma cells have other properties to evade immune destruction, e.g. tumor heterogeneity. We aimed to identify by multiplex immunofluorescence melanoma cell subpopulations, to reveal those that are insufficiently targeted by current immunotherapies. In **chapter 8**, we describe a method to improve the quality of multiplex immunofluorescence staining. **Chapter 9** describes melanoma phenotypic changes in immunotherapy-treated melanoma patients. Finally, **chapter 10** and **11** discusses the results of this thesis and present a conclusion, respectively.

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Part 1

Involvement of the immune system in skin pigment cell disorders

Chapter 2

Immunophenotypic analysis reveals differences in circulating immune cells in peripheral blood of segmental and non-segmental vitiligo patients

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Abstract

Accumulating studies have indicated immune-based destruction of melanocytes in both segmental vitiligo (SV) and non-segmental vitiligo (NSV). Whereas SV often occurs unilaterally during childhood and stabilizes after an initial period of activity, the disease course of NSV is usually slowly progressive, with new lesions occurring bilaterally during life. This suggests involvement of distinct pathophysiology pathways, specifically increased systemic immune activation in NSV patients, but not in SV patients. This research aimed to identify differences in immune cells in blood of patients with SV and NSV, through immunophenotyping of circulating cells. Regulatory T cells (Tregs) were unaffected in patients with SV compared to healthy controls, but decreased in NSV patients. In NSV patients, the reduction in Tregs was associated with presence of other systemic autoimmune comorbidities, which were less present in SV. Likewise, absence of a melanocyte-specific antibody response in patients with SV, suggests less involvement of B cell immunity in SV. These data show that, in contrast to NSV, no increased systemic immunity is found in SV patients, indicating that SV pathogenesis is associated with a localized cytotoxic reaction targeting epidermal melanocytes.

Introduction

Vitiligo is the most common skin depigmenting disorder characterized by white patches resulting from the loss of pigment producing cells, melanocytes (Bergqvist and Ezzedine, 2020). It affects approximately 0.5% of the general population with no apparent differences in rates of occurrence according to sex, skin type or ethnicity (Boniface et al., 2018). An international consensus classified vitiligo into two subtypes (Ezzedine et al., 2012). The commonest form, non-segmental vitiligo (NSV), shows symmetrical depigmentation of the body. Contrary, segmental vitiligo (SV) is less common (± 10%) and is characterized by a unilateral distribution. Additionally, NSV shows an unpredictable disease course, while SV typically stabilizes a few months after onset. Altogether, this suggest distinct pathophysiology pathways might be involved, that could clarify differences in clinical presentation and disease course.

Initially, somatic mosaicism, neurogenic mechanisms and oxidative stress were suspected to be the underlying cause of SV (Speeckaert et al., 2020). Only recently, immune-mediated pathophysiology of SV has been recognized. Increasing evidence has shown immune-based cytotoxic destruction of melanocytes in SV, with lesional IFN-γ-producing melanocyte-antigen reactive $CD8^+$ T cell infiltrates migrating to the basal layer (Attili and Attili, 2013, Shin et al., 2016, van Geel et al., 2010). Although NSV is closely associated with other autoimmune disorders, e.g. thyroid disease and alopecia areata, systemic autoimmune comorbidities are less common in patients with SV (Dahir and Thomsen, 2018, Speeckaert et al., 2020). Nevertheless, localized skin inflammation, e.g. linear morphea, is repeatedly observed in SV patients, implying a local inflammatory response (Speeckaert et al., 2020). The comparison between SV and NSV provides a unique setting to study whether anti-melanocyte immunity remains localized in SV and spreads systemically in NSV.

A study on gene expression profiles of SV, NSV patients and healthy individuals showed that differentially expressed genes in SV were involved in the adaptive immune response, while in NSV regulation of the innate immune response, B cell differentiation and activation was more prominent, implying SV and NSV may utilize different immune responses and mechanisms for melanocyte destruction (Wang et al., 2016). Concomitantly, blood of NSV patients showed a decrease in regulatory T cells (Tregs) and an increase in unswitched memory B cells compared to healthy control blood, which was related to disease activity (Raam et al., 2018). Considering the positive correlation between switched memory B cells and circulating T follicular helper (cTfh) cells, active NSV patients show activation of germinal centers and faster B cell isotype switching (Raam et al., 2018). Despite data showing involvement of B cells and germinal center reactions, the correlation to a humoral response remained unstudied.

To our knowledge, analysis of circulating immune cells, involvement of a humoral response and germinal center reactions in SV have not been fully characterized. Whereas associated autoimmune diseases are more common in NSV (Alkhateeb et al., 2003, Gill et al., 2016, Hadi et al., 2020, Spritz and Andersen, 2017), evidence points to a temporary cytotoxic response targeting melanocytes in SV, suggesting differences in systemic immune cell dysregulation between SV and NSV patients.

This study aimed to compare differences in cellular and humoral adaptive immunity and innate immunity in human blood of SV and NSV patients that can contribute to clinical presentation and disease progression. Our results show no increased systemic immunity in SV patients, in contrast to NSV patients, and points to localized immune-based cytotoxic destruction of melanocytes.

Results

Demographics and clinical characterization of study subjects

Characteristics of the participants are shown in **table 1**. SV patients had an average age of 34 years, which is higher than the average age of the general SV population because of the [3] 18 years age inclusion criterium. SV had stabilized in our patient cohort, for at least 12 months. To focus our comparative study on the differences between SV and NSV without interference of active versus stable vitiligo disease activity, we compared stable SV patients to stable NSV patients. The majority of included SV patients had a type 2 skin type and only one patient had alopecia areata as autoimmune comorbidity. NSV patients had stable disease, an average age of 43 years, the majority of patients had a type 2 or 3 skin type and 6 of the 22 patients (27.2%) showed autoimmune comorbidities. SV and NSV patients did not differ in age of onset, disease duration and affected body surface area. Healthy controls were comparable concerning age, gender and skin type as the vitiligo patients but without vitiligo.

Table 1. Patient characteristics

	Healthy donor			**Segmental vitiligo**			**Non-segmental vitiligo**			
	N	%	IQR/SD[1]	N	%	IQR/SD	N	%	IQR/SD	*p-value*[2]
Total	22			12			22			
Age										
< 25	2	9.1		3	25		1	4.5		
25-50	17	77.3		8	66.7		14	63.6		
> 50	3	13.6		1	8.3		7	31.8		
Mean	36		(23 - 48)	34		(22 - 46)	43		(29 - 57)	> 0.05
Gender										> 0.05

Male	6	27.3	9	75		11	50		
Female	16	72.7	3	25		11	50		
Skin type[3]									> 0.05
Type 1	0		0			1	4.5		
Type 2	13	59.1	8	66.7		12	54.5		
Type 3	7	31.9	3	25		8	36.4		
Type 4	1	4.5	1	8.3		0			
Type 5	1	4.5	0			1	4.5		
Type 6	0		0			0			
Comorbidities[4]									
Thyroid disease			0			3	13.6[6]		
Alopecia Areata			1	8.3		0			
DM type I			0			4	18.2[6]		
RA			0			0			
SLE			0			0			
Psoriasis			0			0			
Other[5]			0			1	4.5[6]		
Vitiligo age of onset (years) mean			25		± 14.8	33		± 15.6	> 0.05
Disease duration (years) median			4		(2.3 -12.3)	8	(4 - 12.5)		> 0.05
% Affected body surface area median			0.75		(0.5 - 1.9)	1	(0.9 - 3.9)		> 0.05

[1] Interquartile range/standard deviation
[2] P-value after Student's *t* test or Mann-Whitney test
[3] Skin type according to the Fitzpatrick skin scale
[4] Abbreviations: DM, diabetes mellitus; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus
[5] Other specified: Addison's disease, Arthritis psoriatica, auto-immune hepatitis, IBD, Coeliac, CREST, Morphea, Pernicious anemia, PMR, Sarcoidosis, Scleroderma, Sjogren's Syndrome
[6] One NSV patient showed multiple autoimmune comorbidities (hypothyroidism, DM type I and colitis ulcerosa)

Non-segmental vitiligo patients with secondary autoimmune comorbidities have less circulating regulatory T cells

Perturbations in Treg cell numbers and function in vitiligo remain indistinct. Some studies report systemically reduced Tregs in NSV (Ben Ahmed et al., 2012, Dwivedi et al., 2013, Giri et al., 2020, Hegab and Attia, 2015, Lili et al., 2012, Raam et al., 2018), while other demonstrate abundance of Tregs in NSV trends towards an increase (Abdallah and Saad, 2009, Moftah et al., 2014). Moreover, Treg involvement in SV remains unstudied. We, therefore, studied systemic Treg and type 1 regulatory T (Tr1) cell numbers and IL-10 production in our SV and NSV patient cohort and healthy donor samples. The gating strategy for regulatory T cells is depicted in **figure S1**. Circulating Tregs were significantly decreased in blood of patients with NSV compared to SV and healthy controls (**Figure 1A**). IL-10 production by Tregs, measured as IL-10^{+} cells after intracellular FACS staining, however, was not affected in these patients (**Figure 1A**). The level of Tregs in SV patients did not differ from healthy controls (**Figure 1A**). We verified these findings on the RNA expression level and in an independent patient cohort, using the RNAseq dataset from Wang *et al.* (2016). This dataset contains 20 SV, 20 NSV patients and 20 healthy control individuals (within each patient group, 5 patients were pooled into a new sample, giving a total of 4 samples) (Wang et al., 2016). Gene expression profiles of NSV, SV and healthy individuals were then analyzed for the presence of a Treg gene expression signature. For this, we made use of a gene signature used to discriminate between Tregs (CD25^{high}) and conventional CD4^{+} T cells (CD25^{-}) (Niedzielska et al., 2018). This gene signature comprises 25 genes, of which 21 genes that are upregulated and 4 genes that are downregulated on Tregs compared to conventional CD4^{+} T cells (**Table S1**). Similar to cellular Treg analysis, NSV patients showed decreased expression of the Treg signature compared to healthy individuals ($p<0.05$) (**Figure 1B**). Because of the large spread among SV samples, comparing Treg levels in NSV and SV patients did not reach significance ($p=0.08$) (**Figure 1B**).

Since systemic autoimmune comorbidities might influence circulating Treg numbers, we compared Treg levels in NSV patients with or without comorbidities as indicated in **table 1**. NSV patients with autoimmune comorbidities showed significantly less Tregs than NSV patients without autoimmune comorbidities **(Figure 1C**), suggesting impaired Treg numbers

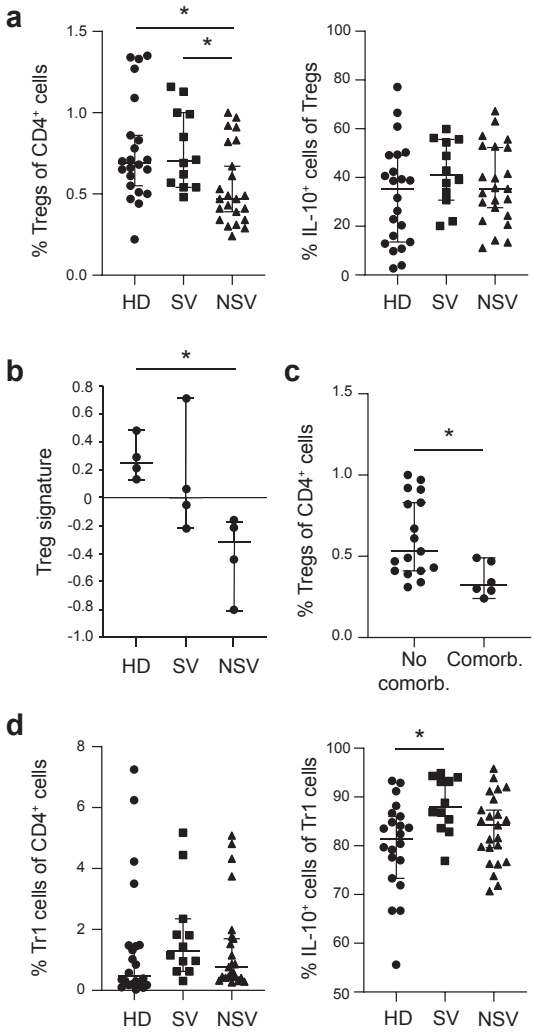

Figure 1. Comparison of circulating regulatory T cell subpopulations in vitiligo patients. (A) The percentage of Tregs among CD4⁺ T cells (left) and IL-10-producing cells among Tregs (right) in healthy control individuals (HD, n=22), segmental vitiligo (SV, n=12), and non-segmental vitiligo patients (NSV, n=20). (B) Expression of the Treg core gene signature in HD (n=20), SV (n=20) and NSV (n=20) (within each group, 5 samples were pooled into a new sample). The genes included in this Treg core signature are included in **Table S1**. (C) The percentage of Tregs among CD4⁺ T cells in NSV patients, without (no comorb., n=16) or with autoimmune comorbidities (comorb., n=6). (D) The percentage of Tr1 cells among CD4⁺ T cells (left) and IL-10-producing cells among Tr1 cells (right) in HD, SV and NSV patients. Results are shown as individual dot plots with a line at median and 95% confidence interval (A, C, D) or median and minimum and maximum (B). ANOVA and Student's *t* test significant as indicated; **P* <0.05, ***P* < 0.01, ****P* <0.001 and *****P* <0.0001.

might be the consequence of secondary autoimmune responses and not specific to vitiligo pathogenesis. Treg levels in NSV patients without autoimmune comorbidities still trended towards a decrease compared to healthy controls, but this did not reach significance in our patient cohort size (p=0.09, data not shown). Nevertheless, gene expression patterns of NSV patients showed a less pronounced Treg signature, even when autoimmune conditions were absent (the patient cohort of Wang *et al*. (2016) only includes patients with no history of any other autoimmune condition) (**Figure 1B**).

In contrast to Tregs, the percentage of Tr1 cells did not demonstrate significant differences between the studied groups, nonetheless IL-10-producing Tr1 cells were increased in patients with SV (**Figure 1D**). To conclude, these results further support that Tregs, but not Tr1 cells, are negatively affected in (part of) NSV patients, which may facilitate the development of autoimmune comorbidities. In contrast, Tregs remain unaffected in patients with SV, consistent with less systemic autoimmune comorbidities in SV patients.

Antibody responses against melanocyte antigens are present only in non-segmental vitiligo patients

Antibody responses against melanocyte antigens, e.g. tyrosinase and TRP-2, have been found in the sera of patients with NSV (Kemp et al., 2007). Sera from SV patients, however, have rarely been tested for the presence of autoantibodies. To test reactivity to melanocyte antigens in SV and NSV patients, sera were evaluated for antibody reactivity against TYR, TYRP1, TYRP2, PMEL, TH, MART-1 and MCHR1, respectively. Antibodies against selected melanocyte antigens were present in the circulation of a significant proportion of patients with NSV (**Figure 2**). Eight out of 22 (36%) NSV patients were found to have anti-melanocyte antibody responses (**Table S2**). Moreover, 3 out of 8 patients showed antibody reactivity to several melanocyte antigens (**Table S2**). NSV patients with antibody responses against aforementioned antigens were not significantly different from NSV patients without autoantibodies regarding age, sex distribution, skin type, presence of secondary autoimmune comorbidities, vitiligo age of onset, disease duration and % affected body surface area (**Table S3**). Contrary, none of the SV patients showed an antibody response (**Figure 2**), indicating that the presence of a humoral immune response to melanocyte antigens is limited to NSV patients.

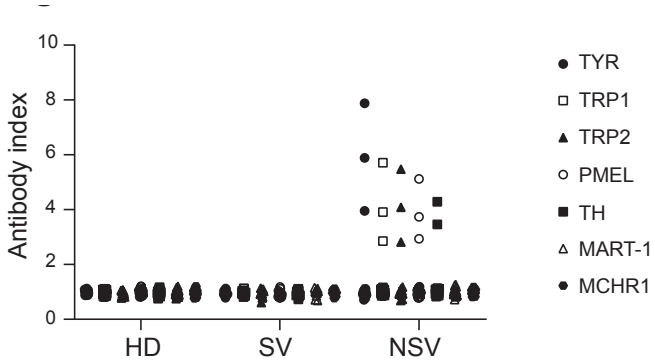

Figure 2. Presence of melanocyte-specific antibody responses in vitiligo patients. Sera of healthy controls (HD, n=30), segmental vitiligo (SV, n=12), and non-segmental vitiligo patients (NSV, n=20) were analyzed in radiobinding assays for presence of antibodies against tyrosinase (TYR), TRP1, TRP2, Pmel, tyrosinase hydroxylase (TH), MART-1 and MCHR1, respectively. The antibody index for each individual patient is shown and is calculated as cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by the group of healthy control sera. Each serum was tested in at least three independent experiments. Next, the mean antibody index was calculated from these values. Patient sera with an antibody index above the upper limit of normal (mean antibody index + 3x standard deviation of the healthy control individuals) were regarded as positive for antibody reactivity.

Segmental vitiligo patients have less circulating antibody-producing plasmablasts

We subsequently analyzed whether the absence of antibody responses in SV is also reflected in less B cell activation and plasma cell differentiation. The gating strategy for B cells is depicted in **figure S1**. The percentage of total B cells ($CD3^-$ $CD19^+$) was comparable between vitiligo patients and healthy individuals (**Figure 3A**). Similarly, naïve B cells, unswitched and switched memory B cells did not demonstrate significant differences between de studied groups (**Figure 3A**), implying B cells seem to mature similarly in vitiligo patients and healthy individuals. Likewise, transitional B cells did not differ between SV and NSV patients, nor from healthy controls (**Figure 3B**). Plasmablasts, however, were significantly decreased in blood from patients with SV compared to healthy donor blood (**Figure 3C**). Concomitantly, plasmablasts showed a trend towards a decrease in SV patients compared to NSV patients, suggesting SV patients have less circulating antibody-producing cells.

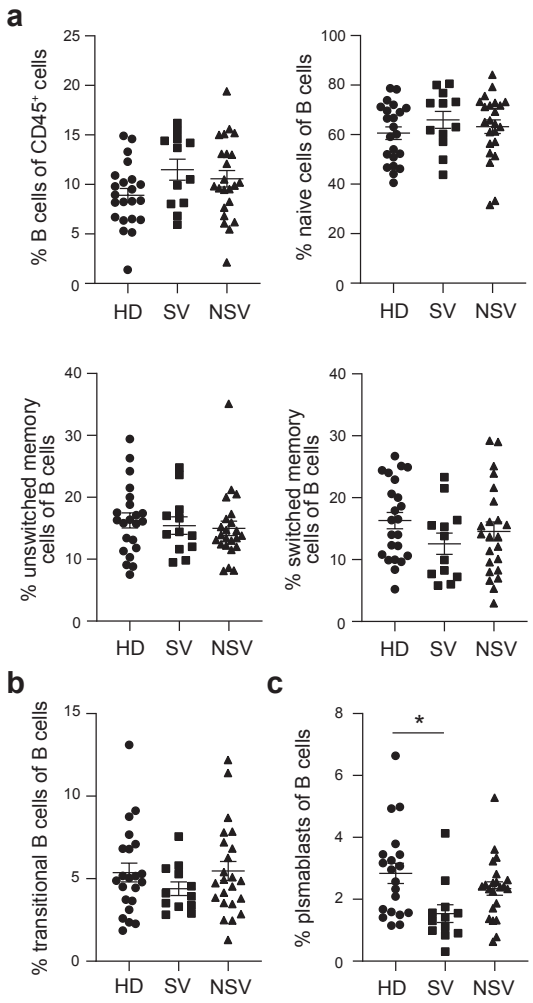

Figure 3. Distribution of peripheral B cell subsets in vitiligo patients. (A-C) The percentage of B cells among lymphocytes, naïve B cells, unswitched memory B cells and switched memory B cells among B cells (A), transitional B cells (B) and plasmablasts (C) among B cells in healthy controls (HD, n=22), segmental vitiligo (SV, n=12), and non-segmental vitiligo patients (NSV, n=20). Results are shown as individual dot plots with means ± SEM. ANOVA significant as indicated; *P <0.05, **P < 0.01, ***P <0.001 and ****P <0.0001.

Segmental vitiligo patients have more cTfh2 and cTfh17 cells, but no increase in active cTfh cells

Our data so far indicates that SV patients have no melanocyte-specific antibody response and a diminished humoral response, illustrated by less circulating plasmablasts (**Figure 2** and **3C**). To verify if this reduced

plasmablast differentiation stems from reduced germinal center help, we analyzed the presence of cTfh cells. Levels of both cTfh cells and active cTfh cells (PD-1^{+} ICOS^{+}) were unaffected in NSV compared to healthy control individuals. Instead, the numbers of cTfh cells were significantly increased in SV compared to NSV patients (**Figure 4A**). Similarly, cTfh cells with an active phenotype were increased in patients with SV compared to both NSV patients and healthy controls ($p<0.05$) (**Figure 4A**).

Based on the expression of CXCR3 and CCR6, cTfh cells can be classified into cTfh1, cTfh2, and cTfh17 cells (**Figure S1**) (Koutsakos et al., 2019). These cells differ in their ability to provide help to naïve and memory B cells and, thus abundance of specific cTfh cell subsets might be as important as total cTfh cell levels. In blood, cTfh1 cells did not show significant differences between the studied groups (**Figure 4B**). Contrary, cTfh2 and cTfh17 cells, which are superior IL-21 producers compared to cTfh1 cells and especially provide help to naïve B cells, were significantly increased in patients with SV compared to NSV patients and healthy controls ($p<0.05$) (**Figure 4B**). More importantly, patients with SV did not show an increase in active cTfh2 and cTfh17 cells (**Figure 4C**). Concomitantly, active cTfh cell subsets did not differ between NSV and healthy individuals (**Figure 4C**). As provision of B cell help is limited to activated cTfh cells and the percentage of active cTfh1, cTfh2, and cTfh17 cells do not differ between SV and NSV, it seems that both vitiligo cohorts show normal germinal center help that is not different from healthy controls.

As for Treg numbers, systemic autoimmune comorbidities might affect cTfh cell levels and activation status. NSV is closely associated with other autoimmune conditions, while these are less common in patients with SV (Dahir and Thomsen, 2018, Speeckaert et al., 2020). These comorbidities may involve humoral responses and increased B cell help from cTfh cells. Indeed, when NSV patients were divided into subgroups according to the presence of secondary autoimmune comorbidities, it was evident that systemic autoimmune comorbidities were associated with increased percentages of active cTfh2 cells ($p<0.05$) and cTfh17 cells ($p=0.07$), but not with cTfh1 cells (**Figure 4D**). Collectively, our results demonstrate increased cTfh2 and cTfh17 cell numbers in SV, but no increased germinal center reactions in human vitiligo. Consistent with an increase in activated cTfh2 and cTfh17 cell subsets cell activation in NSV patients with secondary autoimmune comorbidities seems to result from those, rather than skin autoimmunity.

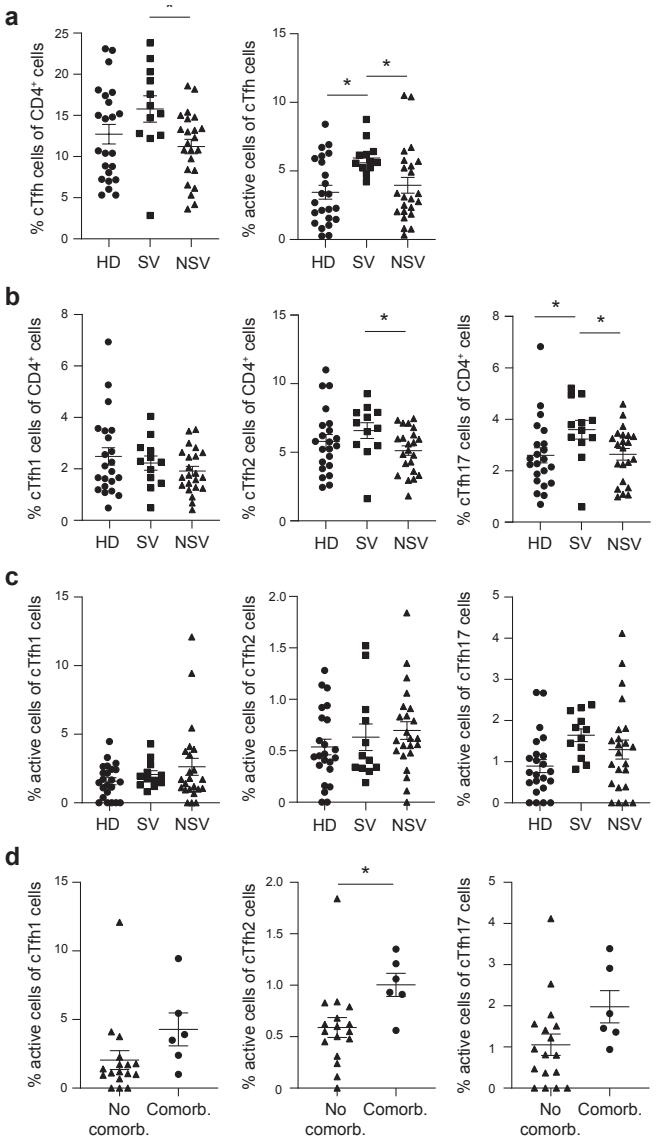

Figure 4. Comparison of cTfh cell subsets in vitiligo patients. (A-C) The percentage of cTfh cells among $CD4^+$ cells and active ($PD\text{-}1^+$ $ICOS^+$) cTfh cells (A), cTfh cell subsets (B) and active cells within $CD4^+$ cells and cTfh cell subsets, respectively, (C) in healthy controls (HD, n=22), segmental vitiligo (SV, n=12), and non-segmental vitiligo patients (NSV, n=20). (D) The percentage of active cells among cTfh cell subsets in NSV patients, without (no comorb., n=16) or with secondary autoimmune comorbidities (comorb., n=6). Results are shown as individual dot plots with means ± SEM. ANOVA and Student's *t* test significant as indicated; **P* <0.05, ***P* < 0.01, ****P* <0.001 and *****P* <0.0001.

Vitiligo patients do not differ from healthy controls in circulating NK cells

Besides aberrations in the adaptive immune response, innate immunity is suggested to be involved in NSV pathogenesis as well, but left unstudied in patients with SV. The gating strategy for NK cells is depicted in **figure S1**. Systemic NK cells levels were not different in both vitiligo subtypes compared to healthy control blood (**Figure 5A**). Expression of the activating NK cell receptor, NKG2D, on NK cells and cytotoxic $CD56^{dim}$ NK cells was significantly decreased in NSV patients compared to healthy controls, but unaffected on cytokine-producing $CD56^{bright}$ NK cells (**Figure 5B**). In blood of SV patients, however, no difference in NKG2D expression was seen compared to healthy control blood. As NKG2D can be expressed by $CD8^+$ T effector memory cells and increased expression has been observed active NSV skin (Jacquemin et al., 2020), we analyzed NKG2D expression by peripheral $CD3^+$ T cells. No significant differences in the proportion of $NKG2D^+$ $CD3^+$ T cells were seen in the blood of patients with NSV compared to SV and healthy controls (data not shown). As NK cells were unaffected in patients with SV, innate immunity seems to be less involved in SV pathogenesis.

Discussion

This study provides important insights into the differences between segmental vitiligo (SV) and non-segmental vitiligo (NSV) pathogenesis, and shows that, in contrast to NSV, SV does not involve systemic immune activation. We found that regulatory T cells are less abundant in patients with NSV than in healthy controls, but did not differ in SV patients. Furthermore, a humoral response and germinal center reactions were not observed in patients with SV. This is consistent with less autoimmune comorbidities in patients with SV and points to a local autoimmune reaction.

Previous studies have shown presence of melanocyte-specific antibodies in part of NSV patients (Kemp et al., 2007), while this remained largely unstudied in SV patients. Our present work demonstrates that the involvement of a humoral response against melanocyte antigens is restricted to NSV patients. Absence of a melanocyte-specific antibody response is consistent with the observation that there is no systemic immune activation

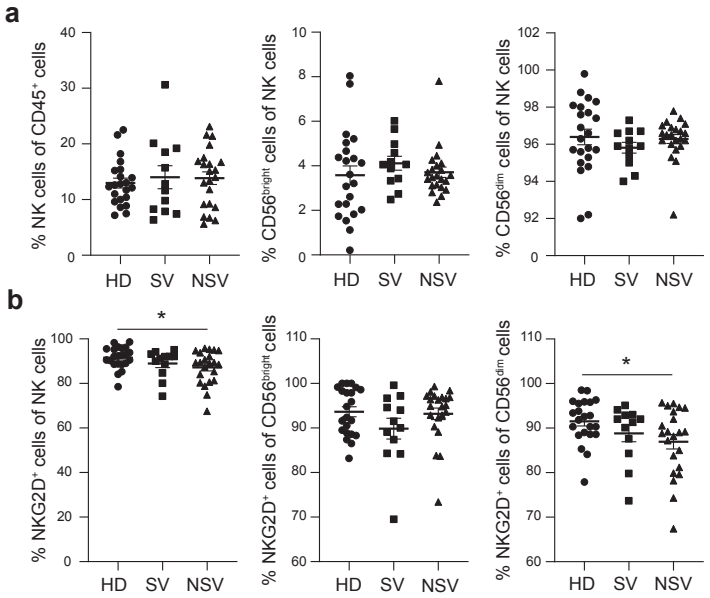

Figure 5. Distribution of circulating NK cells in vitiligo patients. (A-B) The percentage of NK cells among $CD45^+$ cells, $CD56^{bright}$ and $CD56^{dim}$ cells among NK cells (A) and $NKG2D^+$ cells among NK cells, $CD56^{bright}$ and $CD56^{dim}$ cells in healthy controls (HD, n=22), segmental vitiligo (SV, n=12), and non-segmental vitiligo patients (NSV, n=20). (B). Results are shown as individual dot plots with means ± SEM. ANOVA significant as indicated; *P <0.05, **P < 0.01, ***P <0.001 and ****P <0.0001.

in patients with SV. Although autoantibodies were found in part of NSV patients, we hypothesize this is an underestimation, for we tested only 7 common melanocyte autoantibody targets. Indeed, in immunoprecipitation experiments with melanocyte extracts, 100% of NSV patients and 0% of healthy controls were found to have anti-melanocyte antibodies in their sera (Naughton et al., 1983). Also, incidence and level of autoantibodies have been correlated with disease activity and the extent of the disease, meaning active vitiligo patients and patients with 5-10% skin depigmentation are more likely to have circulating anti-melanocyte antibodies (Harning et al., 1991, Naughton et al., 1986).

Regulatory T cells induce anergy in melanocyte-specific T cells in healthy individuals (Maeda et al., 2014). More importantly, it is assumed that melanocyte-reactive $CD8^+$ T cells escape anergy by loss of co-inhibitory CTLA-4 expression in NSV. Similarly, we demonstrate that Tregs numbers are decreased in patients with stable NSV, most prominently in patients with

secondary autoimmune comorbidities, as hypothesized earlier (Le Poole and Mehrotra, 2017). Even in the absence of autoimmune comorbidities, we found that NSV patients still show a trend towards a decrease in Treg numbers and signature. In a previous study, abundance and activity of circulating Tregs in NSV patients was shown to be similar to healthy controls, but reduced in skin of NSV patients, explained by failure of Tregs to home to the skin in vitiligo (Klarquist et al., 2010). Contrary to our cohort, half of these patients showed progressive disease and were under treatment at the moment of collecting blood samples. Therefore, these results might be explained by different disease activity. This highlights the importance of reporting these patient characteristics in great detail. Since numerous studies have not clearly reported the presence or absence of secondary autoimmune comorbidities or disease activity while studying regulatory T cells in NSV, it is difficult to place results into context, as greatest differences are seen in those with other auto-immunities.

Involvement of NK cells in vitiligo has been suggested by previous studies. RNA analysis of NSV skin biopsies revealed high expression of genes of the innate immune system, especially NK cells compared to healthy skin and more NK cells were found in both lesional and non-lesional NSV skin (Yu et al., 2012). Likewise, NK cells were shown to be significantly increased in the blood of stable NSV patients compared to healthy controls (Tulic et al., 2019). We, however, together with others (Raam et al., 2018) did not detect increased peripheral NK cell levels. Nevertheless, NKG2D expression by NK cells was shown to be significantly decreased in NSV patients, especially on $CD56^{dim}$ cells and *KLRC4-KLRK1*, which encodes NKG2D, was found to be downregulated in blood of SV patients (Wang et al., 2016). In lesional NSV skin, the stress molecules MICA/MICB (ligands for the activating NKG2D receptor) were shown to be expressed in dermal areas, but not in non-lesional or healthy skin (Raam et al., 2018). Also, IFN-γ-producing innate lymphoid cells were shown to initially induce CXCR3B-mediated melanocyte apoptosis (Tulic et al., 2019). Recently, increased NKG2D expression was found on skin-resident NK cells, NKT cells and $CD8^+$ effector memory T cells, especially in patients with active disease (Jacquemin et al., 2020). Unlike increased NKG2D expression by $CD8^+$ effector memory T cells in skin of active NSV patients, no significant difference in the proportion of these cells were seen in the blood of patients with NSV compared to healthy controls (Jacquemin et al., 2020). It is, therefore, suggested that a skin factor

(possibly IL-15, IFN-α) is responsible for the promotion of NKG2D expression (Jacquemin et al., 2020). In addition, NKG2D upregulation in vitiligo occurs in response to insults and stress, which primarily occurs in lesional skin (Plaza-Rojas and Guevara-Patino, 2021). The involvement of NK cells in NSV and SV pathogenesis therefore remains indistinct, but suggests skin-resident NK cells to be involved in initial initiation of the anti-melanocyte autoimmunity during active disease, rather than long lasting systemic NK cell involvement.

To our knowledge, this is the first immunophenotypic analysis of circulating immune cells in blood of SV patients, as compared to NSV patients and healthy individuals. Our results strengthened the notion that immunity plays an important role in vitiligo pathogenesis. Most importantly, our study highlights the immunological difference between NSV and SV. NSV is characterized by systemic immune activation, decreased Treg levels and the development of autoimmune comorbidities. In contrast, the absence of systemic immune activation in patients with SV, indicates that SV pathogenesis is associated with a localized cytotoxic reaction against epidermal melanocytes.

Materials and methods

Patient material

This study was conducted in accordance with the Declaration of Helsinki. All subjects signed written informed consent approved by the Institutional medical Ethics Review Committee (NL 64983.018.18). Peripheral blood samples were obtained from patients with SV (n = 12) or stable NSV (n = 22) aged ≥ 18 years of the outpatient clinic at the Amsterdam University Medical Center, according to current vitiligo classification and disease activity scoring (Rodrigues et al., 2017, van Geel et al., 2019). Exclusion criteria were disease activity in the past 12 months during standard of care treatment. Likewise, we recruited healthy control subjects (n = 20) aged ≥ 18 years. The demographic characteristics of patients with vitiligo and healthy controls are represented in **table 1**. Peripheral blood mononuclear cells (PBMC) were purified from whole blood by density gradient centrifugation (LymphoPrep, Stemcell Technologies, Vancouver, Canada) and cryopreserved prior to analysis.

Antibodies and flow cytometry

Fluorochrome-conjugated antibodies are specified in **table S4**. Cell surface staining was performed in FACS buffer (PBS supplemented with 1% bovine serum albumin and 0.05% NaN_3). Subsequently, cells were fixed in True-Nuclear™ Fix (Biolegend, San Diego, CA) and stained intranuclear in True-Nuclear™ Perm Buffer (Biolegend), according to the manufacturer's instructions. FACS acquisition were performed on a FACSCanto II B (BD Biosciences, Franklin Lakes, NJ) using BD FACSDiva software (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Gene expression analysis

The R2 Genomics Analysis and Visualization platform (http://r2.amc.nl) was used for analysis of gene expression profiles of NSV, SV patients and healthy individuals (GSE80009) (Wang et al., 2016).

Radioligand binding assays

Antibodies in serum samples were detected using radioligand binding assays (RBAs). Plasmids pcDNA3-TH, pcDNA3_TYR, pcDNA3-PMEL17, pcDNA3-MCHR1 and pcDNA-Melan-A (MART-1) were used according to the manufacturer's instructions in an *in vitro* TnT® T7-coupled Reticulocyte Lysate System (Promega, Madison, WI) with [35S]-methionine to produce radiolabeled full-length TYR, TRP1, TRP2, Pmel, TH, MART-1 and MCHR1, respectively. Next, radiolabeled antigens were used in RBAs with patient sera (n=34) and healthy control (n=30) at a 1:100 dilution. The antibody index is calculated as cpm immunoprecipitated by tested serum/mean cpm immunoprecipitated by the group of healthy control sera (Kemp et al., 2002). Each serum was tested in at least three independent experiments. The mean antibody index was calculated from these values. Patient sera with an antibody index above the upper limit of normal (mean antibody index + 3x standard deviation of the healthy control individuals) were regarded as positive for antibody reactivity.

Statistical analysis

Statistical analysis was performed using Graphpad Prism software (Graphpad Software Inc., San Diego, CA). Comparisons were made with ANOVA analysis,

Student's *t* test or Mann-Whitney test. Tukey's multiple comparisons corrections were applied for ANOVA analysis. P-values less than 0.05 were considered statistically significant; *P <0.05, **P < 0.01, ***P <0.001 and ****P <0.0001.

Data availability statement

No publicly available datasets were generated during the current study. Dataset GSE80009 was used for gene expression analysis.

Author contributions

Conceptualization: MW, RML; Methodology: MW, NOPVU, SC, RML; Formal Analyses: MW; Investigation: MW, NFP, NOPVU, VSN, SC, EHK; Visualization: MW; Supervision: MWB, RML; Funding Acquisition: RML; Writing – original draft: MW, RML; Writing – review and editing: MW, NFP, NOPVU, VSN, SC, EHK, MWB, RML.

Conflict of interest

The authors state no conflicts of interest.

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Supplementary materials

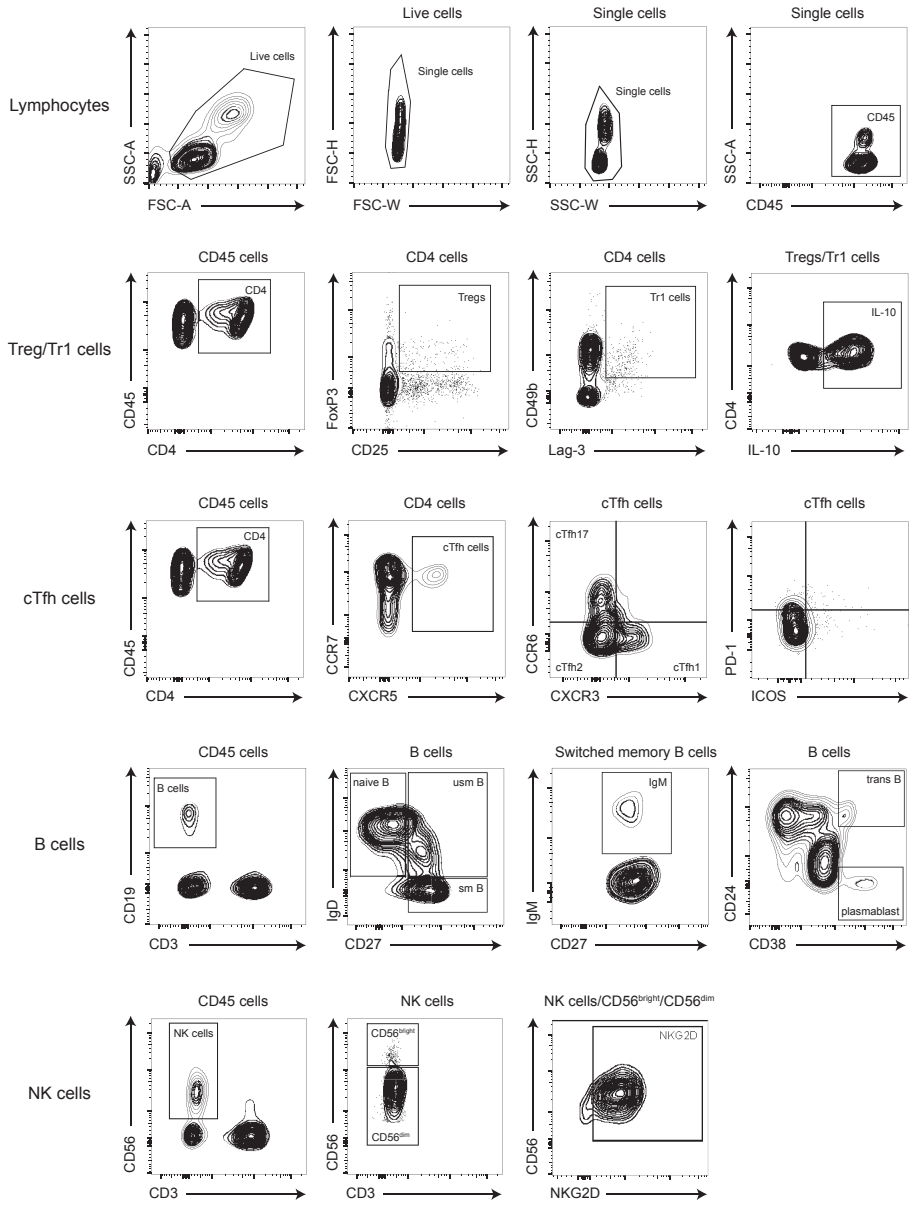

Figure S1. Gating strategy for lymphocyte subpopulations. Regulatory T cells, cTfh cells, B cells and NK cells were analyzed in 4 separate flow cytometry panels. In each panel, lymphocytes were selected as CD45⁺ cells (upper row). Tregs were considered

to be $CD25^+$ $FoxP3^+$ cells from $CD4^+$ cells and Tr1 cells were gated as $CD49b^+$ $Lag\text{-}3^+$ cells from $CD4^+$ cells (second row). In both Tregs and Tr1 cells IL-10-producing cells were studied. cTfh cells were gated as $CXCR5^+$ cells from $CD4^+$ cells (third row). Based on the expression of CCR6 and CXCR3, we identified cTfh1 cells ($CCR6^-$ $CXCR3^+$), cTfh2 cells ($CCR6^-$ $CXCR3^-$) and cTfh17 cells ($CCR6^+$ $CXCR3^-$). In all cTfh subsets we studied the activation status by the markers PD-1 and ICOS. B cells were identified as $CD19^+$ $CD3^-$ cells from $CD45^+$ cells (fourth row). B cells were then classified into naïve B cells (IgD^+ $CD27^-$), unswitched memory B cells (IgD^+ $CD27^+$) and switched memory B cells (IgD^- $CD27^+$). Within the switched memory B cell population we analyzed the immunoglobulin subtype by staining for IgM. From B cells, we could also identify transitional B cells ($CD24^+$ $CD38^+$) and plasmablasts ($CD24^-$ $CD38^+$). Finally, NK cells were considered to be $CD3^-$ $CD56^+$ cells (bottom row). Within the NK cell population, we separated $CD56^{bright}$ and $CD56^{dim}$ NK cells. Finally, we analyzed the expression of NKG2D on these cells.

Table S1. Differentially expressed genes between $CD25^{high}$ Tregs and conventional $CD25^-$ $CD4^+$ T cells

Gene	Upregulated/downregulated on $CD25^{high}$ Tregs
FOXP3	Upregulated
IKZF2	Upregulated
IL2RA	Upregulated
CTLA4	Upregulated
TIGIT	Upregulated
TNFRSF18 (GITR)	Upregulated
TNFRSF4 (OX40)	Upregulated
LAG3	Upregulated
HAVCR2 (TIM-3)	Upregulated
LRRC32 (GARP)	Upregulated
ICOS	Upregulated
IL-10	Upregulated
EBI3 (IL35B)	Upregulated
IL1RL1 (ST2)	Upregulated
BATF	Upregulated
LAYN	Upregulated
CSF2RB	Upregulated
TRIB1	Upregulated
ENTPD1 (CD39)	Upregulated
UTS2	Upregulated
RTKN2	Upregulated
IL7R	Downregulated
ENC1	Downregulated
NKG7	Downregulated
CD40LG	Downregulated

Table S2. Antibody indexes for sera from antibody-positive NSV patients

	TYR	TRP1	TRP2	PMEL	TH	MART-1	MCHR1
Patient 19	1,1	1,02	0,69	1,06	**3,46**	0,98	0,99
Patient 21	**5,89**	**3,91**	**4,09**	0,92	0,92	1,10	1,09
Patient 23	**3,95**	**2,85**	**2,82**	1,15	1,09	0,99	0,94
Patient 24	1,02	1,10	0,88	**2,94**	1,06	1,00	0,91
Patient 29	**7,88**	**5,71**	**5,48**	0,98	1,02	0,88	1,02
Patient 30	0,83	0,85	1,09	1,01	**4,29**	1,07	0,94
Patient 33	1,06	1,16	0,96	**5,12**	0,86	0,91	0,98
Patient 40	1,04	0,91	0,93	**3,74**	1,14	1,05	1,11

Table S3. Patient characteristics

	Antibody-positive NSV			Antibody-negative NSV		
	N	%	IQR/SD[1]	N	%	IQR/SD
Total	8			14		
Age						
< 25				1	7	
25-50	6	75		8	57	
> 50	2	25		5	36	
Mean	46		(29 - 63)	42		(30-54)
Gender						
Male	2	25		9	64	
Female	6	75		5	36	
Skin type[2]						
Type 1	0			1	7	
Type 2	5	62.5		7	50	
Type 3	3	37.5		5	36	
Type 4	0			0		
Type 5	0			1	7	
Type 6	0			0		
Comorbidities	1	12.5		5	36	
Vitiligo age of onset (years) mean	32		± 13.9	34		± 19.1

Disease duration (years) median	7	(4 - 11.3)	8	(4.5 - 20)
% Affected body surface area median	1	(1 - 4)	1.5	(0.5 - 4.6)

[1] Interquartile range/standard deviation
[2] Skin type according to the Fitzpatrick skin scale

Table S4. Used antibodies

Marker	Clone	Fluorochrome	Company	Catalogue no.
CD45	2D1	BV510	Biolegend	368526
CD3	SK7	FITC	Biolegend	344804
CD56	HCD56	BV421	Biolegend	318327
CD16	3G8	PE-Cy7	Biolegend	302015
Granzyme B	QA16A02	APC/Fire™ 750	Biolegend	372210
CD94	DX22	PE	Biolegend	305506
NKG2D	1D11	APC	Biolegend	320808
NKp44	P44-8	PerCP-Cy5.5	Biolegend	325113
CD27	O323	PE-Cy7	Biolegend	302838
IgD	IA6-2	PE	Biolegend	348204
CD19	HIB19	APC	Biolegend	302212
CD24	ML5	PerCP-Cy5.5	Biolegend	311116
CD38	HIT2	BV421	Biolegend	303526
IgM	MHM-88	APC/Fire™ 750	Biolegend	314546
CXCR5	J252LD4	PE	Biolegend	356904
CXCR3	G025H7	PerCP	Biolegend	353740
CCR7	G043H7	BV421	Biolegend	353208
CCR6	G034E3	APC	Biolegend	353416
ICOS	C398.4A	APC/Fire™ 750	Biolegend	313536
CD49b	P1E6-C5	APC	Biolegend	359310
CD25	BC96	APC/Fire™ 750	Biolegend	302642
CD4	SK3	FITC	Biolegend	344604
Lag-3	11C3C65	PerCP	Biolegend	369312
CD127	A019D5	PE	Biolegend	351304
IL-10	JES3-9D7	PE-Cy7	Biolegend	501420
FOXP3	206D	BV421	Biolegend	320124
PD-1	MIH4	PE-Cy7	eBioscience	25-9969-42

Chapter 3

Skin-resident memory T cells as a potential new therapeutic target in vitiligo and melanoma

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Abstract

Tissue-resident memory T (T_{RM}) cells are abundant in the memory T cell pool and remain resident in peripheral tissues, such as the skin, where they act as alarm sensors or cytotoxic killers. T_{RM} cells persist long after the pathogen is eliminated and can respond rapidly upon re-infection with the same antigen. When aberrantly activated, skin-located T_{RM} cells have a profound role in various skin disorders, including vitiligo and melanoma. Autoreactive T_{RM} cells are present in human lesional vitiligo skin and mouse models of vitiligo, which suggests that targeting these cells could be effective as a durable treatment strategy for vitiligo. Furthermore, emerging evidence indicates that induction of melanoma-reactive T_{RM} cells is needed to achieve effective protection against tumor growth. This review highlights seminal reports about skin-resident T cells, focusing mainly on their role in the context of vitiligo and melanoma, as well as their potential as therapeutic targets in both diseases.

Introduction

The presence of a pathogen stimulates naïve T cells to differentiate into memory and effector T cells in order to eliminate pathogen-infected cells. Memory T cells can be sub-grouped into central memory T (T_{CM}) cells, effector memory T (T_{EM}) cells and migratory memory T (T_{MM}) cells (Sallusto, Lenig, Forster, Lipp, & Lanzavecchia, 1999; Watanabe et al., 2015). The T_{CM} cell pool predominates in secondary lymphoid organs and express markers, such as chemokine receptor CCR7 and the vascular addressin L-selectin (CD62L). In contrast, T_{EM} cells migrate into the non-lymphoid tissues to clear the infection with their high cytokine production capacity and perforin expression. T_{EM} cells express low CCR7 and CD62L levels, but can express high levels of the tissue-homing addressin and E-selectin ligand Cutaneous Lymphocyte Antigen (CLA), which enables them to enter into the skin (Farber, Yudanin, & Restifo, 2014; Mueller, Gebhardt, Carbone, & Heath, 2013). Expression of CCR7 and absence of CD62L characterizes migratory memory T (T_{MM}) cells, which recirculate between blood and tissues and are excluded from the lymph nodes (Watanabe et al., 2015).

Memory T cells were initially considered to be circulatory and to enter the tissues only when needed, to clear an infection. Work over the past

years has defined another pool of memory T cells, called resident memory T (T_{RM}) cells (Gebhardt et al., 2009; Masopust et al., 2010; Wakim, Waithman, van Rooijen, Heath, & Carbone, 2008). T_{RM} cells do not recirculate, but reside permanently in tissues such as skin, intestine, lung, brain and female reproductive tract, where they provide rapid protective immunity against re-infecting pathogens (Jiang et al., 2012; Lefrancois & Masopust, 2002; Mueller & Mackay, 2016). Upon viral or bacterial infection, antigen-specific primary and memory $CD8^+$ T cells become present throughout the body. Resident memory $CD8^+$ T cells isolated from non-lymphoid tissues showed higher antigen-specific response than circulatory memory cells isolated from lymphoid tissues (Masopust, Vezys, Marzo, & Lefrancois, 2001). T_{RM} can even respond more rapidly to tissue infection than circulatory memory cells (Ariotti et al., 2014; Clark, 2010; Schenkel et al., 2014). The T_{RM} cell population within each tissue is capable of recognizing the specific pathogens that most commonly affect those tissues, and T_{RM} cells remain in place long after pathogen elimination (Gebhardt et al., 2009; Jiang et al., 2012; Mackay et al., 2012).

Besides eliminating pathogens, T_{RM} cells may also contribute to various disorders when aberrantly activated. These cells can develop not only after pathogen infection, but also after sensitization to otherwise harmless environmental or self-antigens (Clark, 2015). The involvement of T_{RM} cells has been demonstrated in various skin diseases, such as psoriasis (Cheuk et al., 2014; Clark, 2011; Matos et al., 2017; Suarez-Farinas, Fuentes-Duculan, Lowes, & Krueger, 2011), fixed drug eruptions (Shiohara, 2009), allergic contact dermatitis (Gaide et al., 2015; Honda, Egawa, Grabbe, & Kabashima, 2013), cutaneous T cell lymphoma - a malignancy of T_{RM} cells (Campbell, Clark, Watanabe, & Kupper, 2010) and vitiligo (Boniface et al., 2018; Cheuk et al., 2017; Richmond, Strassner, Rashighi, et al., 2018; Richmond, Strassner, Zapata, et al., 2018).

Interestingly, auto-immunity and tumor immunity are often linked, as exemplified by the association between vitiligo and melanoma. Overwijk *et al*. (2003) showed that the same specific lymphocytic response could promote tumor destruction and vitiligo, in the exact same mouse (Overwijk et al., 2003). Adaptive transfer of gp100-specific $CD8^+$ T cells in mice bearing B16 melanoma cured the mice of the tumor, but also caused vitiligo. The vitiligo started at the former tumor site, and even one year after therapy these

mice remained tumor-free with progressive vitiligo. Gp100 is a member of a family of "self" (i.e., unmutated), melanoma/melanocyte differentiation antigens that are widely expressed by melanoma cells. Hence, vitiligo was caused by activated anti-melanoma immunity that not only targeted malignant cells, but also healthy melanocytes. A subsequent study reported that tumor-bearing mice with vitiligo generated 10-fold larger $CD8^+$ memory T cell populations that are specific for shared melanoma/melanocyte antigens than mice without vitiligo (Byrne et al., 2011). These responses were not observed in melanocyte-deficient mice. $CD8^+$ T cells in mice with vitiligo acquired phenotypic and functional characteristics of T_{EM} cells, suggesting that they were supported by ongoing antigen stimulation. Conversely, melanocyte-deficient mice did not generate such protective responses, indicating a requirement for melanocyte destruction as antigen source in maintaining $CD8^+$ T cell immunity to melanoma.

In humans, it has been observed that vitiligo can occur in melanoma patients spontaneously or during immunotherapy treatment and correlates with prolonged survival (Boasberg et al., 2006; Gogas et al., 2006; Quaglino et al., 2010; Teulings et al., 2015). Conversely, vitiligo patients have 3-fold lower probability of developing melanoma during their lifespan than non-vitiligo patients (Paradisi et al., 2014; Teulings et al., 2013). Recent work has indicated the pathogenic involvement of T_{RM} cells in human vitiligo (Boniface et al., 2018; Cheuk et al., 2017; Richmond, Strassner, Rashighi, et al., 2018; Richmond, Strassner, Zapata, et al., 2018) and data on this is still emerging. Other studies have demonstrated a protective role for T_{RM} cells in melanoma (Boddupalli et al., 2016; Edwards et al., 2018; Enamorado et al., 2017; Gálvez-Cancino et al., 2018; Malik et al., 2017; Murray et al., 2016; Park, Buzzai, et al., 2018). The present review highlights seminal papers on skin-resident memory T cells in the context of vitiligo and melanoma and addresses the potential significance of these cells for the treatment of vitiligo and melanoma.

Features of skin-resident memory T cells

Phenotypic characteristics of skin-resident T_{RM} cells

The human skin contains approximately one million T cells per cm^2, which amounts to almost 20 billion T cells in total (Clark et al., 2006). This is nearly

twice as many T cells as those circulating in the blood. T_{RM} cells, like all memory T cells, can be distinguished from naïve T cells by expression of CD44, a marker of antigen experience. Furthermore, T_{RM} cells lack expression of CD62L and CCR7; which differentiates them from recirculating T_{CM} and T_{MM} cells (**Figure 1**). The chemokine receptor CCR7 interacts with CCL19 and CCL21, thereby helping T cells to migrate towards lymph nodes. As CCR7 expression is needed for T cell egress from peripheral tissues, $CCR7^-$ T cells in tissue can be considered tissue-resident (Bromley, Thomas, & Luster, 2005; Bromley, Yan, Tomura, Kanagawa, & Luster, 2013; Debes et al., 2005). Another study showed that in normal skin under resting conditions, more than 90% of $CCR7^-$ $CD62L^-$ T cells co-expressing the skin homing molecule CLA are skin-resident (Clark et al., 2006).

To discriminate T_{RM} cells from T_{EM} cells, more phenotypic markers are needed. In human skin, 50-70% of T cells express CD69 and CD103 (Watanabe et al., 2015). Although CD69 has been characterized as a T cell activation marker, it has been shown to be constitutively expressed by a subset of T cells within peripheral tissues under steady-state conditions (**Figure 1**) (Shiow et al., 2006).

A subset of T_{RM} cells also expresses CD103, which is the α-subunit of the α3β7 integrin receptor (**Figure 1**). In healthy human skin, its expression is most prominent on epidermal $CD4^+$ and $CD8^+$ T_{RM} cells, where it enables T_{RM} cell tethering within the epidermal compartment by binding to E-cadherin, which is widely expressed by epithelial cells (Mackay et al., 2012). Nevertheless, binding to E-cadherin is not required for skin residency (Nestle, Di Meglio, Qin, & Nickoloff, 2009). Although $CD4^+$ and $CD8^+$ $CD103^+$ T_{RM} cells are less proliferative than $CD103^-$ T_{RM} cells, $CD103^+$ T_{RM} cells have a larger effector cytokine-production capacity (Watanabe et al., 2015). Relative proportions of resident and recirculating memory T (T_{CIRC}) cells have been measured in highly immunocompromised NOD SCID IL-2Rγ-deficient (NSG) human-engrafted mice and in lymphoma patients upon alemtuzumab treatment, which is an antibody specific to CD52 (expressed by T cells). Alemtuzumab depletes T cells by antibody dependent cellular cytotoxicity. This requires neutrophils and/or natural killer cells, which are relatively abundant in the circulation, but are rare in peripheral tissues. Alemtuzumab, therefore, only depletes T_{CIRC} cells, but not T_{RM} cells, which makes it possible to determine the relative proportions of both subsets. In healthy adult human skin, most T_{RM}

cells are CD103$^-$ CD4$^+$ and reside in the dermis. While CD103$^+$ T_{RM}, both CD4$^+$ and CD8$^+$, are more frequent in the epidermis, recirculating T cells are the minority among both CD4$^+$ and CD8$^+$ T cell populations in skin (Watanabe et al., 2015). The resident T cell populations in human skin thus differ in their migration compartments and functional capacities.

The α-subunit of the α1β1 integrin receptor, CD49a (also known as very-late antigen (VLA)-1) was identified to delineate a subset of CD8$^+$ T_{RM} cells in human skin epithelia that preferentially localize to the epidermis. These cells are poised towards IFN-γ production and acquire high cytotoxic capacity upon IL-15 stimulation (Cheuk et al., 2017). In the same study, CD49a$^-$ CD8$^+$ T_{RM} cells excelled at IL-17 production, and expression of CD49a was restricted solely to CD8$^+$ T cells. Moreover, CD49a binds to collagen IV, a major component of the basement membrane between epidermis and dermis.

Tissue retention and transcriptional signatures shared by T_{RM} cells

Various molecular factors have been implicated in tissue retention. CD69 transcriptionally downregulates the sphingosine-1-phosphate receptor (S1P1), a G protein-coupled receptor for sphingosine 1-phosphate (S1P) (Mackay, Braun, et al., 2015). This limits egress of these memory cells out of tissues, showing that S1P1 downregulation is needed for long-term residency of T_{RM} cells (Skon et al., 2013). Conversely, S1P1, through detection of its ligand S1P in the blood and lymph, is essential for naive lymphocytes to access the circulatory system from the thymus and lymph nodes (Matloubian et al., 2004). Effector T cells also use S1P1 to sense S1P gradients among tissues, lymph and blood, thereby guiding entry in efferent lymphatics from lymphoid tissues (Spiegel & Milstien, 2011).

Expression of S1P1 can also be regulated by the transcription factor Kruppel-like factor 2 (KLF2) (Skon et al., 2013). KLF2 was not expressed by CD69$^+$ mouse memory CD8$^+$ T cells isolated from non-lymphoid tissues. Hence, T_{RM} did not express its target gene *S1pr1* (encoding S1P1), while forced S1P1 expression prevented establishment of T_{RM} cells. Furthermore, cytokines capable of inducing the CD69$^+$ CD103$^+$ resident phenotype (including TGF-β, IL-33 and TNF), provoked KLF2 downregulation and thus downregulation of S1P1.

Key phenotypic properties and location of skin-resident memory T cells

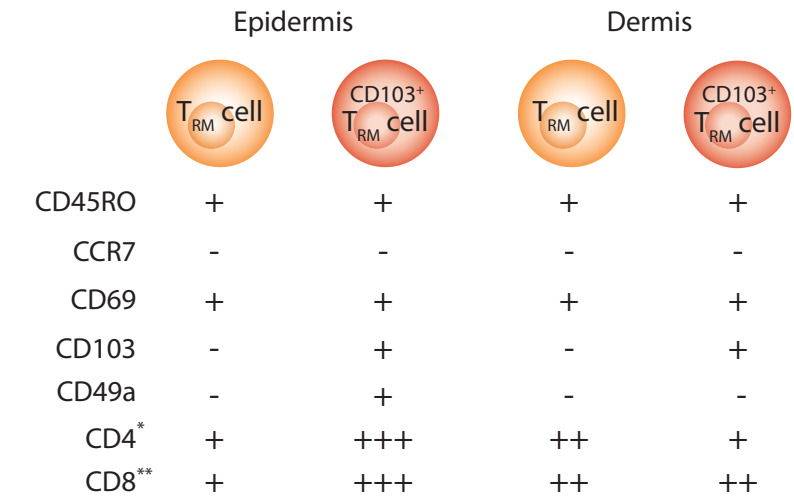


Figure 1. Key phenotypic properties and location of skin-resident T cells. Phenotypic characteristics of skin-resident memory T cells and distribution in human skin are shown. All memory T cells express CD45RO, but the absence of CCR7 and expression of CD69 and CD103 distinguishes T_{RM} cells from circulating memory T cells. CD49a is found on epidermal $CD69^+$ $CD103^+$ $CD8^+$ T cells only. CD69 and CD103 can be found on both $CD4^+$ and $CD8^+$ T cells, but at different levels. $CD4^+$ T cells constitute approximately 75% of the lymphocytes present in both layers of the healthy human skin. However, there are twice as many $CD103^+$ T_{RM} cells (both $CD4^+$ and $CD8^+$) in the epidermis, while in the dermis the majority of T_{RM} cells are $CD103^-$. -, no expression; + expression. Regarding $CD4^+$ and $CD8^+$: + low expression frequency; ++ medium expression frequency; +++ high expression frequency.
* Fraction of $CD69^+CD4^+$ and $CD69^+CD103^+CD4^+$ T_{RM} cells of the total $CD4^+$ T_{RM} cell pool in either the epidermis or dermis are shown.
**Fraction of $CD69^+CD8^+$ and $CD69^+CD103^+CD8^+$ T_{RM} cells of the total $CD8^+$ T_{RM} cell pool in either the epidermis or dermis are shown.

Expression of CD103 (or its ligand, E-cadherin) by T_{RM} cells contributes to their maintenance in some non-lymphoid tissues (Hofmann & Pircher, 2011), but is not a universal mechanism for residency retention in all tissues. For example, Casey *et* al. (2012) showed that while CD103 was required for maintenance of T_{RM} cells in the small intestinal intraepithelial lymphocyte population, it was found to be dispensable for memory cell establishment in the lamina propria lymphocyte population of the same organ.

Other factors involved in tissue retention include inflammatory cytokines such as transforming growth factor (TGF)-β, interleukin (IL)-33 and tumor-necrosis factor (TNF)-α. TGF-β was shown to induce CD103 expression on mouse memory $CD8^+$ T cells, and IL-33 and TNF-α were found to synergize with TGF-β (Casey et al., 2012). This resulted in memory cells that adopted a resident phenotype ($CD69^+$ $CD103^+$) and indicates that tissues can intrinsically support differentiation of T_{RM} cells by the cytokine milieu. Stromal cells control tissue residency of memory T cells by expression of integrins, thereby regulating activation of TGF-β (Mohammed et al., 2016). Moreover, TGF-β and IL-15 signaling were shown to be needed for development of T_{RM} cells in skin (Mackay et al., 2013). IL-15 promoted formation and survival of T_{RM} cells in mice. IL-15 deficient mice had reduced T_{RM} cell formation, and this correlated with reduced Bcl-2 expression, a prosurvival molecule, in $CD103^+$ T_{RM} cells. Similarly, CD69 is rapidly induced in response to type 1 interferon (IFN) and suppresses S1P1 expression (Shiow et al., 2006).

It has been shown that T_{RM} have a transcriptional profile that is distinct from their memory T cell counterparts and includes transcription factors Hobit, Blimp1, and Runx3. In mice, the transcription factor Hobit is specifically upregulated in T_{RM} cells and, together with Blimp1, instructs tissue retention in different epithelial barrier tissues (Mackay et al., 2016). While Hobit was found to be essential for T_{RM} cell development, Blimp1 by itself was not, but synergized with Hobit. Also, Blimp1 was shown to initiate cytotoxic effector function while Hobit was essential in the long-term maintenance of granzyme B-driven cytotoxicity (Kragten et al., 2018). The expression of Hobit is regulated by IL-15 and the transcription factor T-bet (Mackay, Wynne-Jones, et al., 2015). In the absence of IL-15, T_{RM} cells had decreased Hobit levels, and upon IL-15 stimulation, activated $CD8^+$ T cells upregulated Hobit expression in a T-bet-dependent manner (Mackay et al., 2016). Blimp1 expression, however, is not induced by IL-15 or T-bet. Its expression is regulated by the transcription factor Runx3 (D. Wang et al., 2018), which also promotes the expression of the T_{RM} retention markers CD69 and CD103 (Milner et al., 2017).

Data on human T_{RM} cell transcriptional profiles are now emerging. Compared to their circulating counterparts, $CD8^+$ T_{RM} cells isolated from human lungs expressed high levels of *GZMB, IFNG, TNF,* and *NOTCH1* transcripts (Hombrink et al., 2016). Additionally, $CD69^+$ memory cells from lung, spleen, and blood exhibited a transcriptional signature including

CD103 and CD49a, chemokine receptors CXCR6 and CX3CR1, and immune checkpoint PD-1 (Kumar et al., 2017). Despite similar core signatures with mouse T_{RM} cells, human T_{RM} cells lacked expression of Hobit.

Immunosurveillance and protection by T_{RM} cells

Although T_{RM} cells do not recirculate throughout the body, they can migrate slowly within their environment. Antigen-specific $CD8^+$ T cells have been shown to crawl slowly between keratinocytes (Ariotti et al., 2012). This enables T_{RM} cells to identify antigen-expressing target cells at different tissue locations within minutes to hours (Ariotti et al., 2014; Gebhardt et al., 2011). Their ability to scan the environment in which they persist after a primary infection is associated with enhanced pathogen detection upon reinfection by pathogens (Ariotti et al., 2012). T_{RM} cells are located in frontline sites of infection, such as the skin, lungs and intestines and, therefore tend to respond rapidly to pathogen re-challenge.

Additionally, upon antigen resensitization T_{RM} cells trigger rapid innate and adaptive immune responses by secreting cytokines. Initially, T_{RM} cells can attract circulating memory T cells within hours by producing IFN-γ (Schenkel, Fraser, Vezys, & Masopust, 2013). Moreover, T_{RM} cell-derived IFN-γ initiates an anti-pathogen state at the local tissue site (Ariotti et al., 2014). At the same time, activated T_{RM} cells express TNF-α, which is essential for dendritic cell maturation (Schenkel et al., 2014). Also, $CD4^+$ and $CD8^+$ $CD69^+$ T cells are able to produce IL-22, IL-17 and anti-inflammatory IL-10. T_{RM} cells can thus trigger inflammation by pro-inflammatory cytokines, but prevent excessive inflammation through IL-10 (Kumar et al., 2017). Within 12 hours of local reactivation T_{RM} cells express IL-2, which leads to elevated levels of granzyme B secreted by both T_{RM} and natural killer cells (Schenkel et al., 2014). After local pathogen challenge, T_{RM} cells proliferate in situ and recruit memory T cells from the circulation, which subsequently undergo T_{RM} cell differentiation (Park, Zaid, et al., 2018). As a result, secondary T_{RM} cells are generated from pre-existing T_{RM} cells, as well as from recirculating precursors. However, the pre-existing T_{RM} cell populations are not displaced and remain in place in the tissue.

Despite their role in conferring protective immunity, T_{RM} cells can become pathologically activated and can cause tissue-specific autoimmunity and inflammatory disease (Clark, 2015). The clinical characteristics of inflammatory

lesions caused by T_{RM} cells manifest as fixed, delineated zones of lesions, with an abrupt cut-off from non-lesional tissues. The pathogenic role of T_{RM} cells has been shown in many diseases, including psoriasis (Cheuk et al., 2014; Clark, 2011; Matos et al., 2017; Suarez-Farinas et al., 2011) and cutaneous T cell lymphoma (Campbell et al., 2010). Commonly used treatments for psoriasis cannot fully deplete the pathogenic T_{RM} cells from skin lesions (Cheuk et al., 2014; Matos et al., 2017). This appears to explain why psoriatic lesions often reoccur at exactly the same anatomical location after therapy cessation.

The role of T_{RM} cells in autoimmune vitiligo

T_{RM} cells in the pathogenesis of vitiligo

Vitiligo is a common autoimmune disease, affecting approximately 1% of the general population. It results from the loss of epidermal melanocytes (Ezzedine, Eleftheriadou, Whitton, & van Geel, 2015). Genetic predisposition, environmental factors, and metabolic and immune alterations have been implicated in melanocyte destruction (Gauthier, Cario Andre, & Taieb, 2003; Picardo et al., 2015; Spritz, 2012). Previous studies have clarified the autoimmune etiology in human vitiligo. For example, vitiligo patients have melanocyte-specific $CD8^+$ T cells that are capable of killing melanocytes (Ongenae, Van Geel, & Naeyaert, 2003; Palermo et al., 2001; van den Boorn et al., 2009) and initiating antibody responses against melanocyte antigens, such as tyrosinase and TRP-2 (Kemp, Gavalas, Gawkrodger, & Weetman, 2007).

Vitiligo lesions often recur at the same locations as those previously affected, suggesting that T_{RM} cells could be involved. A mouse model of melanoma-associated vitiligo showed that both lesional and non-lesional skin contained resident memory T cells, although they were preferentially localized in hair follicles containing white hairs (Malik et al., 2017). To induce melanoma-associated vitiligo, mice were inoculated with B16 melanoma cells and depleted from regulatory T cells, after which the tumor was surgically removed. Vitiligo-affected skin was shown to have $CD8^+$ T cells recognizing tumor/self-antigens and to exhibit a T_{RM} cell phenotype ($CD44^{hi}$ $CD62L^{lo}$ $CD69^+$ $CD103^+$). In line with this, autoreactive $CD8^+$ T cells with a $CD69^+$ $CD103^{+/-}$ T_{RM} phenotype have been found in the skin of vitiligo patients

(**Figure 2**) (Boniface et al., 2018; Cheuk et al., 2017; Richmond, Strassner, Rashighi, et al., 2018; Richmond, Strassner, Zapata, et al., 2018). Compared to healthy unaffected donor or psoriasis skin, lesional skin from vitiligo patients was shown to be enriched with CD49a$^+$ CD103$^+$ CD8$^+$ (Cheuk et al., 2017) and CD69$^+$ CD103$^{+/-}$ CD8$^+$ T_{RM} cells, independent of disease activity (Boniface et al., 2018). In the same study it was suggested that the remaining CD8$^+$ T_{RM} cells could possibly mediate disease flares or, alternatively, block repigmentation. CD8$^+$ T_{RM} cells may prevent repigmentation by blocking either renewal of epidermal melanocytes or entry from the follicular reservoir of melanocyte precursors.

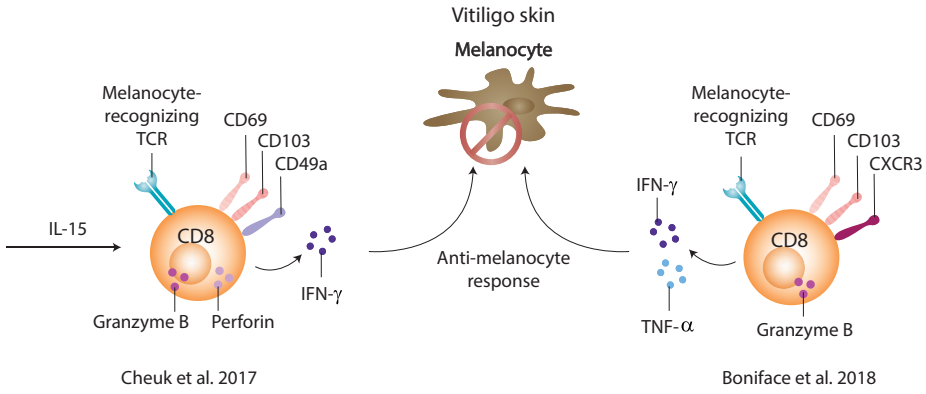


Figure 2. Resident memory T cells in vitiligo. The role of resident memory T cells in human vitiligo is shown. Firstly, Cheuk *et al.* (2017) reported an increase in CD49a$^+$ T_{RM} cells in vitiligo skin, which produce IFN-γ, granzyme B and perforin upon IL-15 stimulation. Furthermore, a substantial proportion of CD49a$^+$ T_{RM} cells recognised melanocyte-antigens, indicating a pathogenic role. Secondly, Boniface *et al.* (2018) showed vitiligo perilesional skin to be enriched with melanocyte-specific CXCR3$^+$CD8$^+$ T_{RM} cells and CD8$^+$ T_{RM} cells were poised for secretion of IFN-γ and TNF-α with moderate cytotoxic activity.

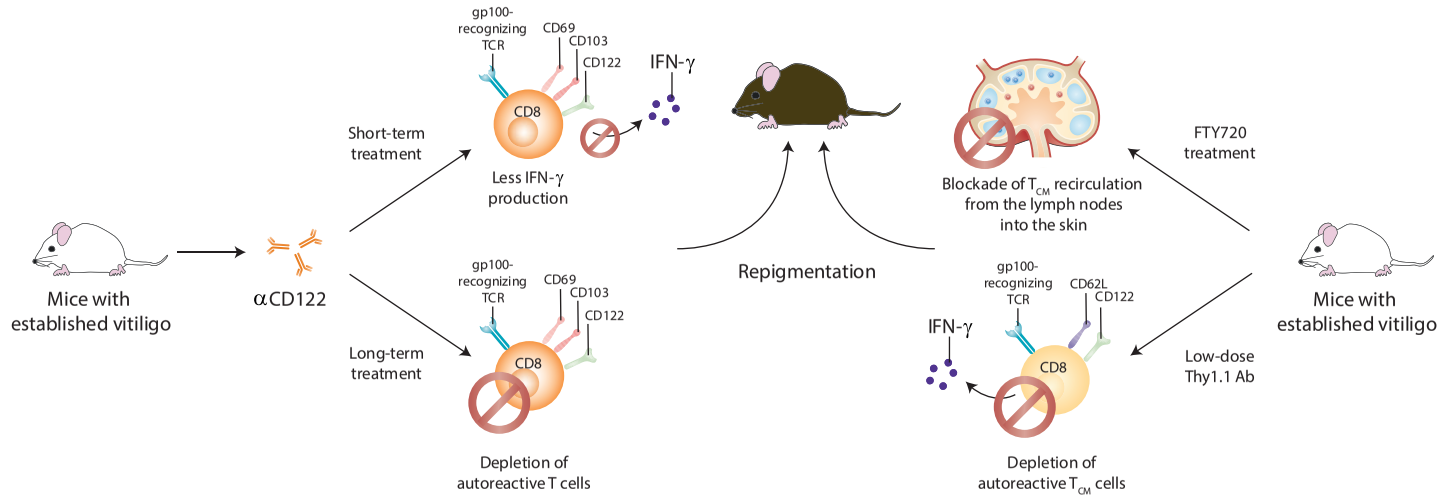
The chemokine receptor CXCR3 (the receptor for the chemokines CXCL9 and CXCL10) was shown to be important for epidermal localization of effector T cells and T_{RM} cell development (Mackay et al., 2013), which led to studies on CXCR3 expression in vitiligo patients. Perilesional skin from patients with progressive disease showed high CXCR3 expression (Bertolotti et al., 2014). Expression of CXCR3 was found on the majority of CD8$^+$ T_{RM} cells in human vitiligo, including melanocyte-specific cells, and these T_{RM} cells

were poised for the secretion of IFN-γ and TNF-α (Boniface et al., 2018). These results indicated that targeting the CXCL9/10-CXCR3 pathway could be an attractive strategy for the treatment of vitiligo. In line with this, another study reported an enrichment of IFN-γ-producing CD49a$^+$ CD103$^+$ CD8$^+$ T_{RM} cells in vitiligo lesions, with a rapid granzyme B and perforin production response upon IL-15 stimulation (Cheuk et al., 2017). Two studies showed that IFN-γ and granzyme B are key cytokines in the pathogenesis of vitiligo because they induce melanocyte apoptosis (Harris et al., 2012; Yang et al., 2015). These results add to improved understanding of the autoimmune response in human vitiligo and suggest a profound role for CD8$^+$ T_{RM} cells in human vitiligo, which explains the interest in targeting this cell subset in the treatment of vitiligo patients.

Therapeutic intervention in vitiligo

Based on the fundamental pathogenic role of T_{RM} cells in human vitiligo, novel strategies specifically targeting T_{RM} cells may improve the treatment outcome of vitiligo. T_{RM} cell formation has been shown to be highly dependent on IL-15, and IL-15 promoted T_{RM} cell function ex vivo (Adachi et al., 2015; Mackay et al., 2013). A subsequent study therefore looked at IL-15 signaling as a therapeutic target for vitiligo (Richmond, Strassner, Zapata, et al., 2018). Treatment with anti-CD122 antibody, a subunit of the IL-15 receptor on human and mouse T_{RM} cells, was shown to reverse disease in mice with established vitiligo (**Figure 3**). A 2-week short-term treatment decreased IFN-γ production, while an 8-week long-term treatment depleted autoreactive CD8$^+$ T_{RM} cells. These findings indicate that targeting IL-15 signaling via CD122 may be an effective strategy to treat vitiligo and possibly other T_{RM} cell-mediated diseases. T_{RM} cell survival and function also depends on the uptake of exogenous lipids and on their oxidative metabolism (Pan et al., 2017). Future studies targeting this pathway might reveal if it can affect or even deplete T_{RM} cells from peripheral tissues.

Besides melanocyte-specific T_{RM} cells, autoreactive T_{CM} cells have been found in the blood of vitiligo patients (Ogg, Rod Dunbar, Romero, Chen, & Cerundolo, 1998; van den Boorn et al., 2009), and these cells have the potential to home to the skin. However, the functional capacity of T_{CM} has remained unknown. A study examining the functional relationship between T_{CM} and T_{RM} in a vitiligo mouse model reported that T_{CM} cells cooperate with T_{RM} cells



Richmond, Strassner, Zapata et al. 2018

Richmond, Strassner, Rashighi et al. 2018

Figure 3. Therapeutic intervention in vitiligo. Potential therapeutic approaches for vitiligo are illustrated. Two murine studies showed that targeting T_{RM} cells can reverse disease in mice with established vitiligo. The left model shows T_{RM} cells express the IL-15 receptor subunit CD122 and treatment with anti-CD122 antibody led to repigmentation. Short-term treatment led to less IFN-γ production by T_{RM} cells and long-term treatment depleted autoreactive T_{RM} cells and other memory T cell pools. The right model shows that T_{RM} and T_{CM} cells cooperate to maintain vitiligo. Treatment of mice with FTY720, which limits T cell access to the skin, or low-dose Thy1.1 antibody, which depletes T_{CM}, resulted in repigmentation.

to maintain disease (**Figure 3**) (Richmond, Strassner, Rashighi, et al., 2018). Both subsets recognized self-antigen and secreted IFN-γ and chemokines. Gp100-specific CD69$^+$ CD103$^+$ CD8$^+$ T_{RM} cells produced CXCL9 and CXCL10, which are chemokines recognized by CXCR3, potentially to recruit T_{CM} cells towards melanocytes in the skin. Treatment of mice with FTY720, as a means of blocking T cell access to the skin, or low-dose Thy1.1 antibody, to deplete T_{CM}, resulted in reversal of disease. This study suggests that circulating and resident T cells cooperate in vitiligo pathogenesis. However, the extent of such a relationship between circulating and resident memory T cell subsets in human vitiligo remains unclear. Better understanding of this relationship may give clues on how pathogenic T cells can most effectively be targeted in vitiligo.

Skin-resident T cell responses in melanoma

Prognostic significance of resident memory-like tumor-infiltrating lymphocytes (TILs)

While T_{RM} cells have been widely characterized in viral infections, their role in mediating tumor immunity is not yet fully known. Studies analyzing the infiltration of T_{RM} cells in human tumors have shed some light on their relevance in anti-tumor immunity. Tumor infiltration of CD8$^+$ T cells exhibiting a resident phenotype (CD69$^+$ CD103$^+$ and/or CD103$^+$) correlates with a more favorable prognosis for various human cancers (Djenidi et al., 2015; Koh et al., 2017; B. Wang et al., 2015; Webb, Milne, Watson, Deleeuw, & Nelson, 2014). Similar correlations were shown for human melanoma (**Figure 4**) (Edwards et al., 2018; Murray et al., 2016). CD103$^+$ CD8$^+$ T cells, residing in the tumor microenvironment, were strongly correlated with increased melanoma-specific survival in immunotherapy naïve stage III melanoma patients (Edwards et al., 2018). High CD103$^+$ CD8$^+$ T_{RM} cell counts led to a 5-year survival rate of 50% compared to 20% in those with lower counts. Also, expression of CD49a by vaccine-induced CD8$^+$ T cells was shown to predict a prolonged overall and disease-free survival in stage III/IV melanoma patients (**Figure 4**) (Murray et al., 2016). CD49a$^+$ CD8$^+$ TILs were found to be enriched in human melanoma metastases in various peripheral tissues. Most interestingly, CD49a was frequently co-expressed with CD69

and CD103, and in vivo blockade of CD49a or CD103 in a C57BL/6 melanoma mouse model significantly impaired control of subcutaneous B16-OVA tumors, supporting the notion of T_{RM} cell-mediated anti-tumor immunity. CD49a$^+$ B16-OVA derived T_{RM} cells produced higher levels of IFN-γ and granzyme B and exhibited a high activation status, which was even more prominent in the CD103$^+$ subset. Moreover, in human melanoma, local IL-15 levels strongly correlated with tumor-resident CD8$^+$ T cell numbers, and high IL-15 levels were associated with a more favorable prognosis (Edwards et al., 2018). IL-15 seems to be essential in retaining T cells within the tumor microenvironment, indicating that IL-15 is worthy of further investigation, as supported by the murine vitiligo data discussed previously (Richmond, Strassner, Zapata, et al., 2018).

Expression of immune checkpoints by resident memory-expressing TILs

Upregulation of immune checkpoints on TILs has emerged as a major barrier to effective anti-tumor immunity. Interestingly, not all TILs express immune checkpoints. Identifying which subpopulations among tumor-infiltrating immune cells – defined both phenotypically and functionally – express immune checkpoints is important in evaluating anti-tumor immunity. Counterintuitively, in human melanoma metastases, tumor-associated CD8$^+$ T cells with a T_{RM} phenotype were shown to express the highest levels of immune checkpoints, such as PD-1 and TIM-3, independent of CD103 expression (**Figure 4**) (Boddupalli et al., 2016). In the same study, TILs simultaneously produced less cytokines, which is consistent with an exhausted phenotype (Baitsch et al., 2011). Another study reported that mainly the CD103$^+$ CD8$^+$ subset within TILs expressed high levels of PD-1, LAG-3, 2B4 and TIM-3 (**Figure 4**) (Edwards et al., 2018). Upon anti-PD-1 therapy, CD103-expressing CD8$^+$ T_{RM} cells significantly expanded, suggesting that these cells have been released from the negative effect of PD-1 checkpoint signaling by anti-PD-1 therapy. Tumor-resident TILs may thus represent a major target for immune checkpoint blockade. However, these findings were based on markers that are redundant with other T cell states. For example, CD69 and PD-1 can be co-expressed as a result of recent antigen stimulation. Similarly, TGF-β has been shown to induce expression of CD103

on $CD8^+$ T cells (El-Asady et al., 2005) and TGF-β is often produced within the tumor environment (Thomas & Massague, 2005). It therefore remains unclear whether inhibitory checkpoint molecules are particularly enriched on resident memory-expressing TIL or whether resident cell markers are expressed as result of environmental factors or antigen stimulation.

Another consideration is that less immune checkpoint expression is found on T_{RM} cells in normal skin or during autoimmune response or infection. In the B16 mouse model of melanoma-associated vitiligo, it was shown that cutaneous tumor/self-antigen-specific $CD8^+$ T_{RM} cells located within depigmented hair follicles, lacked PD-1 and LAG-3 expression (Malik et al., 2017). Likewise, skin $CD8^+$ T_{RM} cells lacked PD-1 expression in a murine model of viral infection (Jiang et al., 2012). In a vitiligo mouse model, however, autoreactive $CD8^+$ T_{RM} cells did express PD-1 (Richmond, Strassner, Rashighi, et al., 2018). This indicates that expression of immune checkpoints has not been fully elucidated and requires more attention in future research.

Clinical implications of resident memory-like TILs

Current data suggest an important role for TILs that express T_{RM}-associated markers in providing anti-tumor immunity and their potential as biomarkers. Immune checkpoints seem to be enriched particularly on T_{RM} cells, suggesting that the T_{RM} subset of TILs may be the major target for immune checkpoint blockade. Hence, cancer immunotherapy vaccination strategies should aim at priming tumor-reactive T_{RM} cells subsets, which could synergize with immune checkpoint blockade.

Human metastatic lesions are enriched with $CD8^+$ T_{RM}-like cells (Boddupalli et al., 2016), and adoptive transfer of resident memory-like TILs might be a promising therapeutic option to melanoma patients. However, individual metastasis in the same patient may contain a distinct repertoire of T_{RM}-like cells. Sequencing of the T cell receptor (TCR) revealed interlesional heterogeneity of TILs, which was also found in the resident T cell population (Boddupalli et al., 2016). This heterogeneity was not due to variance in mutations or neoepitopes in tumor cells. Consequently, patients may experience mixed responses, with some tumor lesions regressing and others progressing, as occasionally observed following immunotherapy. It is therefore logical to explore adoptive transfer of T_{RM}-like TIL isolated from multiple lesions, as this may provide a more diverse repertoire. However,

considering the limited sample number in the study of Boddupalli *et al*. (2016), interlesional heterogeneity should be confirmed in larger studies.

Anti-tumor immunity by T_{RM} cells

Despite the great interest in immunotherapy, its clinical success still requires substantial optimization. To improve the efficacy of cancer immunotherapy, it is important to induce a potent effector response together with a stable, functional memory response, thus protecting the patients from cancer recurrence or relapse. Mouse models of melanoma have shown that tumor-specific T_{RM} cells can protect against highly aggressive melanoma. $CD8^+$ T_{RM} cells driven by a model of autoimmune vitiligo were shown to inhibit melanoma growth in a CD103-dependent manner (Malik et al., 2017). Also, infecting the skin with recombinant vaccinia virus expressing full-length ovalbumin (OVA) protein generated $CD8^+$ T_{CIRC} and T_{RM} cells that delayed the growth of OVA-expressing melanoma (**Figure 4**) (Enamorado et al., 2017). Intraperitoneal vaccination, which generates T_{CIRC} only, or FTY720 treatment, which blocks T cell access to the skin, revealed that either T_{CIRC} or T_{RM} cells were sufficient for protection against B16-OVA re-challenge in the skin, but that the presence of T_{RM} cells improved anti-tumor efficacy (Enamorado et al., 2017). Gálvez-Cancino *et al*. (2018) showed that intradermal administration of vaccines, which are known to induce strong $CD8^+$ T cell responses, efficiently induced T_{RM} cell responses against tumor antigens and self-antigens (**Figure 4**) (Gálvez-Cancino et al., 2018). Moreover, growth of cutaneous melanoma tumors was strongly suppressed, independently of circulating $CD8^+$ T cells and other adaptive immune cells. Similarly, $CD8^+$ T_{RM} cells promoted a melanoma-immune equilibrium in the epidermal layer of the skin (**Figure 4**) (Park, Buzzai, et al., 2018). In the B16/Bl6 mouse melanoma model, approximately 40% of mice that received epicutaneous inoculation of B16 melanoma cells remained free of macroscopic tumor growth. Tumor cells were dynamically surveyed by $CD69^+$ $CD103^+$ $CD8^+$ T_{RM} cells, and T_{RM} cell responses were observed more often and at higher densities in peritumoral skin than in the skin of tumor-bearing mice. In line with the findings of Enamorado *et al*. (2017), melanoma development was also suppressed in the majority of mice, irrespective of depletion of T_{CIRC} cells, but protection was most pronounced in mice harboring both T_{CIRC} and T_{RM} cells. These studies clearly affirm the potential of intradermal vaccine-induced T_{RM} cells

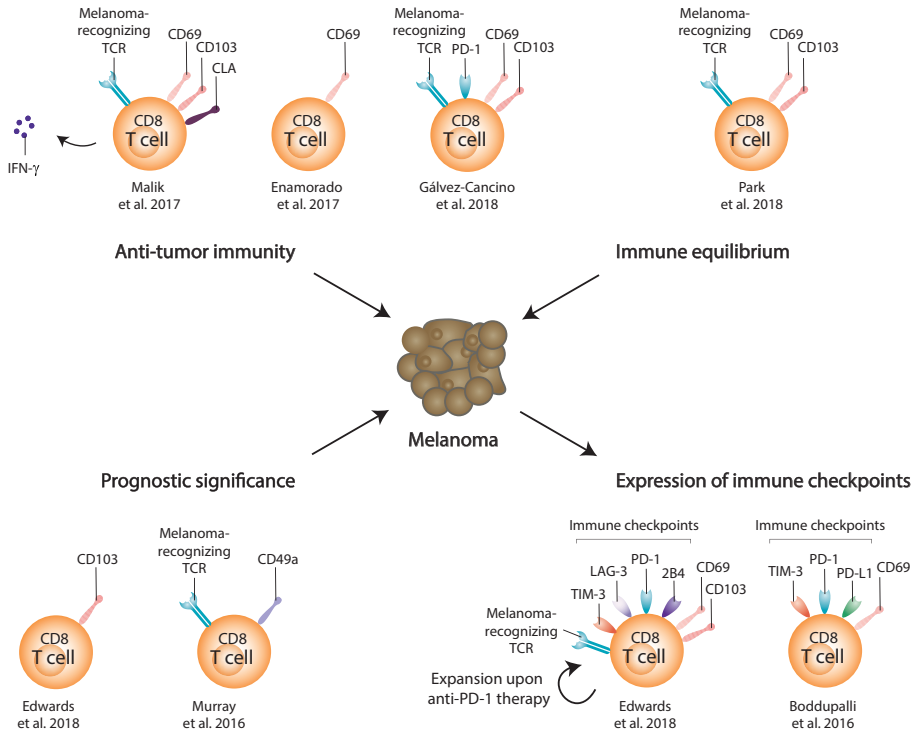


Figure 4. Resident memory T cells in melanoma**.** Skin-resident memory T cells can have various roles in melanoma; (1) T_{RM} cells can mediate anti-tumor immunity, upon vitiligo induction strategies or intradermal vaccine administration (murine data), (2) $CD8^+$ T_{RM} cells promote a melanoma-immune equilibrium in the epidermis (murine transplanted melanoma model), (3) tumor infiltration of T cells expressing either CD103 or CD49a, frequently co-expressed with CD69 and CD103, is correlated with improved survival of melanoma patients and (4) metastatic melanoma patients show expression of the immune checkpoints PD-1, LAG-3, 2B4 and TIM-3 on intratumoral T_{RM} cells, with or without CD103 co-expression.

to achieve potent protection against skin cancer. To effectively protect against malignancies, cancer vaccines should therefore evoke potent T_{RM} cell responses within the tissue.

Conclusions

Emerging evidence has shown that non-recirculating T_{RM} cells constitute a large fraction of the memory T cell pool and are involved in controlling

various infectious diseases, cancer or in mediating autoimmunity. The growing appreciation that T_{RM} cells are central players in immunity to vitiligo and melanoma has led to increased interest in T_{RM} cells as promising targets for future vaccines and immunotherapies.

T_{RM} cells are likely to have a prominent role in disease development and flare-up in human vitiligo. Therefore, targeting T_{RM} cells appears to be an attractive treatment strategy. Blocking the generation, maintenance and coordination of T_{RM} cells efficiently inhibits melanocyte killing in mice models. Future trials in patients will provide important insights into targeting T_{RM} cells for the treatment of human vitiligo.

Although not fully confirmed by all studies so far, inhibitory immune checkpoints appear to be particularly enriched on cells with T_{RM} cell properties in human melanoma. At the same time, however, their expression on T_{RM} cells in the context of autoimmune vitiligo remains unstudied. In vitiligo, the autoimmune reaction is not downregulated, but remains present; future studies might therefore clarify whether immune checkpoint expression is possibly dispensable on vitiligo-associated T_{RM} cells.

The evidence on the contribution of T_{RM} cells in cancer suppression also shows how the manipulation of T_{RM} cells can be beneficial in optimizing the anti-tumor immunity. Vaccination strategies have successfully generated T_{RM} cell populations that have effectively suppressed tumor growth in mouse models of melanoma. However, developing these therapies will require additional experimental studies to obtain more insight into the exact phenotype and function of T_{RM} cells in mice and humans. Furthermore, to validate data from mouse experiments in human clinical trials, it is crucial to study the potential of targeting T_{RM} cells in human disease.

The research highlighted in this review has focused on $CD8^+$ T_{RM} cells as key mediators of anti-tumor immunity. However, the role of $CD4^+$ T_{RM} cells in immunity to cancer remains undefined. Future studies should clarify whether tumor immunity benefits from local helper T_{RM} cells, and whether regulatory T_{RM} cells are detrimental to this immunity. Moreover, studies on vitiligo have not reported data on $CD4^+$ helper or regulatory T_{RM} cell subsets either. With more knowledge becoming available on the involvement of T_{RM} cells in autoimmunity and cancer, future research will hopefully overcome barriers to effectively block or to promote effective responses of T_{RM} cells to vitiligo and melanoma.

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Chapter 4

Presence of skin tissue-resident memory T cells in human nonmalignant and premalignant melanocytic skin lesions and in melanoma

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Abstract

The infiltration of tissue-resident memory (T_{RM}) cells in melanoma correlates with improved survival, suggesting an important role for T_{RM} cells in immunity against melanoma. However, little is known about the presence of T_{RM} cells in nonmalignant and premalignant melanocytic lesions. This study aimed to evaluate the presence of T_{RM} cells in human skin melanocytic lesions, representing the spectrum from healthy skin to metastatic melanoma. FFPE sections from healthy skin, sun-exposed skin, benign nevi, lentigo maligna (LM), primary LM melanoma (LMM), primary cutaneous and metastatic melanoma were analyzed by immunohistochemistry. The number of infiltrating cells expressing T_{RM}-associated markers CD3, CD4, CD8, CD69, CD103 and CD49a was quantified by digital analyses. Multiplex immunofluorescence was performed to analyze coexpression of T_{RM} cell markers. More T cells and $CD69^+$ cells were found in melanoma lesions, as compared with healthy skin and nevi. $CD103^+$ and $CD49a^+$ cell numbers did not significantly differ. More importantly, no differences were seen in expression of all markers between healthy skin and benign nevi. Similar results, except for CD69, were observed in LMM as compared with LM and sun-exposed skin. Interestingly, multiplex immunofluorescence showed that nevi tissues have comparable $CD103^+$ T cell numbers to healthy skin, but comprise more $CD103^+$ $CD8^+$ cells. Expression of T_{RM} cell markers is significantly increased in melanoma as compared with nonmalignant skin. Our data also show that T_{RM} cells are not abundantly present already in premalignant tissues. Further studies on the specificity of T_{RM} cells for melanocyte/melanoma antigens may reveal their significance in cancer immunosurveillance.

Introduction

On infection, naïve T cells differentiate into effector and memory T cells to eliminate pathogen-infected cells. Traditionally, memory T cells were considered to circulate within blood and lymph and enter tissues only when needed, to clear an infection. Studies in the past 2 decades have identified tissue-resident memory T (T_{RM}) cells, representing a subset of long-lived memory cells that stably reside in tissues, including skin, intestine, lung, and brain, and do not recirculate throughout the body. T_{RM} cells are generally defined by the constitutive expression of the early activation marker CD69,

a C-type lectin responsible for tissue retention, which limits migration out of tissues.[1] A subset of T_{RM} cells also expresses CD103, the α-subunit of the α3β7 integrin receptor that controls tethering in the epidermal compartment.[2] Skin epidermal $CD8^+CD69^+CD103^+$ T_{RM} cells can also express the skin retention integrin CD49a.[3] T_{RM} cells remain in place long after pathogen elimination.[2,4,5] On reinfection, T_{RM} cells can mediate superior immunity[4-8] by the production of high levels of effector molecules, for example IFN-γ and TNF-α.[9] T_{RM} cells provide protective immunity and vaccination strategies to generate T_{RM} cells are therefore attractive to protect against pathogens.

Emerging evidence from mice and humans shows that T_{RM} cells are also involved in controlling cancer. Tumor infiltration of $CD8^+$ T cells exhibiting a resident phenotype correlates with a more favorable prognosis in human melanoma.[10,11] In addition, mouse models of melanoma have shown that vaccination could induce tumor-specific T_{RM} cell responses that strongly suppressed growth of established melanomas.[12-14] Finally, $CD103^+$ $CD8^+$ T_{RM} cells that were generated by epidermal inoculation of B16 melanoma cells promote a melanoma-immune equilibrium and correlate with spontaneous disease control in a transplantable cutaneous melanoma mouse model.[15] It thus seems that the induction of T_{RM} cells can enhance the efficacy of antitumor immunity. This also implies that T_{RM} cells may be involved in cancer immunosurveillance. However the presence of T_{RM} cells in premalignant lesions has not been studied so far. In addition, unlike cutaneous melanoma, T_{RM} cell infiltration in lentigo maligna melanoma and its premalignant phase, lentigo maligna, remains unstudied, indicating the rationale to study T_{RM} cell abundance in these tissues as well.

This is an explorative study aimed to investigate the expression of T_{RM} markers by immune cells present in human skin and melanocytic lesions, thereby representing the spectrum from healthy skin and premalignant lesions to metastatic melanoma.

Materials and methods

Patient samples

Samples of skin lesions, obtained from individual subjects, were provided by the Department of Pathology at the Amsterdam University Medical

Centers, Amsterdam, the Netherlands. This study was approved by the Biobank Ethical Committee of the VU University Medical Center. Formalin-fixed paraffin-embedded (FFPE) sections from healthy skin (HS, n=7), chronically sun-exposed skin (n=7), benign melanocytic nevi (n=23), lentigo maligna (LM, n=8), primary lentigo maligna melanoma (LMM, n=7), nodular primary melanoma (n=7), superficially spreading primary melanoma (n=7), cutaneous metastatic melanoma (n=7), and metastatic melanoma (n=7) were included. Biopsy site, sex and age were controlled in the inclusion of patients. The patient characteristics are listed in **Table 1** and **2**.

Table 1. Patient characteristics

Characteristics	**Healthy skin**	**Benign melanocytic nevi**	**Primary melanoma**	**Metastasized melanoma**	***P-value***
Number	7	23	14	14	
Gender					*P > 0.05*
Male	4	11	4	8	
Female	3	11	10	6	
Age (mean ± SEM)	64.00 ± 5.65	33.61 ± 2.46	64.64 ± 4.98	68.38 ± 3.36	*P < 0.0001*
Type nevus					
Combined		20			
Compound		1			
Dermal		2			
Junctional		0			
Breslow (mean ± SEM)			2.06 ± 0.56	2.90 ± 0.75	
Melanoma type					
Nodular			7		
Superficially spreading			7		
Metastasis					
Lymph				7	
Brain				4	
Other				3	

SEM, standard error of mean

Table 2. Patient characteristics

Characteristics	Chronically sun-exposed skin	Lentigo maligna	Primary lentigo maligna melanoma	*P-value*
Number	7	8	7	
Gender				*P > 0.05*
Male	4	4	2	
Female	3	4	5	
Age (mean ± SEM)	74.14 ± 6.54	73.38 ± 3.61	79.88 ± 3.61	*P > 0.05*
Breslow (mean ± SEM)		0.03 ± 0.03	1.66 ± 0.91	

SEM, standard error of mean

Immunohistochemistry

Four-micrometer tissue sections were deparaffinized in xylene and rehydrated with serial passage through graded ethanol. Heat-induced antigen retrieval was performed for 20 minutes at 98°C in either citrate buffer pH 6.0 or in EDTA pH 9.0 buffer. Slides were washed in 1x TBS-Tween20 0.05% (TBST). After epitope retrieval, sections were washed and blocked with Superblock (ScyTek Laboratories, Logan, UT) for 10 minutes, before incubation with primary antibodies for 60 minutes at room termperature. Antibodies used included anti-CD3 (polyclonal) and anti-CD4 (clone SP35) (both Dako, Santa Clara, CA), anti-CD8 (clone SP16, Spring Bioscience, Pleasanton, CA), anti-CD69 (clone 15B5G2), anti-CD103 (polyclonal) (both Novus Biologicals, Centennial, CO), and anti-CD49a (Abcam, Cambridge, UK). Antigen-antibody binding was visualized using 3,3'-diaminobenzidine (DAB) (Dako), Perma Red/AP (Diagnostic Biosystems, Pleasanton, CA) or Vector ImmPACT VIP/HRP (Vector laboratories, Burlingame, CA) chromogen. Sections were counterstained with haematoxylin (Sigma Aldrich, Saint Louis, MO) and mounted for review.

Imaging, scoring and analysis

The immunohistochemically stained tissues sections were analyzed and scanned using a 40x objective Philips IntelliSite Ultra Fast Scanner (Philips Digital Pathology Solutions, Best, the Netherlands). Positive cells were

calculated per mm^2 using Fiji software.[16] Results were expressed as number of cells per mm^2.

Multiplex immunofluorescence

Multiplex immunofluorescence staining was performed using the OPAL 7-color fluorescence immunohistochemistry kit (Akoya Biosciences, Marlborough, MA). Tissue sections were deparaffinized in xylene and rehydrated with serial passage through graded ethanol. Endogenous peroxidase was blocked with 0.3% H_2O_2/methanol for 10 minutes. Antibodies used included anti-PD-1 (clone NAT105, Abcam), anti-CD4 (clone 4B12, Neomarkers, Portsmouth, NH), anti-CD8 (clone C8/144B, Dako), anti-CD103 (clone EPR4166 (2), Abcam), anti-Sox10 (clone EP268, Monosan, Uden, the Netherlands), and anti-CD3 (Dako). Steps were repeated for each primary antibody. Heat-induced antigen retrieval was performed for 10 minutes at 95°C in Tris-EDTA pH 9.0 buffer. Slides were washed in TBST and blocked with Blocking/Ab diluent for 10 minutes, before being stained with primary antibody for 60 minutes at room temperature. Afterwards, slides were incubated with Polymer HRP Ms + Rb for 10 min. Next, slides were incubated with Opal fluorochromes (Opal 520, Opal 620, Opal 480, Opal 570, Opal 690 and Opal 780) for 10 min. Finally, DAPI working solution was applied for 5 minute, and slides were mounted with ProLong Diamond Antifade Mountant (Thermo Fisher Scientific, Waltham, MA).

Imaging

The Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences) was used for multispectral imaging at 40x magnification. Whole slide images were analyzed using Phenochart whole slide viewer (Akoya Biosciences), inForm® tissue analysis software (Akoya Biosciences) and QuPath software (open source software).

Statistical analyses

Statistical analysis was executed using GraphPad Prism software (GraphPad Software Inc, San Diego, CA) and SPSS software (SPSS Inc, Chicago, IL). Significant differences were calculated with Kruskal-Wallis tests (non-parametric, unpaired data), one-way analysis of variance (ANOVA)

(parametric, unpaired data) and unpaired *t* tests. Dunn multiple comparisons corrections were applied for Kruskal-Wallis tests, Tukey multiple comparisons corrections were applied for one-way ANOVA analyses. *P*-values less than 0.05 were considered statistically significant; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$.

Results

Patient characteristics

This study aimed to gain insight into the presence of skin-resident memory T cells in (melanocytic) skin lesions, as compared with their nonmalignant and premalignant skin counterparts. To this end, primary melanoma, which develops de novo or from preexisting melanocytic nevi, and melanoma metastases were studied in comparison to healthy skin and benign melanocytic nevi. The characteristics of the included subjects are given in **Table 1**. Patients with benign melanocytic nevi were significantly younger than other study subjects, with a mean age of 34 years. No significant differences in gender distribution were seen between study subjects. Most of the melanocytic nevi were combined nevi, characterized by 2 or more distinct populations of melanocytes. Equal numbers of nodular and superficially spreading primary melanomas were included. Half of the metastatic tumors were cutaneous metastases, whereas the others were melanoma metastases in the brain or other distant organs.

LM and LMM generally evolve from chronically sun-damaged skin. Therefore, chronically sun-exposed skin was analyzed in parallel as nonmalignant, but sun-damaged prestage of LM and LMM. The characteristics of this cohort are given in **Table 2**. LMM patients had an average age of 80 years, which is slightly higher than the average age of patients and control subjects with LM ($P > 0.05$). Similar to the other cohort, no differences in gender distribution were seen.

Melanoma tissue shows increased T cell infiltration as compared to non-malignant tissue

Primary and metastatic melanoma tissues contained significantly more $CD3^+$ cells than benign nevi and healthy skin tissue (**Fig. 1A**). When studying

the topographical distribution of $CD3^+$ cells, some primary and metastatic melanoma specimens demonstrated an "immune-desert" or "immune-excluded" phenotype, with barely any $CD3^+$ cells at all or solely at the border of the tumor, respectively (**Fig. 1B**, top row). By contrast, other tumors showed a highly infiltrated, "inflamed" phenotype, with $CD3^+$ cells present in between the melanoma cells (**Fig. 1B**, bottom row). Similar to CD3-expressing cells, $CD4^+$ cells were increased in primary and metastatic melanoma as compared with nevi ($P < 0.01$) (**Fig. 1C**). $CD8^+$ cell numbers varied between melanoma tissues studied but were on average not significantly different between the studied tissues (**Fig. 1D**).

Next, we analyzed the expression of markers that generally define skin-resident T cells (CD69, CD103 and CD49a). $CD69^+$ cells were found in all tissue sections but significantly more in melanoma metastasis as compared with healthy skin and nevi (**Fig. 1E**). $CD103^+$ cells were present at high levels in metastatic melanoma only, although only in a fraction of tumors, whereas relatively low $CD103^+$ cell numbers were found in 10 of 14 metastatic melanoma tissues (**Fig. 1F**). Although CD103 expression shows a trend toward an increase in metastatic melanoma, as compared with primary melanoma and nonmalignant tissues, this did not reach significance in our study cohort. Finally, $CD49a^+$ cells were observed, but its expression was mostly seen on dermal fibroblasts and tumor stroma. Moreover, the number of $CD49a^+$ cells/mm^2 did not significantly differ between the studied tissues (**Fig. 1G**).

Our data so far show increased expression of T_{RM} markers in melanoma, with most T cells and $CD69^+$ cells in metastatic melanoma tissues. To verify this in another data set, we analyzed the presence of a T_{RM} gene expression signature on a large set of tumors, using the cutaneous melanoma TCGA dataset.[17] This dataset contains 65 primary cutaneous melanomas and 266 melanoma metastases. For this, we made use of an experimentally determined human core T_{RM} signature gene set.[18] Increased presence of $CD69^+$ cells in melanoma metastases (**Fig. 1E**) suggests increased T_{RM} cell infiltration. Accordingly, melanoma metastases also showed a more pronounced T_{RM} signature compared with primary melanoma ($P < 0.001$) (see **Fig. 1A**, **Supplemental Digital Content 1**). In addition, consistent with the notion that T_{RM} cells provide protective immunity in cancer, high expression of the T_{RM} transcriptional profile significantly associated with improved survival, as compared with tumors with a low T_{RM} gene expression signature (see **Fig. 1B**, **Supplemental Digital Content 2**).

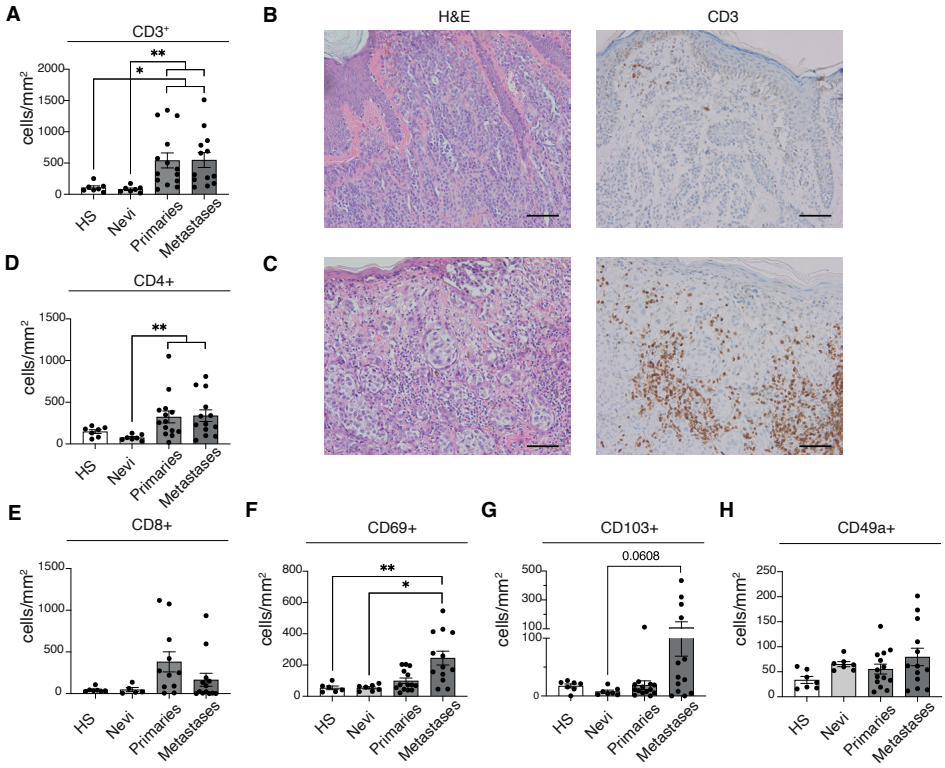

Figure 1. Presence of T cells expression skin-resident T cell markers in melanoma and nonmalignant specimens. A, Expression of CD3 in FFPE sections from healthy skin (HS, n=7), benign melanocytic nevi (n=8), primary melanoma (primaries, n=14) and metastatic melanoma (metastases, n=14). B, Representative image of H&E and CD3 immunohistochemical staining of an "immune-excluded" tumor (top row) and an "inflamed" tumor (bottom row). Scale bar: 50 µm. C-G, Expression of CD4 (C), CD8 (D) and CD69 (E), CD103 (F) and CD49a (G) in FFPE sections from healthy skin (HS, n=7), benign melanocytic nevi (n=8), primary melanoma (primaries, n=14) and metastatic melanoma (metastases, n=14). Results are expressed as number of cells per mm^2. Error bars: mean ± SEM. Kruskal-Wallis tests significant as indicated; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

In summary, melanoma progression coincided with increased T cell infiltration and increased expression of the early activation marker CD69, but not with increased expression of CD103 and CD49a. Concomitantly, no significant differences in expression of T_{RM} cell markers were observed between healthy skin and benign nevi.

Lentigo maligna melanoma shows increased T cell infiltration as compared to non-malignant tissue and pre-malignant lentigo maligna

Unlike nodular and superficial spreading melanoma, the presence of T_{RM} cells in LMM, and consequently also LM and chronically sun-exposed skin, remains unstudied. The number of $CD3^+$ cells/mm^2 did not significantly differ between the studied groups, but showed a trend towards an increase in LMM, as compared with chronically sun-exposed skin (**Fig. 2A**). All LMM specimens showed an inflamed phenotype, with $CD3^+$ cells infiltrating the tumor. (**Fig. 2B**). In contrast to CD3-expressing cells, $CD4^+$ cell numbers, were significantly increased in LMM as compared with LM ($P < 0.01$) (**Fig. 2C**). In sun-exposed skin there was more variation in the number of $CD4^+$ cells and, therefore, did just not significantly differ from LMM ($P = 0.0569$) (**Fig. 2C**). Likewise, LMM tissue contained significantly more $CD8^+$ cells compared with LM ($P < 0.01$) (**Fig. 2D**). Similar to $CD4^+$ cells, $CD8^+$ cells also varied in sun-exposed skin and nearly reached significance as compared with LMM ($P = 0.0829$) (**Fig. 2D**). Interestingly, T cell infiltration did not differ between nonmalignant, sun-exposed skin and LM tissue (**Fig. 2A, C-D**), fluctuating around 150 T cells per mm^2.

Similar to general T cell markers, $CD69^+$ cells were present at high levels in half of lentigo maligna tumors, but due to this variation not statistically different from LM and nonmalignant tissues (**Fig. 2E**). Finally, CD103 and CD49a expression were comparable between the studied groups (**Fig. 2F-G**), suggesting that T cell numbers, but not skin-resident T cells numbers, are increased upon progression of LM into LMM. Similar to the other cohort, no differences in expression of T and T_{RM} cell markers were observed between nonmalignant, chronically sun-exposed skin and premalignant LM.

Nevi tissue shows similar $CD103^+$ T cell numbers as healthy skin but comprise more $CD103^+$ $CD8^+$ cells

Although significant expression of CD3, CD4, CD8, and CD69 was seen in malignant tissue, no differences in expression were seen between nonmalignant and premalignant specimens (**Figs. 1, 2**). Because $CD69^+$ $CD103^+$ T_{RM} cells can actively suppress cancer progression in a melanoma mouse model,[15] we wondered whether ratios of T_{RM} cells differed between

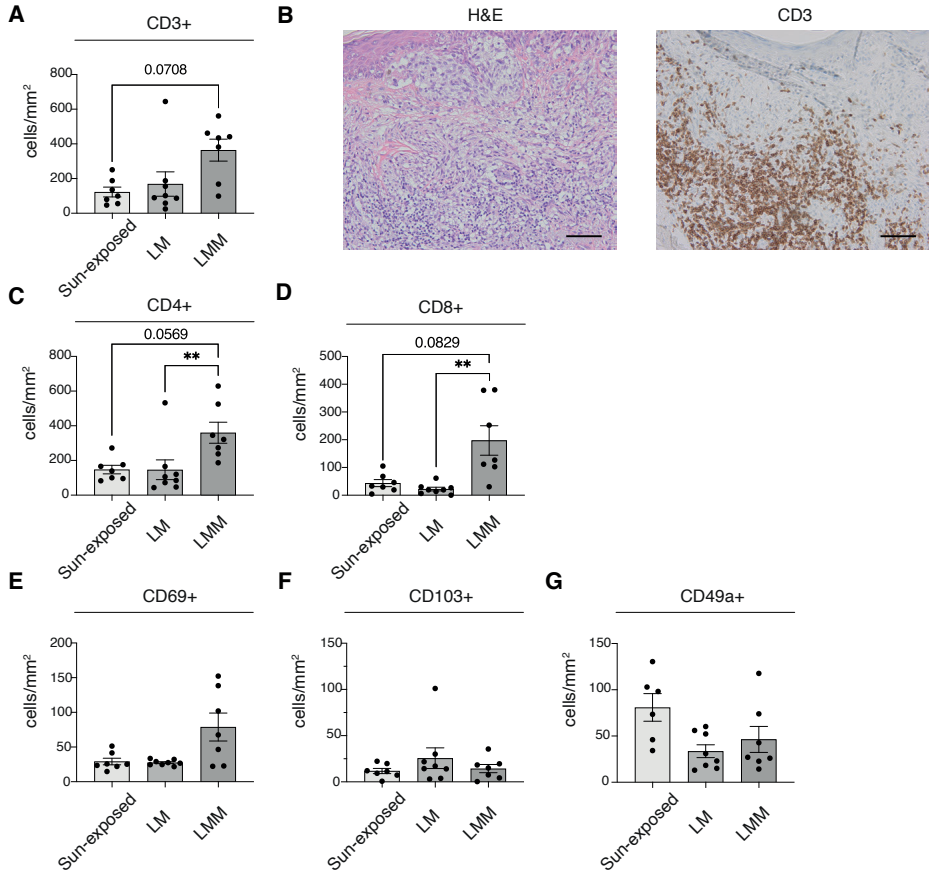

Figure 2. Presence of T cells expressing skin-resident T cell markers in lentigo maligna melanoma and nonmalignant or premalignant specimens. A, Expression of CD3 in FFPE sections from chronically sun-exposed skin (n=7), lentigo maligna (LM, n=8) and primary lentigo maligna melanoma (LMM, n=7). B, Representative image of H&E and CD3 immunohistochemical staining of a LMM, demonstrating an "inflamed" tumor. C-G, CD4 (C), CD8 (D) and CD69 (E), CD103 (F) and CD49a (G) in FFPE sections from chronically sun-exposed skin (n=7), lentigo maligna (LM, n=8) and primary lentigo maligna melanoma (LMM, n=7). Results are expressed as number of cells per mm^2. Error bars: mean ± SEM. Kruskal-Wallis tests significant as indicated; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

healthy and (potentially) premalignant tissues. Hypothetically, T_{RM} cells might inhibit neoplasms of proliferating melanocytes from development to melanoma. Although most dysplastic nevi are stable and rarely progress into a melanoma,[21] a fraction of dysplastic nevi can develop into melanoma very rapidly in as little as 8 weeks.[19] This is in contrast with LMM, where the risk of a

LM to progress into an overt LMM is only 3.5% per year, which equates to over 28 years,[20] which is why T_{RM} cells were studied more thoroughly in healthy skin and nevi tissues.

As aforementioned markers are not exclusively expressed by skin-resident memory T cells, multiplex immunofluorescence staining was performed to identify true T_{RM} cells and to determine T_{RM} cell ratios (**Fig. 3A**). We included merely CD103 and not CD69 or CD49a in multiplex immunofluorescence experiments because CD49a expression was mostly seen on dermal fibroblasts and is only expressed by epidermal $CD103^+$ $CD69^+$ $CD8^+$ T cells. Costaining for CD69 was unfortunately not feasible because of high background fluorescence. Because $CD103^+$ T_{RM} cells have a more significant effector cytokine-production capacity compared with $CD103^-$ T_{RM} cells and $CD103^+$ $CD3^+$ T cells can be considered T_{RM} cells, because almost all $CD103^+$ $CD3^+$ cells in human skin also express CD69[22], we chose to analyze CD103 expression.

The spatial distribution of T_{RM} cells among healthy skin and nevi tissues did not change (**Fig. 3A**). In healthy skin, the majority of CD103-expressing T_{RM} cells were found epidermally, consistent with the literature. Nevertheless, a fraction of T_{RM} cells, mostly $CD4^+$ cells, were found dermally. Similarly, in nevi tissues, T_{RM} cells were identified in the epidermis, but also in nest in between the neoplastic melanocytes (**Fig. 3A**). Absolute and relative numbers of $CD103^+$ $CD3^+$ cells did not differ between healthy skin and benign nevi (**Fig. 3B**). Consistent with data on healthy skin,[23,24] approximately 10% of $CD3^+$ cells were $CD103^+$ in human nevi (**Fig. 3B**). Whereas the fraction of $CD103^+$ cells within the $CD4^+$ population did not differ between healthy skin and nevi (**Fig. 3B**), significantly increased CD103 expression was found on $CD8^+$ T cells in nevi (**Fig. 3B**). At the same time, nevi sections contained slightly less $CD4^+$ and a bit more $CD8^+$ T cells compared with healthy skin (**Fig. 3B**). Contrary to 10% of $CD103^+$ $CD8^+$ cells in healthy skin, around 25% of $CD8^+$ cells in nevi expressed CD103 (**Fig. 3B**). Within the total population of T cells, $CD103^+$ $CD8^+$ and $CD103^+$ $CD4^+$ cells did not significantly differ between tissue sections (**Fig. 3C**). As can be seen in the pie chart (**Fig. 3D**), most of the T cells in the skin are $CD4^+$ and only a quarter represent $CD8^+$ cells. Most importantly, $CD103^+$ $CD8^+$ cells account for 7% of the total T cell population in nevi (**Fig. 3D**), as compared with only 3% in healthy skin. However, this increase in $CD103^+$ $CD8^+$ cells may be too small to observe within the total $CD3^+$ population.

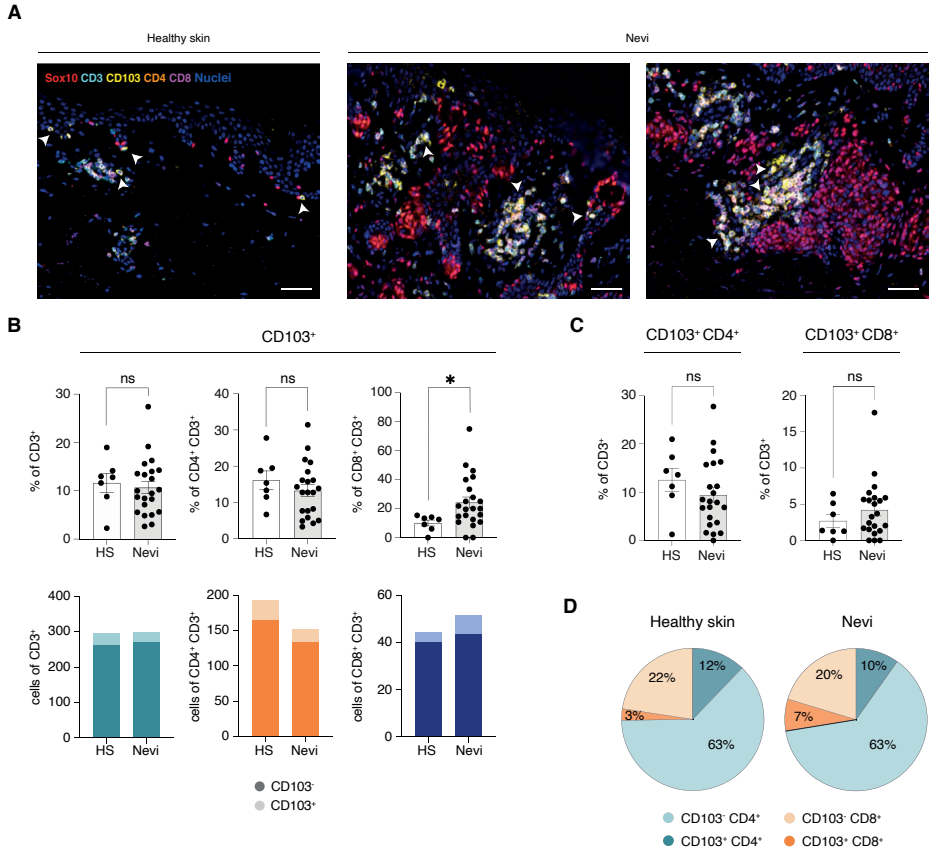

Figure 3. Co-expression of skin-resident T cell markers on T cells in healthy skin and benign nevi. (A) Multiplex immunofluorescence staining of healthy skin (left) and benign melanocytic nevi (right). The color of each of the markers is indicated. Scale bar 50 µm. (B) Percentage (upper row) and absolute number (bottom row) of $CD103^+$ cells among $CD3^+$ (left), $CD4^+$ $CD3^+$ (middle) and $CD8^+$ $CD3^+$ (right) T cells in healthy skin (HS, n=7) and benign melanocytic nevi (n=23). (C) % of $CD103^+$ $CD4^+$ (left) and $CD103^+$ $CD8^+$ (right) cells of $CD3^+$ T cells in healthy skin (HS, n=7) and benign melanocytic nevi (n=23). (C) Pie chart showing mean proportions of $CD103^+$ $CD4^+$ (green), $CD103^-$ $CD4^+$ (light green), $CD103^+$ $CD8^+$ (orange) and $CD103^-$ $CD8^+$ (light orange) in healthy skin (left) and benign melanocytic nevi (right). Error bars: mean ± SEM. Unpaired *t* tests significant as indicated; **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001.

Discussion

The growing appreciation that T_{RM} cells are involved in controlling cancer, specifically melanoma, has led to increased interest in T_{RM} cells as promising

targets for immunotherapy. Although it is known that high infiltration of resident memory-like tumor infiltrating T cells correlates with improved survival in melanoma, the presence of T_{RM} cells in nonmalignant and premalignant skin specimens is largely unexplored. Concomitantly, to date, the presence of T_{RM} cells in LMM remains unstudied. This study therefore aimed to investigate the presence of T cells expressing T_{RM} markers in human skin specimens, representing the spectrum from healthy skin to metastatic melanoma and nonmalignant, chronically sun-exposed skin to LMM.

We demonstrated increased numbers of T cells and $CD69^+$ cells in melanoma, as compared with nonmalignant tissues. The numbers of $CD103^+$ and $CD49a^+$ cells did not significantly differ, although CD103 expression nearly reached a significant increase in metastatic melanoma. More importantly, no differences were seen in expression of all markers between healthy skin and benign nevi. This is consistent with previous data on $CD3^+$ T cell infiltration that shows a gradual increase in the numbers of infiltrating T cells from normal skin to metastatic melanoma,[25] suggesting increased tumor antigenicity.[26,27] Nevertheless, Hussein et al (2006) also demonstrate a significant increase in T cells in benign nevi as compared with healthy skin (21.8 vs. 3).[25] Because clinical characteristics are missing in this paper, it remains unclear which type of nevi were included, which might be different from the combined nevi that were predominantly included in this study.

Similar to primary and (cutaneous) metastatic melanoma, the number of $CD4^+$ and $CD8^+$ cells/mm^2 were significantly higher in LMM, as compared with LM and chronically sun-exposed skin. Neither CD69, nor CD103, nor CD49a expression was different between nonmalignant skin, LM or LMM. Data on T cell infiltration in LM and LMM are limited, but a case report demonstrates a mild superficial T cell infiltrate in LM.[28] Despite a high mutational load due to chronic ultraviolet radiation exposure,[29] which theoretically would implicate the formation of neoantigens and increased immunogenicity, LM seems to be low immunogenic and T cell infiltration is only increased in overt LMM. Nonetheless, the potential immunogenic character of LM may be overruled by the immunosuppressive influence of chronic sun-exposure and ultraviolet-induced damage, that may hamper the local immune infiltration and function.

Although absolute numbers of CD103-expressing cells and T cells did not differ between healthy skin and benign nevi, the proportion of $CD103^+$

CD8⁺ cells was significantly different between the tissues. Contrary to 10% of CD103⁺ CD8⁺ cells in healthy skin, around 25% of CD8⁺ cells in nevi expressed CD103. Hypothetically, these CD103⁺ T_{RM} cells might inhibit neoplasms to develop into melanomas. This theory is supported by the discovery that T_{RM} cells can suppress tumor growth in mice and that such lesions can remain clinically occult.[15] Nevertheless, antigen specificity remains unstudied and thus the question arises whether melanocyte/melanoma-specific CD103⁺ CD8⁺ T_{RM} cells are increased within these specimens. Moreover, a fraction of nevi progresses into a malignancy, and it remains unclear if nevi did not progress because of high T_{RM} cell infiltration or because of melanocyte senescence.

In conclusion, our results show that LMM development and melanoma progression coincides with increased expression of T_{RM} cell markers (CD69 and CD103) and that these markers do not differ between nonmalignant and (possible) premalignant specimens. Our data also show that T_{RM} cells are not already abundantly present in premalignant tissues. This suggests that T cell infiltration and T_{RM} cell differentiation do not yet increase at the premalignant stage but may coincide with melanoma development. Interestingly, the proportion of CD103⁺ CD8⁺ T_{RM} cells is significantly increased in benign melanocytic nevi as compared with healthy skin. Nevertheless, further studies on the specificity of T_{RM} cells for melanocyte/melanoma antigens may reveal their functional significance in cancer immunosurveillance.Acknowledgements

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Supplemental methods

TCGA data analysis

The R2 Genomics Analysis and Visualization platform (http://r2.amc.nl) was used for analysis of cutaneous melanoma samples in The Cancer Genome Atlas (TCGA) dataset.[17]

Supplemental digital content

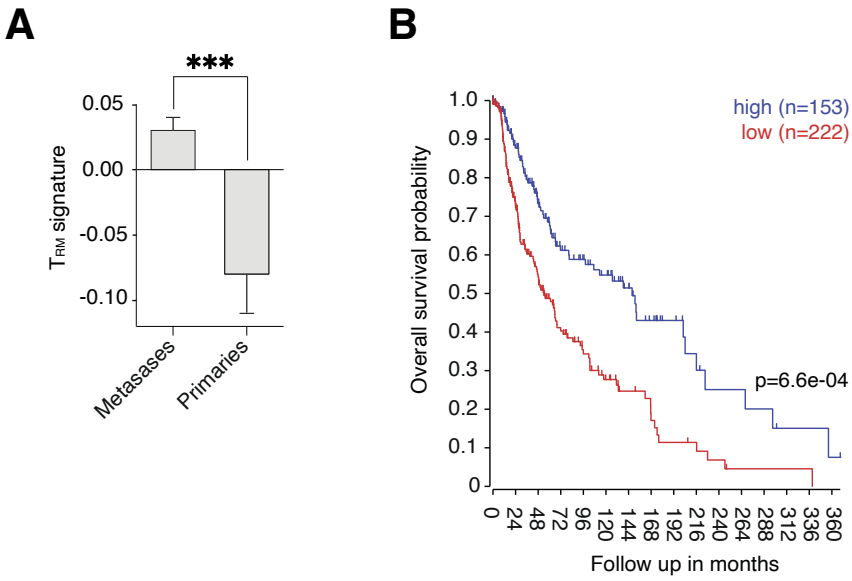

Figure 1. High expression of a T_{RM} transcriptional profile is associated with improved survival in melanoma patients. A, Boxplots showing the variation in the T_{RM} core gene signature between primary and metastasized tumors. B, Post-accession survival curve of 375 cutaneous melanoma patients in the TCGA dataset, subgrouped in high or low T_{RM} core gene signatures. median ± IQR. ANOVA significant as indicated; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.

Part 2

Involvement of melanocytes and melanoma cells in immune evasion

Chapter 5

Instant isolation of highly-purified human melanocytes from freshly-prepared epidermal cell suspensions

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To the editor,

Melanocytes are melanin (pigment)-producing cells that are primarily located in the stratum basale of the epidermis comprising 5% of the epidermal cells in normal human skin (Haass & Herlyn, 2005). Melanocytes show high expression of CD117 (c-Kit) (Cario & Taieb, 2019) and can be visualized in skin sections by specific immunohistochemical staining for this marker (**Figure 1A**). To date, human melanocyte monocultures can be obtained from epidermal cell suspensions by suppressing growth of contaminating keratinocytes and fibroblasts (Cario & Taieb, 2019; Godwin et al., 2014; Hsu, Li, & Herlyn, 2005; Zhang et al., 2018). Although this procedure ultimately provides pure melanocyte cell populations, it is quite time consuming (it takes several weeks of *in vitro* culture) and there is a potential risk that melanocytes undergo phenotypical and functional changes during culturing. The aim of this study was to establish a technique for instant isolation of large numbers of highly-pure primary human melanocytes from freshly-prepared epidermal cell suspensions by means of flow-cytometer cell sorting, in line with earlier studies in mice (Diwakar, Zhang, Jiang, & Hornyak, 2008; Kawaguchi et al., 2008), given that within the non-leukocyte ($CD45^-$) epidermal cell population only melanocytes show cell-surface expression of CD117. In order to thoroughly exclude epidermal T cells ($CD3^+$) and Langerhans cells ($HLA\text{-}DR^+$) we also included antibodies to these cell types in our protocol.

Donor human skin 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 research was conducted in accordance with the Declaration of Helsinki. Thin skin sheets of 0.3 mm prepared by an electrodermatome were incubated in 0.2% grade II Dispase (Sigma-Aldrich) for 2 h at 37°C or overnight at 4°C. Next, the epidermis was separated from the dermis, minced by scissors and digested in 0.1% trypsin, 0.04% EDTA in HBSS (Gibco) for 15 minutes at 37°C. After passing through a 70 µm filter, the epidermal cells (viability > 98%) were fluorescently labelled using APC/Cy7-conjugated mouse anti-human CD45 (clone 2D1, BioLegend), PE/Dazzle594-conjugated mouse anti-human CD3 (clone UCHT1, BioLegend), PE-conjugated mouse anti-human HLA-DR (clone G46-6, BD Biosciences), and PE/Cy5.5-conjugated mouse anti-human CD117 (clone 104D2D1, Beckman Coulter). FACS acquisition and sorting were

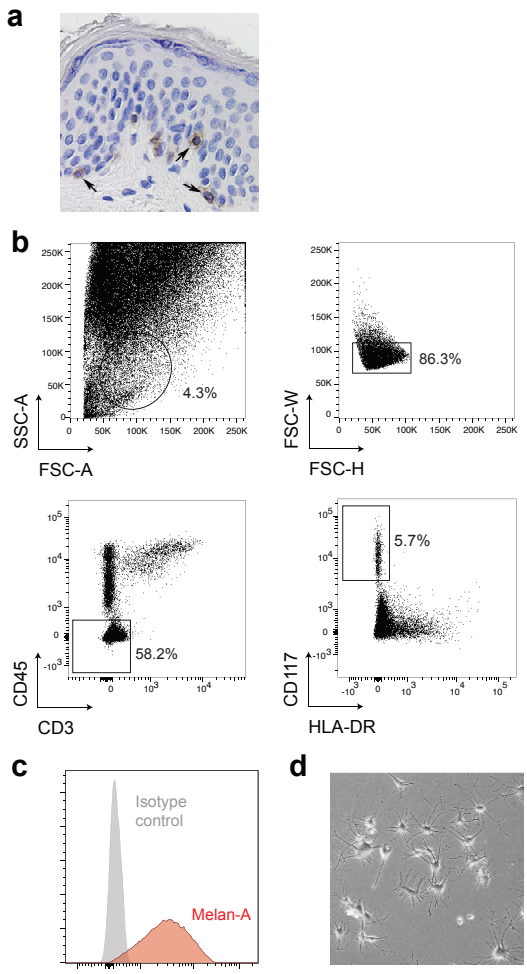

Figure 1. Isolation of melanocytes from freshly-prepared epidermal cell suspensions. (A) Melanocytes are identified as CD117$^+$ cells in formalin-fixed paraffin-embedded tissue section of healthy human skin by immunohistochemical staining (polyclonal anti-CD117 and DAB chromogen from Dako). (B) Gating strategy to purify melanocytes from epidermal cell suspensions being defined as CD45$^-$CD3$^-$HLA-DR$^-$CD117$^+$ cells. First, the epidermal cell suspension was gated on forward scatter (FSC-A) and side scatter (SSC-A) to select cells of interest based on size and granularity. Next, FSC height versus width was used to enrich for single cells, thereby removing any doublets, clumps or debris. To exclude leukocytes (CD45$^+$) and in particular T cells (CD3$^+$) during sorting, we gated on CD45$^-$ CD3$^-$ cells. Finally, from this gate, melanocytes were isolated as CD117$^+$ cells which were lacking Langerhans cell marker HLA-DR. Data are representative of 7 independent experiments. (C) All purified CD117$^+$ cells showed positive intracellular staining with FITC-conjugated anti-Melan-A. Data are representative of 3 independent experiments. (D) Plated purified CD117$^+$ cells acquired a multi-dendritic morphology characteristic for cultured melanocytes.

performed on a FACSAria IIu (BD Biosciences) using BD FACSDiva software (BD Biosciences) and data was analyzed using FlowJo software (Tree Star). Putative melanocytes were isolated as $CD45^-$ $CD3^-$ HLA-DR^- $CD117^+$ cells (gating strategy see **Figure 1B**).

FACS analysis showed a population of approximately 10% of $CD117^+$ cells within the $CD45^-CD3^-$ cell population (5.7% of 58.2% $CD45^-$ $CD3^-$ cells in **Figure 1B**). To demonstrate that these $CD117^+$ cells indeed represent melanocytes, they were sort-purified and intracellularly stained with FITC-conjugated mouse anti-human Melan-A (clone A103, Santa Cruz). This melanocyte differentiation antigen, also known as melanoma antigen recognized by T cells (MART)-1 antigen, is specific to identify melanocytes. We found that all $CD117^+$ epidermal cells expressed Melan-A (**Figure 1C**), suggesting that sorting for $CD45^-$ $CD3^-$ HLA-DR^- $CD117^+$ epidermal cells yields pure melanocytes. Further, when the purified $CD117^+$ cells were plated in M254 (Gibco) medium supplemented with HMGS (Gibco) they became flattened and adherent to the plastic culture plate and acquired a multi-dendritic morphology which is characteristic for cultured melanocytes (**Figure 1D**). Typical yield of melanocytes from an epidermal single-cell suspension is around 3,300 per cm^2 skin.

Melanin production is the key property of melanocytes in vivo. To test whether the purified $CD117^+$ cells are still capable of producing melanin, cells were seeded at 1 x 10^5 cells per well in M254 with HMGS in a 12-well plate and cultured for 24 h at 37°C in the absence or presence of 1 mM L-DOPA to stimulate melanin synthesis. Cells were imaged at 15-minute intervals with a 40x objective using a Leica IRBE inverted microscope and custom-written software. During this time-lapse imaging, cells were maintained at 37°C and 5% CO_2, showing that the cultured $CD117^+$ cells were actively moving and migrating when left unstimulated (**Supplementary video 1**). L-DOPA stimulated melanocytes, however, barely migrated but produced melanin, causing darkening of the medium, as observed macroscopically (**Figure 2A**) and microscopically by comparison of the first and last frame of the time-lapse imaging (**Supplementary video 2, Figure 2B**). After 24 h, the culture medium was collected and the adherent cells were washed in PBS. Next, 100 µl 1 M sodium hydroxide was added to both cells and 100 µl culture medium to dissolve melanin at 70°C for 90 min. To determine the melanin concentration, the cell lysate and 24-h culture medium were transferred to a 96-well flat-bottom microplate and the optical density was measured at

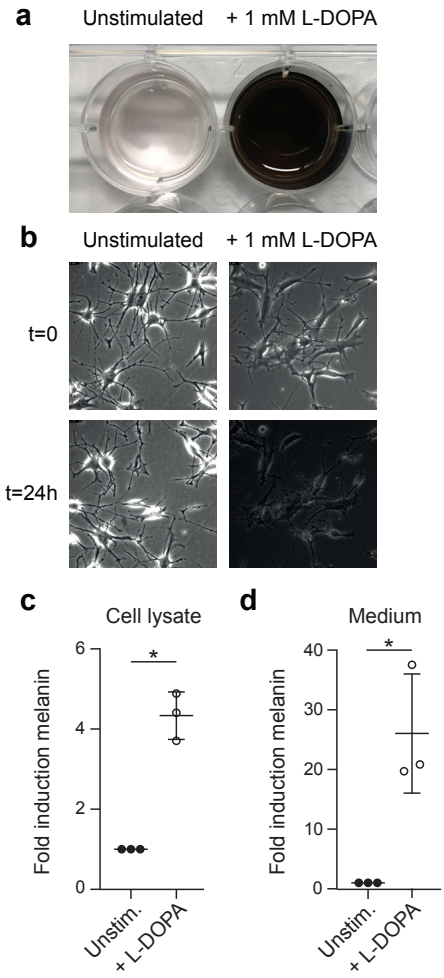

Figure 2. Isolated primary human melanocytes are functionally active. (A-D) Sorted primary melanocytes were stimulated with 1 mM L-DOPA or left unstimulated for 24 hours. L-DOPA-treated cells produced melanin, causing dark brown coloring of the culture medium, as can be observed macroscopically (A) and by comparing the first frame (B, top-right) with the last frame (B, bottom-right) of time-lapse microscopy. L-DOPA stimulation significantly increased the intracellular melanin concentration 4 fold, compared to unstimulated condition (C), whereas the melanin concentration in the medium was increased 26-fold (D). *$p < 0.05$

405 nm in a Versamax ELISA microplate reader. The melanin concentration was calculated by a calibration curve of synthetic melanin (Sigma-Aldrich) ranging from 0.5 to 500 µM melanin. We found that the cellular melanin content was increased 4-fold in L-DOPA treated cells compared to untreated

cells (**Figure 2C**). Notably, a 26-fold increase was observed in the medium of L-DOPA treated cells, compared to medium of untreated melanocytes (**Figure 2D**). This data shows that the isolated CD117$^+$ cells are functional in producing and secreting melanin. A possible limitation of our study could be the use of PMA (component of HMGS) which as non-physiological additive may influence normal cell function during *in vitro* maintenance. Further, the purified melanocytes were cultured only shortly (24 h), which was sufficient to demonstrate melanin production, but not long enough to examine cell expansion and the need of additives such as PMA.

To date, research on primary human melanocytes is hampered by the lack of a good protocol to isolate these cells. Current protocols for melanocyte purification require culturing of epidermal cell suspensions for several weeks (including at least one passage and *in vitro* proliferation of melanocytes) in order to eliminate contaminating keratinocytes and fibroblasts and obtain melanocyte monocultures. Here, we propose a new isolation protocol for the instant isolation of highly-purified primary human melanocytes by taking advantage of their cell-surface expression of CD117. With our protocol it is possible to obtain reasonable numbers of purified fresh melanocytes, all of which express Melan-A, show a multi-dendritic phenotype *in vitro* and produce melanin upon stimulation. One benefit of our protocol is that melanocytes can be obtained within several hours, allowing direct RNA microarray analysis or proteomic profiling and functional studies of freshly-isolated human melanocytes, which can be considered as proper equivalents of melanocytes *in vivo*.

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Conflicts of interest

None declared.

Supporting information

Additional supporting information may be found online in the Supporting Information section.

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Chapter 6

Targeting the PD-1/PD-L1 axis in human vitiligo

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Abstract

Autoreactive $CD8^+$ T cells play a pivotal role in melanocyte destruction in autoimmune vitiligo. Immunotherapy for melanoma often leads to autoimmune side-effects, among which vitiligo-like depigmentation, indicating that targeting immune checkpoints can break peripheral tolerance against self-antigens in the skin. Therapeutically enhancing immune checkpoint signaling by immune cells or skin cells, making self-reactive T cells anergic, seems a promising therapeutic option for vitiligo. Here we review the current knowledge on the PD-1/PD-L1 pathway in vitiligo as new therapeutic target for vitiligo therapy.

Background

Vitiligo is a common, acquired skin disease characterized by loss of epidermal melanocytes, resulting in sharply, depigmented macules (1). While there is still an extensive debate on the initiating steps, experts agree on immune-mediated melanocyte destruction, with melanocyte-specific $CD8^+$ T cells being able to kill melanocytes (2-4) and anti-melanocyte antibodies found in the sera of patients with vitiligo (5). Especially IFN-γ holds a crucial role in the pathogenesis of vitiligo (6). Moreover, regulatory T cells are impaired, both in numbers and function, in patients with vitiligo (7-9). Recently, tissue-resident memory T (T_{RM}) cells were shown to have a prominent role in disease development and flare-up in human vitiligo. Vitiligo affected skin was shown to be enriched for $CD8^+$ T_{RM} cells compared to healthy, unaffected donor skin, together indicating immune disturbance in patients with vitiligo (10, 11).

Current treatment modalities aim to block melanocyte destruction and/or induce repigmentation of the skin. Topical corticosteroids or immunosuppressants have shown good outcome, while this might not be the most effective approach (12). Moreover, clinical efficacy of current treatment modalities is not satisfactory and, therefore, new therapeutic strategies should be evaluated and tested for vitiligo patients.

Immune checkpoints play a pivotal role in immune evasion by tumors and immunotherapy for melanoma shows that targeting of immune checkpoints is sufficient to break peripheral tolerance and re-activate melanoma-specific cytotoxic T cells in a proportion of patients. Clinically opposite to melanoma, vitiligo patients might benefit from induced

peripheral tolerance, turning the autoimmune response into an anergic response. In this review, we therefore propose that exploiting the PD-1/PD-L1 axis may be effective as a treatment strategy for vitiligo.

Autoimmunity and tumor immunity

Similarities exist between skin autoimmunity and tumor immunity, observed in vitiligo and melanoma. Immunization of mice bearing B16 melanoma with gp100 antigen and tumor-specific $CD8^+$ T cells resulted in tumor destruction, but also caused vitiligo development (13). Similarly, in melanoma patients vitiligo can occur spontaneously or upon immunotherapy. Immunotherapy for melanoma often leads to autoimmune side-effects, including vitiligo-like depigmentation. This depigmentation is caused by activated anti-melanoma immunity, that targets not only malignant cells, but also healthy melanocytes and correlates with prolonged survival (14-18). Conversely, vitiligo patients have 3-fold less risk of developing melanoma during life (19, 20). Nevertheless, this risk is suggested to be influenced by individual skin type (21). Melanocytes are far more protective in dark skin types and, therefore, loss of melanocytes leads to a greater skin cancer risk in these patients. Contrary, in patients with light skin types loss of protective melanocytes seems to be less important and increased immunosurveillance contributes to an overall decreased risk of developing skin cancer. Melanoma susceptibility in vitiligo patients may, therefore, be adjusted for race. Also, immunotherapy for melanoma using a skin-depigmenting compound induced local and systemic anti-melanoma immunity (22). Conversely, a proportion of metastatic cutaneous melanoma patients develop vitiligo-like depigmentation (16, 17). Considering this melanoma/vitiligo relationship, vitiligo patients could benefit from anti-melanocyte tolerance, observed in melanoma, and exploiting immune checkpoints might influence peripheral tolerance against self-antigens in the skin.

PD-1/PD-L1 pathway

PD-1/PD-L1 pathway

Programmed cell death 1 (PD-1 or CD279) is an inhibitory cell-surface molecule that suppresses T cells. PD-1 has emerged as a key player in immune

regulation and is constitutively expressed by regulatory T cells and can be, upon activation, expressed by effector T cells, natural killer cells, and B cells (23). Immune suppression requires PD-1 binding on T cells to PD-1 ligands (PD-L1 and PD-L2) on other cell types. PD-L1 is constitutively expressed by various immune cells, e.g. lymphocytes, DCs and macrophages, and its expression can be induced on non-immune cells, including cancer cells. Ligation of PD-1/PD-L1 represses the activation and function of autoreactive T cells, inhibits their proliferation and induces apoptosis, thereby regulating both central and peripheral tolerance to hamper and regulate inflammatory responses and autoimmune diseases (24).

Melanoma immunotherapy

Signaling via the PD-1/PD-L1 axis is commonly involved in tumor immune evasion. In melanoma, tumor-associated PD-L1 can functionally suppress T cell responses to melanoma and promote T cell apoptosis (25). Consequently, PD-L1^{+} melanomas have been identified as a subset that tends to be more aggressive and is associated with poor prognosis (26).

Targeting these immune checkpoint molecules has become one of the therapeutic options (27). PD-1-targeting monoclonal antibodies have been approved for clinical use and are currently among the first-line treatment options for advanced melanoma patients and many other cancers (28, 29). In a fraction of melanoma patients (ranging from 3.4 to 28%), vitiligo-like depigmentation (or melanoma-associated leukoderma) occurs as an adverse effect of immune checkpoint inhibition, indicating the breaking of tolerance to melanocytic self-antigens (30, 31). Nevertheless, skin depigmentation has now been reported in anti-PD-1/PD-L1 treated patients with other metastatic cancers as well (32-37). Skin depigmentation is significantly associated with a favorable prognosis in melanoma patients (17, 18). Likewise, PD-L1-targeting antibodies, e.g. atezolizumab, are currently being investigated in metastatic melanoma patients (38). Considering vitiligo-like depigmentation in (melanoma) patients receiving anti-PD-1 monotherapy, vitiligo patients may benefit from induced peripheral tolerance to self-proteins by exploiting the T cell immunosuppression mediated by these molecules.

PD-1/PD-L1 in autoimmune diseases

Emerging evidence demonstrates that impaired PD-1/PD-L1 function is involved in a variety of autoimmune diseases, among which type 1 diabetes, inflammatory bowel diseases, and rheumatoid arthritis (39) Manipulating PD-1/PD-L1 signaling appears to elicit significant outcomes in disorders with aberrantly-regulated immune system function. More specifically, PD-L1-expressing DCs can induce anergy of otherwise active T cells and, thereby, hinder induction of autoimmune encephalomyelitis, an animal model of multiple sclerosis (40). Also, intravenous injection of recombinant adenovirus expressing full-length mouse PD-L1 (Ad.PD-L1) gene can suppress lupus-like syndrome in BXSB mice, when combined with anti-ICOSL(B7h) antibody to block ICOS-mediated co-stimulation (41). In this study, it was suggested that the protective effect of Ad.PD-L1 was through suppression of autoreactive T cells at the target organ. Furthermore, severe psoriatic inflammation was induced in PD-1-null mice and PD-L1-Ig fusion protein was shown effective in inhibiting inflammatory skin γδ-low T cell activity *in vitro* (42), indicating the rationale to test the therapeutic potential of increasing PD-1/PD-L1 signaling in skin autoimmunity. Raising our knowledge on PD-1/PD-L1 functions and signaling may thus enable us to develop new therapeutic strategies to manipulate the inhibitory function of PD-1.

PD-1/PD-L1 in vitiligo

PD-1/PD-L1 expression in vitiligo

While abundantly studied in melanoma, the PD-1/PD-L1 axis in vitiligo has received far less attention thus far, but deserves exploration as a therapeutic target. Similarly to melanoma cells, PD-L1 can be expressed by melanocytes, especially in the case of inflammatory responses (43). PD-L1-expressing melanocytes co-localize with infiltrating immune cells and PD-L1 strongly correlates with immune cell infiltration in both nevi and malignant melanoma. Also, CD45 and IFN-γ mRNA were detected in PD-L1^{+} melanomas, while IFN-γ could not be detected in PD-L1^{-} tumors. Expression of PD-L1 can be upregulated on various cell types upon stimulation with pro-inflammatory cytokines, including IFN-γ (44). Although IFN-γ-producing CD8^{+} T cell are abundantly present in lesional vitiligo skin, cytokine-induced

PD-L1 expression might not be occurring or insufficient in driving a negative feedback loop.

Vitiligo patients also have increased PD-1 expression on peripheral $CD3^+$ $CD4^+$ and regulatory T cells (45) compared to healthy individuals, suggesting involvement of PD-1 in disease regulation and pathogenesis. Blocking PD-L1 signaling *in vitro* led to expansion of regulatory T cells (46), implicating that PD-1/PD-L1 engagement may negatively regulate regulatory T cell function. The authors, therefore, hypothesized that PD-1^+ regulatory T cells may become exhausted by PD-L1-expressing autoreactive T cells, hence leading to a deficiency of regulatory T cells in vitiligo (45). PD-L1 expression by autoreactive T cells, however, hasn't been shown. Similarly, vitiligo patients show increased PD-1 expression on $CD8^+$ T cells compared to healthy donor $CD8^+$ T cells (47), implying that PD-1 expression is due to excessive activation of autoreactive $CD8^+$ T cells. PD-1 expression on $CD8^+$ T cells appears to positively correlate with disease activity (47), suggesting a more vigorous attempt to control the inflammatory situation by increasing PD-1 expression. *In situ*, PD-1 is significantly expressed in marginal and lesional infiltrates when compared to non-lesional skin in patients with active non-segmental vitiligo (48). Because no CD4/PD-1 and CD8/PD-1 double staining was done, it remains unstudied which skin T cells express PD-1. Together, these results suggest a role for PD-1/PD-L1 in the lack of peripheral tolerance in vitiligo, providing a rationale to target this axis in vitiligo treatment.

Vitiligo is a polygenic disorder, implying simultaneous contributions of multiple genetic risk factors and environmental triggers. Large-scale genome-wide association studies have identified approximately 50 genetic loci that contribute to vitiligo risk, of which a large fraction encode proteins involved in immune regulation (49-52). To date, no single-nucleotide polymorphisms in the *PD-1* (*PDCD1*) or *PD-L1* (*CD274*) gene has been reported in human vitiligo. Nevertheless, *PD-1* polymorphisms that give a higher risk of developing other autoimmune disorders, among which rheumatoid arthritis and systemic lupus erythematosus, have been reported (53). Additionally, microsatellite polymorphisms in the *CTLA*-4 gene, encoding the CTLA-4 immune checkpoint molecule, have been demonstrated in European-derived vitiligo patients (54). Likewise, a missense polymorphism in the *PTPN22* gene has been associated with susceptibility to autoimmune disorders, including vitiligo (55). Lymphoid protein tyrosine phosphatase

(LYP), encoded by the *PTPN22* gene, is an important downregulator of T cell activation. Hypothetically, patients carrying a *PTPN22* polymorphism initially might be less sensitive to T cell inhibition (e.g. by PD-1/PD-L1 signaling), since the LYP missense polymorphism drastically reduce the binding of LYP to C-terminal Src kinase (CSK) *in vitro*. As a result downregulation of T cell activation is disrupted so that T cells lacking the LYP-CSK complex remain hyper-reactive.

Targeting PD-1/PD-L1 in vitiligo

Therapeutically enhancing immune checkpoint signaling on self-reactive T cells, making them anergic, seems a promising therapeutic option for vitiligo. Maio *et al*. (2018) studied PD-1/PD-L1 signaling as a therapeutic target for vitiligo and showed that treatment with PD-L1 fusion protein reversed depigmentation in a Pmel-1 vitiligo mouse model (56). Around 60% of the original pigment loss was restored upon treatment. PD-L1 fusion protein reversed depigmentation in Pmel-1 vitiligo mice via a marked increase in regulatory T cells in the skin and a decrease in melanocyte-reactive T cells. Fortunately, no significant side effects were observed, among which skin oncogenesis, indicating that targeting the PD-1/PD-L1 axis can be effective as a treatment strategy for T cell-induced vitiligo. The suppression of depigmentation was relatively stable until 8 weeks after last treatment, but, none of the mice regained permanent repigmentation. This limited (long-term) repigmentation in Pmel-1 vitiligo mice might be due to loss of a pigment cell reservoir, suggesting that the presence of a viable melanocyte reservoir might be crucial for the efficacy of PD-L1 fusion protein and other immune suppressive therapies. More specifically, human vitiligo lesions in hairless skin and those containing white hair do not have a strong capacity for repigmentation (57). PD-1/PD-L1 therapy, therefore, might be most effective in an early or progressive stage of vitiligo, supported by the observation that disease duration negatively affects prognosis to treatment (58). Nevertheless, patients with long-duration vitiligo may repigment well, as long as their lesions contain pigmented hair. Patients with active vitiligo might therefore be the best responders to immunomodulating agents, whereas patients with long-duration disease might need additional melanocyte transplantation (57).

Concomitantly, UV phototherapy, especially narrow-band ultraviolet B (NB-UVB) therapy, might be necessary to obtain more complete repigmentation. Among current topical and systemic treatment modalities, NB-UVB phototherapy has emerged as one of the safest and most effective therapies in vitiligo (59). Therapeutically enhancing immune checkpoint signaling may induce peripheral tolerance, yet melanocytes might still need additional stimulation to restore skin pigmentation. At the same time, NB-UVB therapy likely affects PD-L1 expression. UV-B radiation was shown to induce PD-L1 expression in human melanoma cell lines in an NF-κB-dependent manner (60). Similarly, UV-B treatment induced NF-κB activation in human primary epidermal melanocytes, implying PD-L1 upregulation is a conserved stress response to UV exposure in human melanocytes and melanoma cells that can inhibit effector T cell activity. Altogether, combined use of local agonistic PD-1/PD-L1 treatment and NB-UVB therapy seems promising in inducing local melanocyte tolerance and significant repigmentation.

Better understanding of the PD-1/PD-L1 may provide clues on how this pathway can most effectively be targeted in vitiligo. Topical treatment modalities, optionally with laser assisted delivery, or local injection are preferred over systemic therapy, partly to avoid systemic tolerance. Vitiligo patients show local autoreactivity resulting in depigmented skin patches, while tolerance to melanocyte antigens seems to predominate in other parts of the skin, leaving those pigmented. The feasibility of local vitiligo treatment, however, depends on the vitiligo disease extent. At the same time, as mentioned before, therapy is preferably given at an early disease stage, to acquire full melanocyte tolerance, long-term efficacy and possibly curation.

Local immunotherapy might decrease immunosurveillance, carrying a potential risk for oncogenesis. Vitiligo patients, however, have a 3-fold less risk of developing melanoma during life (19, 20). We, therefore, hypothesize that agonistic PD-1/PD-L1 therapy will increase the probability of melanoma development to the risk of healthy, control individuals. Nonetheless, clinicians should be aware of potential tolerance to e.g. transformed melanocytes or keratinocytes.

Discussion

Mounting evidence has shown that signaling via the PD-1/PD-L1 axis is commonly involved in melanoma immune evasion and targeting these immune checkpoint molecules can reactivate potent effector immune responses against the tumor. The growing appreciation of similarities between vitiligo (autoimmunity) and melanoma (tumor immunity), has led to increased interest in PD-1/PD-L1 as promising targets for future immunotherapies in human vitiligo.

Although not fully confirmed so far, PD-1/PD-L1 seems to be involved in loss of peripheral tolerance in human vitiligo, with PD-1 being expressed on regulatory and $CD8^+$ T cells, and *in vivo* PD-L1 protein therapy reversing depigmentation in murine vitiligo. At the same time, however, PD-1/PD-L1 expression on other cell types, among which dendritic cells (DCs), B cells, keratinocytes and fibroblasts, remain understudied and might be relevant. Recently, efficacy of anti-PD-1/PD-L1 therapy in cancer was attributed to PD-L1 expression on DCs, rather than tumor cells (61-64). PD-1/PD-L1 blockade enhanced maturation of DC in tumor-draining lymph nodes and increased T cell priming (61, 62). In **figure 1**, we propose a model of how PD-1/PD-L1 signaling in vitiligo maintains an inflammatory environment. Vitiligo patients show increased PD-1 expression on peripheral regulatory $CD4^+$ and cytotoxic $CD8^+$ T cells. Concomitantly, PD-1^+ mononuclear cells have been identified in (peri-)lesional vitiligo skin.

Though (peripheral) T cells have been demonstrated to be PD-1^+, PD-L1 expression remains largely understudied. PD-L1 can be expressed by melanocytes, especially in the case of inflammatory responses (43). IFN-γ-producing $CD8^+$ T cell are abundantly present in affected skin, but to date, (cytokine-induced) PD-L1 expression by melanocytes in (peri)lesional vitiligo skin remains unclear. If expressed, PD-$L1^+$ melanocytes seem to be ineffective in inhibiting melanocyte-specific T cells. Concomitantly, PD-L1 expression on myeloid cells, among which DC, in vitiligo skin has received no attention thus far. PD-$L1^+$ myeloid cells might be important in inhibiting autoreactive T cells as well. Future studies should clarify PD-L1 expression levels *in situ* to determine if these are diminished in human vitiligo and, thus, insufficiently inhibit autoreactive $CD8^+$ T cells. Finally, it has been hypothesized that PD-L1-expressing melanocyte-specific T cells cause exhaustion of PD-1^+ regulatory T cells, hence leading to uncontrolled autoimmunity (45). It is, therefore,

important to study if PD-L1$^+$ cells decrease peripheral tolerance by inhibiting CD4$^+$ regulatory T cell function. Most importantly, however, are the PD-1 expression levels on skin-resident T cells, for skin autoimmunity appears locally and PD-L1-induced peripheral tolerance is only relevant when PD-1$^+$ T cells are present.

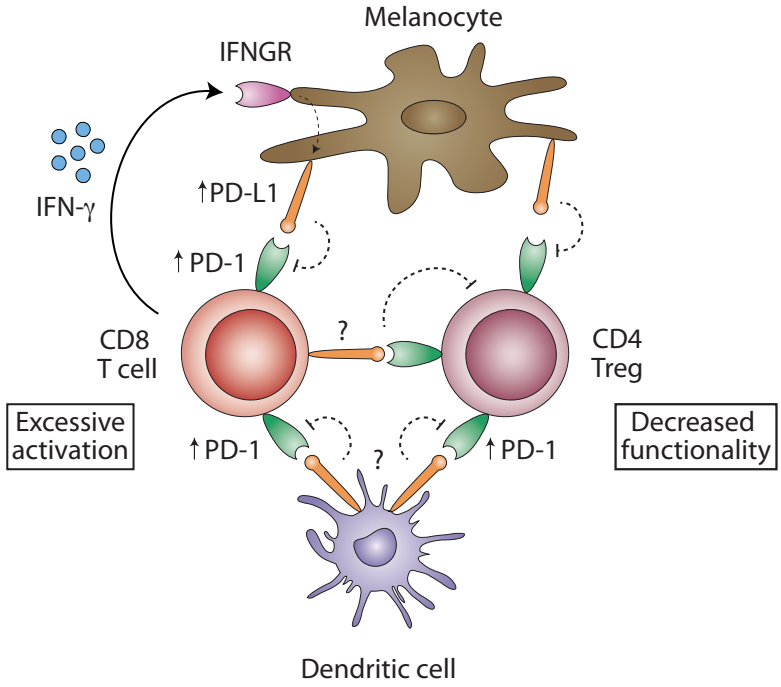

Figure 1. Proposed model of PD-1/PD-L1 signaling in vitiligo. Blood regulatory CD4$^+$ and cytotoxic CD8$^+$ T cells show increased levels of PD-1 protein. IFN-γ-producing CD8$^+$ T cell are abundantly present in affected skin and may induce PD-L1 expression on melanocytes, albeit possibly ineffective in inhibiting autoreactive T cells. Consequently, melanocyte-reactive CD8$^+$ T cells remain activated. PD-L1 expression on DCs remains unstudied in vitiligo and might be important in effectively inhibiting autoreactive CD8$^+$ T cells. PD-L1$^+$ DC might inhibit not only T cell priming in the tumor-draining lymph nodes, but also T cell effector function. PD-L1 expression *in situ* remains unclear but might be expressed by melanocytes, DC or melanocyte-specific T cells, turning PD-1$^+$ regulatory CD4$^+$ T cells to functional exhaustion. Overcoming excessive activation of autoreactive CD8$^+$ T cells and decreased functionality of regulatory CD4$^+$ T cells in vitiligo might be achieved by interfering with PD-1/PD-L1 signaling.

Given that PD-1 is expressed by both peripheral regulatory CD4$^+$ and cytotoxic CD8$^+$ T cells in vitiligo patients, we reasoned that agonistic PD-1/PD-

L1 therapy might suppress melanocyte-reactive T cells, but concomitantly suppress regulatory T cells. Counterintuitively, injection of PD-L1 fusion protein in Pmel-1 vitiligo mice led to a marked enrichment of regulatory T cells in the skin and a reduction in effector T cells (56), implying PD-L1 negatively affects autoreactive T cells, but not regulatory T cells. PD-1 expression by regulatory T cells, however, was left unstudied in this mouse model. This suggests that effector T cells were the main PD-1$^+$ T cell population. Considering the presence of PD-1$^+$ CD4$^+$ regulatory T cells in blood of vitiligo patients, PD-L1 fusion protein might give different results in the human setting, justifying further research on the effect of increased PD-1/PD-L1 signaling on both T cell subsets to determine treatment outcome. Since topical or local treatment modalities are preferred, we hypothesize that PD-1 expression by skin-resident regulatory or memory T cells are most relevant for therapy outcome, rather than peripheral PD-1 expression.

With more knowledge becoming available on PD-1 and PD-L1 expression levels among different immune cell subsets, targeting this pathway might induce effective long-term melanocyte-specific tolerance in human vitiligo.

Author contributions

MW wrote the manuscript with input from all authors. All authors contributed to the article and approved the submitted version.

Conflict of Interest

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

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Chapter 7

Impaired IFN-γ-induced PD-L1 expression on human vitiligo melanocytes

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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 signaling 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 signaling might have therapeutic potential in vitiligo.

$PD\text{-}1^+$ 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. Most 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 signaling may therefore be a therapeutic option for vitiligo patients.

Introduction

Vitiligo is a cutaneous pigment disorder characterized by white skin patches due to loss of melanocytes, the pigment producing cells of the skin.[1] Research has clarified immune-mediated melanocyte destruction in vitiligo affected skin, caused by autoreactive melanocyte-specific $CD8^+$ T cells.[2] Moreover, antibody responses against melanocyte antigens, such as tyrosinase and

TRP-2, can be found in the sera of patients with vitiligo.[3] Additionally, an imbalance of $CD4^+/CD8^+$ T cell ratio and regulatory T cells might contribute to T cell mediated pathogenesis of vitiligo.[4] Finally, T cells with a resident phenotype (tissue-resident memory T (T_{RM}) cells) contribute to disease development and flare-up in human vitiligo. $CD8^+$ T_{RM} cells were enriched in vitiligo affected skin, compared to healthy donor skin[5,6], indicating immune disturbance in patients with vitiligo.

Immune checkpoints, *e.g.* programmed cell death 1 (PD-1), modulate immune responses by regulating peripheral tolerance, thereby preventing unwanted inflammatory responses and autoimmunity.[7] Immunotherapy for melanoma shows that anti-PD-1 antibodies can break peripheral tolerance and thereby reactivate anti-tumor responses in patients.[8] Moreover, in 2-43% of melanoma patients treated with immunotherapy, vitiligo-like depigmentation occurs, which indicates the presence of immune reactivity against both melanoma cells and melanocytes.[9-13] Vitiligo development in melanoma patients has been associated with a favorable prognosis.[9-12] 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.[14] Impaired PD-1/PD-L1 function is involved in a variety of autoimmune diseases, e.g. type I diabetes and rheumatoid arthritis[15], indicating the rationale to test the therapeutic potential of increasing PD-1/PD-L1 signaling in autoimmunity. Considering that IFN-γ and type I interferons play a crucial role in vitiligo pathogenesis[2,16,17] and both are known to induce immune evasion by PD-L1 expression in melanoma[18,19], the question arises to what extent PD-L1 upregulation and resulting immune regulation occurs in vitiligo.

To our knowledge, *in situ* PD-1 expression and (cytokine-induced) PD-L1 expression in vitiligo remains incompletely studied. This study, therefore, aimed to study *in situ* PD-1/PD-L1 expression in human vitiligo specimens. Secondly, we aimed to investigate the role of PD-1/PD-L1 checkpoint signaling 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 points to an insufficient ability of melanocytes of vitiligo patients to protect themselves against autoreactive T cells.

Methods

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 of the Netherlands Institute for Pigment Disorders at the Amsterdam University Medical Centers. Two or four millimeter 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.[20] 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**.

Table 1. Patient characteristics

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

Immunohistochemistry

Immunohistochemical staining was performed on frozen tissue sections. Acetone-fixed 4 µm sections were pre-incubated with Superblock (ScyTek Laboratories, Inc., Logan, UT) before incubation with primary antibodies for 60 minutes. Antibodies included anti-human CD3 (clone SK7, BD Biosciences, Franklin Lakes, NJ), anti-human CD3 (clone SP7, Immunologic, Duiven, The Netherlands), anti-human PD-1 (clone NAT105, Abcam, Cambridge, UK), anti-human PD-L1 (clone MIH1, eBioscience, San Diego, CA) and anti-human Melan-A (clone A19-P, Biorbyt Ltd, UK). Antigen-antibody binding was visualized using PermaRed/AP (Diagnostic Biosystems, Pleasanton, CA), Vector® NovaRED or Vector® Blue (both Vector Laboratories, Inc. Burlingame, CA) chromogen. Sections were counterstained with hematoxylin (Klinipath, Amsterdam, the Netherlands) and mounted for review. Sections of tonsil specimens with confirmed high expression of the target molecules served as positive control.

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 mm^2 was determined for each staining. Images (magnification 400x) were acquired on a Leica DM microscope using Leica software (Leica Biosystems, Wetzlar, Germany).

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 Miltenyi Biotec, Bergisch Gladbach, Germany). Thereafter, cells were collected by filtering through a 70 µm cell strainer. Alternatively, skin biopsies were incubated in 50 U/ml dispase (Sigma-Aldrich, Saint Louis, MO) 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, Waltham, MA) for 5 minutes at 37°C. Trypsin was neutralized by FCS and cells were collected by filtering through a 70 µm cell strainer.

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 Thermo Fisher Scientific). Primary fibroblasts 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. 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 (Amsterdam, The Netherlands).

Cytokine exposure

One day prior to exposure, cells were seeded in a 6-well plate at a density of 2×10^5 cells per well in a total volume of 2 ml medium with supplements and culture conditions as stated above. After 24 hours, cells were left unexposed or exposed for 48 hours with 500 U/ml IFN-γ (R&D Systems, Minneapolis, MN), IFN-α1b, IFN-α2a, IFN-α2b or IFN-β1a (all 1000 U/ml, all Immunotools, Friesoythe, Germany). After 48 hours, cells were harvested for quantitative PCR and flow cytometry analysis.

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, Hilden, Germany) following the manufacturer's protocol. RNA was transcribed into cDNA according to manufacturer's instructions using the Promega AMV Reverse Transcriptase kit (Promega, Madison, WI). An additional on-column DNase treatment was performed using the RNAse-Free DNAse (Qiagen). Gene expression

levels of *PD-L1* were measured by quantitative PCR (qPCR) performed on a Bio-Rad CFX Connect (Bio-Rad Laboratories, Inc., Hercules, CA). The following oligonucleotides sequences were used: β-*actin* forward 5-GATCGGCGGCTCCATCCTG, reverse 5-GACTCGTCATACTCCTGCTTGC, *HSRPS18* forward 5-AGTTCCAGCATATTTTGCGAG, reverse 5-CTCTTGGTGAGGTCAATGTC, and *PD-L1* forward 5-TGAACTGACATGTCAGGCTG, reverse 5- TACCACTCAGGACTTGATGG (all Biolegio Lab Equipment B.V., Nijmegen, The Netherlands). Expression levels were calculated according to the $2^{\Delta\Delta CT}$ method and normalized to the reference genes (β-*actin* and *HSRPS18*).

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, San Diego, CA), FITC-conjugated mouse anti-human Melan-A (clone A103, Santa Cruz, CA). Cell surface staining was performed in FACS buffer (PBS supplemented with 1% bovine serum albumin and 0.05% NaN_3). Subsequently, cells were fixed in Fixation Buffer and stained intracellularly in Perm/Wash Buffer (both Biolegend), according to the manufacturer's instructions. Antibody binding was analyzed on a FACS Canto II and data was analyzed using FlowJo software (both BD Biosciences).

Skin explant assay

Cryosections of previously described skin explant assays were used.[21] 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, East Rutherford, NJ), 20 U/ml interleukin-2 (Eurocetus, Amsterdam, The Netherlands), 5 ng/ml interleukin-15 (Strathmann Biotec GmbH & CO. KG, Germany), 15 µg/ml gentamycin (Duchefa, Haarlem, The Netherlands), 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% CO_2. 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 Miltenyi Biotec), according to the manufacturer's instructions. Next, non-lesional skin biopsies were co-cultured in a 96-well round bottom plate in the absence or presence of 3-5x10^5 autologous perilesional $CD8^+$ T cells for 48 hours 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, Alphen aan den Rijn, The Netherlands) for further immunohistochemical analysis on cryosections of the explants.

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]

Statistical analysis

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

Results

Significant $PD\text{-}1^+$ T cells, but limited $PD\text{-}L1^+$ 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, $PD\text{-}1^+$ cells were present in 13/13 (100%) perilesional vitiligo skin biopsies and the vast majority (75%) of $PD\text{-}1^+$ cells co-localized with $CD3^+$ cells, indicating the presence of PD-1-expressing T cells (**Table 2**, **Figure 1B-C**). Whereas $PD\text{-}1^+$ cells

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)

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.

Type I and II interferon induce PD-L1 expression in healthy donor skin cells

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-γ[2,21] and that IFN-γ can induce PD-L1 expression in melanoma.[18] 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.[19] Exposure to IFN-γ, but also type I interferons (including IFN-α1b, IFN-α2a/b and IFN-β1a), increased constitutive

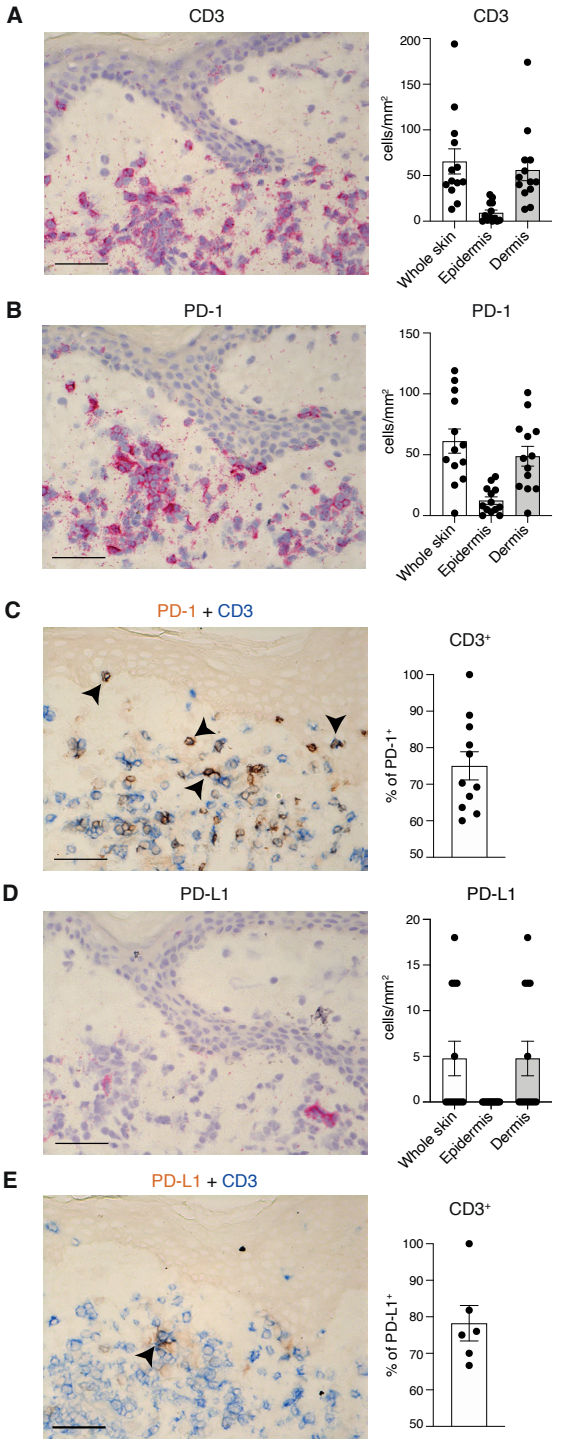

Figure 1. Significant PD-1⁺ T cells, but limited PD-L1⁺ cells in perilesional vitiligo skin biopsies. (A-E) Immunohistochemistry and positive cells/mm² in whole skin, epidermal and dermal skin of CD3 (A), PD-1 (B), and PD-L1 (D) in perilesional biopsies of vitiligo patients. Immunohistochemistry of PD-1 (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.

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 level (**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 (**Supplementary figure S1-S2**). These data show that pro-inflammatory IFN-γ and type I interferons can induce PD-L1 upregulation in healthy donor (skin) cells.

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)[22]. 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, that originate from healthy melanocytes, were not suitable for use, because of HPV E7 induced PD-L1 expression.[23] 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

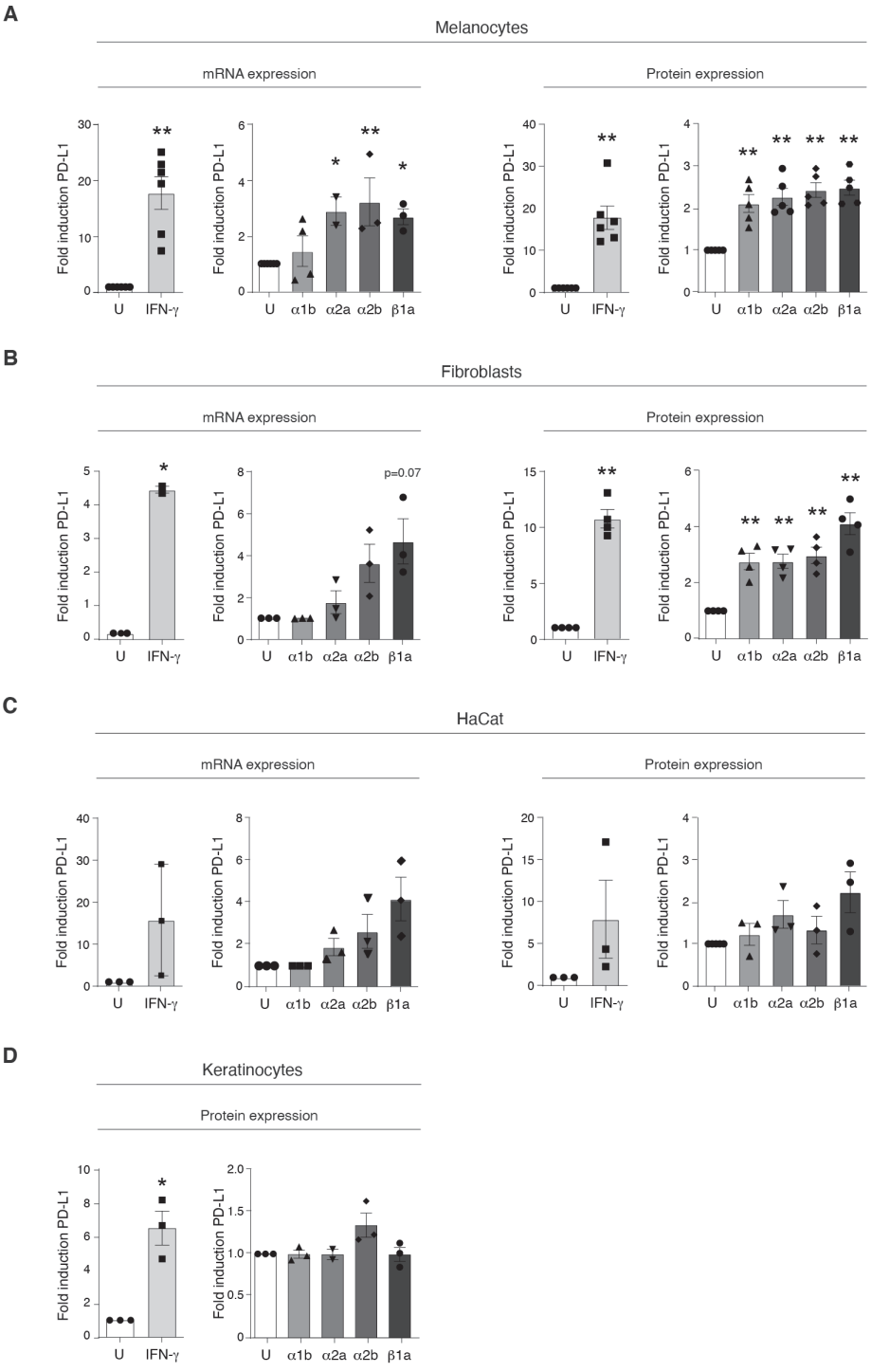
A
Melanocytes
mRNA expression
Protein expression
Fold induction PD-L1
U
IFN-γ
α1b
α2a
α2b
β1a
B
Fibroblasts
p=0.07
C
HaCat
D
Keratinocytes

Figure 2. PD-L1 expression is upregulated by human skin cells after cytokine exposure. (A-C) Primary human melanocytes (n=6) (A), fibroblasts (n=4) (B), HaCat cells (n=3 independent experiments) and primary keratinocytes (n=3) (C) were exposed with IFN-γ (500 U/ml), IFN-α1b, IFN-α2a, IFN-α2b, IFN-β1a (all 1000 U/ml) or left unexposed for 48 hours. Thereafter, PD-L1 expression was measured on mRNA level by qPCR analysis (left panels) or protein level by flow cytometry (right panels). Fold induction of PD-L1 expression was calculated compared to unexposed cells. Error bars: mean ± SEM. ANOVA shows significance to unexposed cells as indicated; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

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 cell are abundantly present in vitiligo-affected skin, *in situ* cytokine-induced PD-L1 expression was analyzed 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 has shown that CD8$^+$ T cells, obtained from vitiligo perilesional skin, infiltrate autologous, non-lesional skin biopsies and mediate melanocyte destruction.[21] 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.

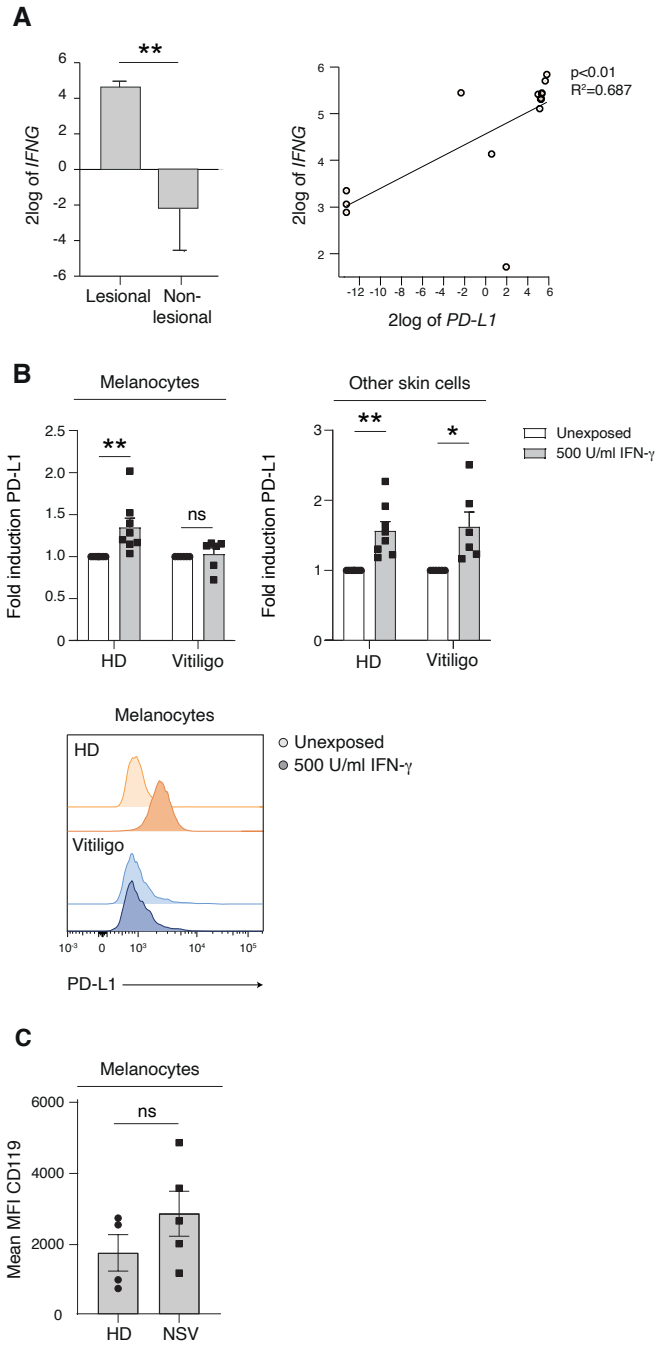

Figure 3. PD-L1 expression by vitiligo melanocytes remains unchanged after IFN-γ exposure. (A) Bar plot showing 2log of *IFNG* mRNA expression in lesional and non-lesional skin biopsies (left) and correlation analyses of 2log of *IFNG* mRNA expression and 2log of *PD-L1* mRNA expression in lesional skin 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.

Discussion

This study reveals important insights into PD-1/PD-L1 signaling 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-L-1 pathway. These data indicate an 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 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 of healthy donors[24,25], whereas the percentage of regulatory T cells was significantly decreased in vitiligo patients.[24] 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.[26] A recent study demonstrated an impaired regulatory T cell suppressive function in blood of patients with vitiligo[27] and, therefore, it can be postulated that perilesional PD-1-expressing cells are mainly CD4$^+$ T cells, that have lost their suppressive function. Nevertheless, considering the reduction in regulatory T cells[28] and abundance of melanocyte-specific CD8$^+$ resident memory T (T_{RM}) cells in depigmented vitiligo skin[5,6], that have been shown to express PD-1[29], it can be postulated that PD-1$^+$ cells in vitiligo skin are mainly melanocyte-reactive CD8$^+$ T_{RM} cells. Whereas PD-1 signaling may involve low cytotoxic potential of skin T_{RM} cells[29], others have demonstrated that PD-1-expressing T_{RM} cells remained reactive and capable of providing immune protection or mediating immune pathology.[30,31] Consistent with this, treatment with PD-L1 fusion protein repressed the numbers of melanocyte-reactive T cells and increased regulatory T cells numbers in a Pmel-1 T cell receptor transgenic vitiligo mouse model.[32] PD-L1 fusion protein treatment specifically repressed the activation of Vβ12-expressing T cells, as measured by IFN-γ production,

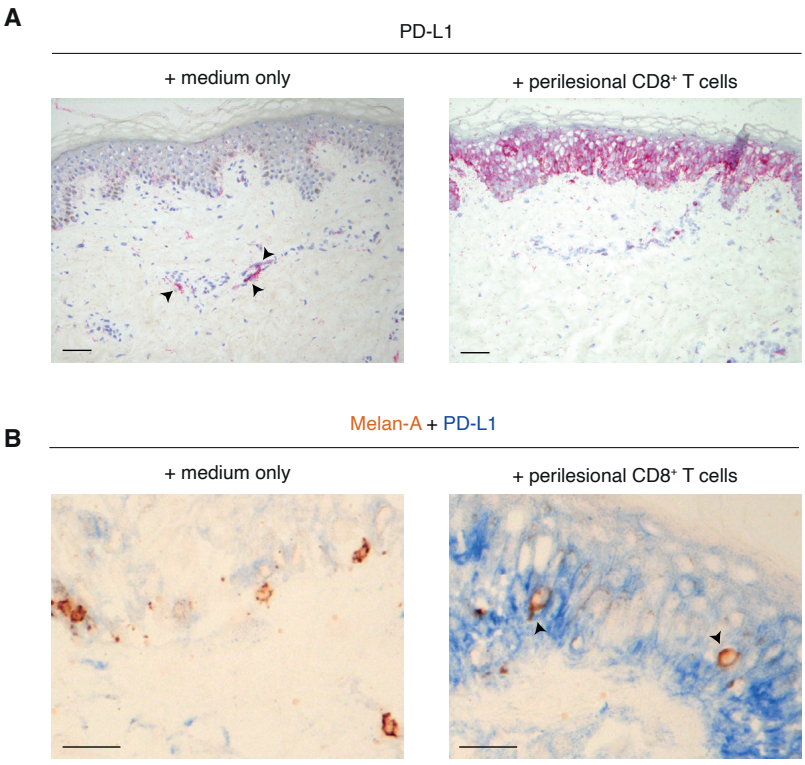

Figure 4. Increased epidermal PD-L1 expression by skin cells, but not by melanocytes in a vitiligo skin explant assay. (A) Immunohistochemistry of PD-L1 in non-lesional vitiligo skin biopsies co-cultured with medium only (left photo) or with autologous perilesional $CD8^+$ T cells (right photo). (B) Immunohistochemistry of PD-L1 (in blue) and Melan-A (in orange) in a non-lesional vitiligo skin biopsy co-cultured with medium only (left photo) or autologous perilesional $CD8^+$ T cells (right photo). Representative immunohistochemical staining is shown. Bars equal 100 µm.

and reversed depigmentation in Pmel-1 mice.[32] Although the main PD-1-expressing T cell subset in vitiligo remains indistinct, targeting PD-1/PD-L1 signaling will either increase regulatory $CD4^+$ T cell numbers and function, or repress cytotoxic $CD8^+$ T cell activity, both positively affecting peripheral tolerance.

The current 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 signaling pathways regulating PD-L1 expression.[33] Also, there is no scRNAseq 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 signaling to induce peripheral tolerance to melanocytes in vitiligo.

Acknowledgements

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Author contributions

MW, GK, EPMT, KJW, AL, 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, MWB and RML designed experiments and participated in manuscript revision. All authors have read and approved the final manuscript. RML supervised the project.

Conflict of interest

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

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supplemental methods

Cell culture

Human melanoma cell lines Mel-WBO, Mel-136.2 and BLM were maintained in IMDM (Thermo Fisher Scientific) and Mel-92.2, Mel-AKR, Mel-GBU, Mel-ICL, Mel-JUSO, Mel-MOU in RPMI-1640 (Thermo Fisher Scientific) both supplemented with 8% FCS, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin (all Thermo Fisher Scientific).

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (LymphoPrep, STEMCELL Technologies) from anonymous healthy donor blood provided by Sanquin Blood Supply Foundation (Amsterdam, The Netherlands), according to the Declaration of Helsinki Principles. Immature monocyte-derived DC (moDC) were generated as described (de Jong et al. 2002). MoDC were left immature or cultured for 48 hours in the presence of IMDM supplemented with 10% FCS, 500 U/ml GM-CSF (Miltenyi Biotec) and 20 µg/ml Poly I:C (Sigma-Aldrich) to mature.

Plasmacytoid DC (pDC) and conventional DC (cDC) were enriched from PBMC by depletion of $CD3^+$, $CD14^+$, $CD16^+$, $CD19^+$ and $CD20^+$ cells by immunomagnetic bead selection using anti-FITC beads (Miltenyi Biotec), followed by fluorescence-activated cell sorting using PE-Cy7-conjugated mouse anti-human CD123 (clone 6H6), PE-Dazzle™ 594-conjugated mouse anti-human CD1c (clone L161)(both Biolegend), and FITC-conjugated mouse anti-human lineage cocktail 1 (BD Biosciences). FACS sorting was performed on a FACSAria IIu using BD FACSDiva software (both BD Biosciences) The following FITC-conjugated antibodies were used: mouse anti-human CD3 (clone SK7), mouse anti-human CD14 (clone MφP9), mouse anti-human CD16 (clone 3G8)(all BD Biosciences), mouse anti-human CD19 (clone HIB19, Biolegend), and mouse anti-human CD20 (clone L27, BD Biosciences).

Skin biopsies were dissociated into single-cell suspensions by enzymatic degradation using the Whole Skin Dissociation Kit and mechanical dissociation using the gentleMACS™ Dissociator (both Miltenyi Biotec, Germany). Thereafter, cells were collected by filtering through a 70 µm cell strainer.

Supplemental material

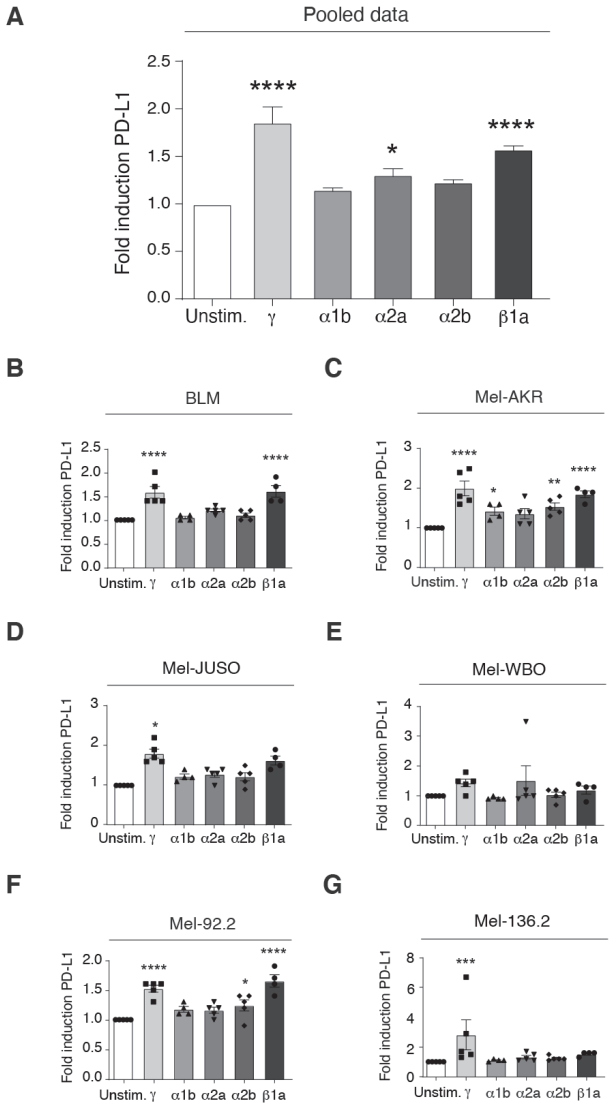

Figure S1. PD-L1 expression is upregulated by melanoma cell lines after cytokine stimulation. Melanoma cell lines were stimulated with IFN-γ (500U/ml), IFN-α1b, IFN-α2a, IFN-α2b, IFN-β1a (all 1000 U/ml) or left unstimulated for 48 hours. Thereafter, PD-L1 expression was measured by flow cytometry. Fold induction of PD-L1 expression was calculated compared to unstimulated cells. (A) Pooled data of melanoma cell lines. (B-G) PD-L1 expression after stimulation in BLM cells (B), Mel-AKR cells (C), Mel-JUSO cells (D), Mel-WBO cells (E), Mel-92.2 cells (F) and Mel-136.2 cells (G). Error bars: mean ± SEM. Data shown are the results of 5 independent experiments. ANOVA significant to unstimulated cells as indicated; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

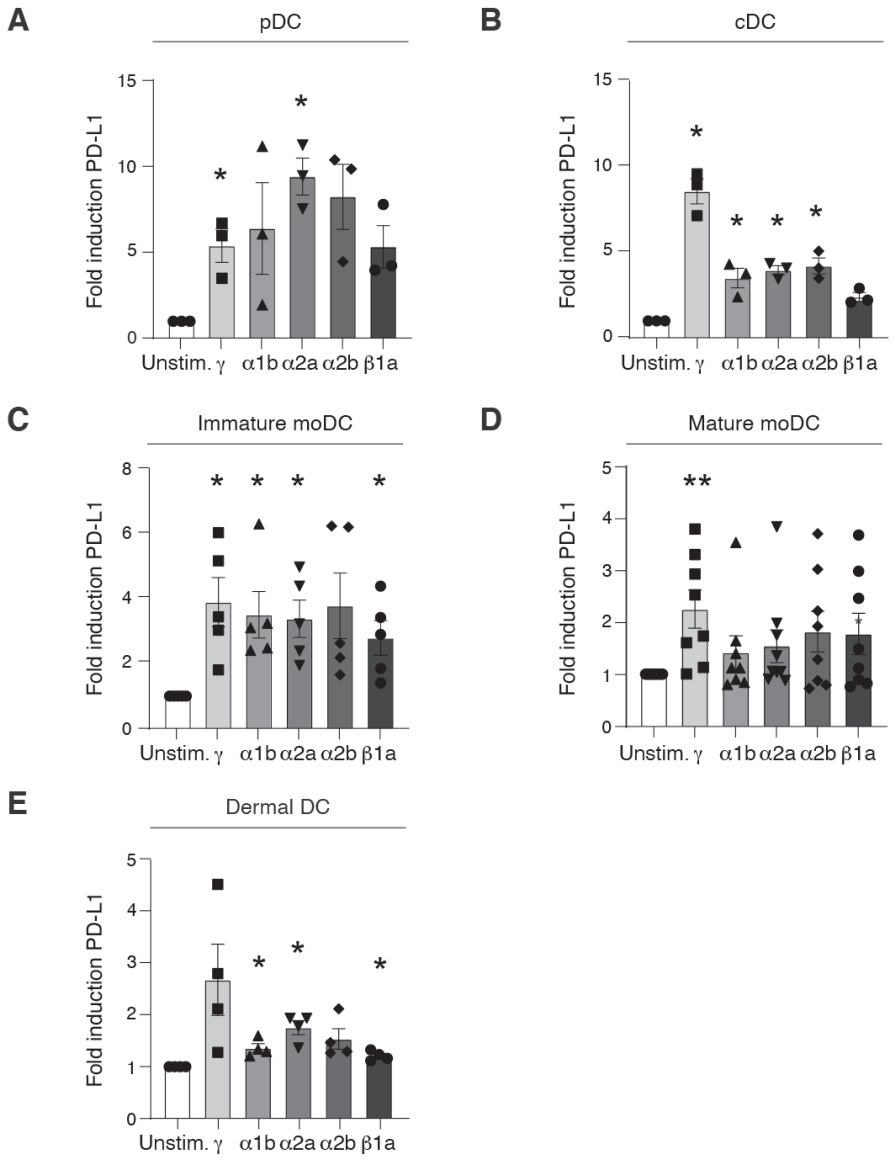

Figure S2. Cytokine stimulation upregulates the expression on PD-L1 on dendritic cells. (A-E) Human blood pDC, (A), cDC (B) (both n=3), immature (C) and mature moDC (D), and human skin suspensions (E) (n=4-8) were stimulated with IFN-γ (500U/ml), IFN-α1b, IFN-α2a, IFN-α2b, IFN-β1a (all 1000 U/ml) or left unstimulated for 48 hours. Thereafter, PD-L1 protein expression was analyzed by flow cytometry. Fold induction of PD-L1 expression was calculated compared to unstimulated cells. Error bars: mean ± SEM. ANOVA significant to unstimulated cells as indicated; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Chapter 8

Improvement of Opal multiplex immunofluorescence workflow for human tissue sections

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Abstract

The Opal multiplex technique is an established methodology for the detection of multiple biomarkers in one section. The protocol encompasses iterative single stainings and heating-mediated removal of the primary and secondary antibodies after each staining round, leaving untouched the Opal fluorophores which are deposited onto the antigen of interest. According to our experience, repetitive heating of skin sections often results in tissue damage, indicating an urgent need for milder alternatives to strip immunoglobulins. In this study we demonstrate that considerable heating-related damage was found not only in skin but also in tissues of different origin, mostly characterized by low cell density. Importantly, the morphology remained fully intact when sections were repetitively exposed to β-mercaptoethanol-containing stripping buffer instead of multiple heating cycles. However, target epitopes appeared sensitive at a differential degree to multiple treatments with stripping buffer, as shown by loss in staining intensity, but in all cases the staining intensity could be restored by increment of the primary antibody concentrations. Application of β-mercaptoethanol-containing stripping buffer instead of heating for antibody removal markedly improved the quality of the Opal multiplex technique, as a substantial higher number of differently-colored cells could be visualized within a well-conserved morphological context.

Introduction

Formaldehyde reacts with end groups found in biological molecules, resulting in various hydroxymethyl adducts attached at the nitrogen, oxygen, sulfur or carbon atoms in these end groups (1). This initial reaction is followed by a slowly-proceeding process of crosslinking of these formaldehyde-modified end groups, thereby forming methylene bridges (1). The formalin-fixation induced adducts and cross-links mask epitopes in tissue specimens and compromise antibody binding during subsequent immunohistochemical staining, leading to weak or false negative detection of certain proteins. A possible way to retrieve compromised antigenic sites in formalin-fixed paraffin-embedded (FFPE) specimens is treatment with heated antigen-retrieval buffer (e.g. citrate buffer pH 6 or Tris-EDTA pH 9.0) to remove adducts and break crosslinks. The technique to unmask epitopes with heated buffer

is discovered 25 years ago (2) and is generally referred to as heat-induced epitope retrieval (HIER). The mechanism of unmasking epitopes is not fully understood but careful selection of the best HIER conditions (temperature, buffer, pH and exposure time) is critical for optimal antigen retrieval (1-3).

Multiplex immunohistochemistry is a technique to simultaneously visualize multiple biomarkers in a single tissue section and is important, not only for clinical purposes (accurate diagnosis, evaluation of therapy choice and prognosis), but also for the extension of our general understanding of the immunobiology of tissues, in particular our insight in the phenotype, utilized signaling pathways, and cell cycle and activation state of cells in situ. Multicolor staining allows to detect co-expression of different molecules on individual cells, to simultaneously assess the distribution, abundancy and heterogeneity of expression of various cell types in healthy versus diseased tissue, and to recognize spatial relationships between various cells. Numerous different strategies for multiple immunostaining of FFPE tissue have been described, generally comprising iterative immunostainings on the same section, including digitization of the stained specimen and subsequent removal of immunoglobulins (plus non-permanent dyes in some protocols) after each staining round (4-7).

Despite the diversity in multiplexing staining methods, all protocols have in common that retrieval of epitopes in sections from FFPE tissue is an obligatory step after deparaffinization. In some protocols HIER is not only applied for the antigen retrieval but also used to strip primary and secondary antibodies after each staining round in order to make a section ready for the next staining cycle (8, 9). In this way a specimen is exposed several times to HIER which may be detrimental to the tissue, i.e. loss of epitopes and architecture and even (partial) detachment of tissue from the object glass (10, 11). In skin sections, for example, the dermis often detaches upon multiple HIER exposures, while the epidermis is often delaminated. Application of stripping buffers has been suggested to avoid the issue of tissue destruction by HIER (4, 5).

The Opal method is a powerful multiplex immunofluorescence technique enabling to distinguish up to 6 markers by covalently depositing distinct Opal fluorophores onto the detected antigens, using HIER for antibody removal after each staining cycle. Despite the great potency of this method, it is less suitable for vulnerable tissue specimens or delicate

antigenic determinants which are damaged by repetitive HIER treatments. This study was aimed to optimize the Opal workflow for HIER-sensitive FFPE human tissue sections, using β-mercaptoethanol-containing stripping buffer instead of HIER to strip immunoglobulins. This adjustment maintained the possibility to identify up to 6 biomarkers, while conserving the morphological context of the tissue and avoiding HIER-induced artifacts.

Methods

Patient material

Human abdominal skin was obtained as anonymized discarded tissue from corrective plastic surgery of the abdomen (n=4). Biopsies from the skin were fixed in formalin and embedded in paraffin according to standard procedures. Formalin-fixed paraffin embedded (FFPE) whole tissue sections from lung (n=1), tonsil (n=1), kidney (n=1), melanoma (n=6) and colon (n=1) were kindly provided by the department of Pathology at the Amsterdam University Medical Centers, Amsterdam, the Netherlands. In addition, an FFPE tissue microarray (TMA) including human tissues from the female reproductive system (n=7), digestive system (n=20), endocrine system (n=3), lymphoid tissues (n=6), kidney (n=3), lung (n=2), skin (n=2) and muscle (n=1) was also provided by the department of Pathology. The TMA consisted of triplicate 0.6 mm cores. The institutional Medical Ethics Review Committee granted a waiver for the anonymous use of human leftover FFPE material of diagnostic procedures. The study was carried out in agreement with the Dutch law (Medical Research Involving Human Subjects Act) and following the Declaration of Helsinki principles. According to the Dutch law, researchers are allowed to use anonymous human tissue without patient consent. Tissue sections of 4 µm thickness were used for immunohistochemical and immunofluorescence stainings.

Antibodies

Primary antibodies used for both immunohistochemical and immunofluorescence stainings included mouse anti-human Melan-A (clone A103, Dako/Agilent, Santa Clara, CA), mouse anti-human pan cytokeratin (clone C11, Abcam, Cambridge, UK), mouse anti-human CD8 (clone C8/144B,

Dako/Agilent), mouse anti-human CD45 (mix of clones 2B11 and PD7/26, Dako/Agilent), rabbit anti-human CD3 (polyclonal, Dako/Agilent), and mouse anti-human mast cell tryptase (clone AA1, Dako/Agilent).

Heat-induced epitope retrieval

FFPE tissue sections were deparaffinized in xylene and rehydrated by serial passage through graded concentrations of ethanol. Endogenous peroxidase in tissues was blocked with 0.3% H_2O_2/methanol for 10 min. Next, HIER was performed for 5 min at 95°C in Tris-EDTA pH 9.0 buffer using the Lab Vision™ PT Module™ (Thermo Fisher Scientific, Waltham, MA). In some experiments Citrate pH 6.0 buffer was used instead of Tris-EDTA. Multiple HIER treatments (up to 6 times) in Tris-EDTA pH 9.0 buffer was performed to mimic repetitive antibody stripping in order to study possible heating-induced damage. Thereafter, sections were stained with hematoxylin (Klinipath/VWR International, Amsterdam, The Netherlands) and eosin (Merck Millipore, Burlington, MA) and mounted for review. Images were acquired on a Leica DM microscope using Leica software (Leica Biosystems, Wetzlar, Germany).

Immunohistochemistry

After standard HIER in Tris-EDTA pH 9.0 buffer, sections were washed in cold running tap water, then 3x in Tris-buffered saline–0.05% Tween20 (TBST) and blocked with Superblock (Scytek Laboratories, Logan, UT) for 10 min, before incubation with a primary antibody for 60 min. Antibodies were diluted in Normal Antibody Diluent (Immunologic/VWR International, Radnor, PA). Next, tissue sections were washed and incubated for 30 min with poly-HRP anti-mouse or a poly-HRP anti-rabbit (Immunologic, Duiven, The Netherlands). Epitope–antibody binding was visualized using NovaRED as chromogen (Vector laboratories, Burlingame, CA). Sections were counterstained with hematoxylin (Klinipath/VWR International) and mounted in glycerol-gelatin (Sigma Aldrich, Saint Louis, MO). Images were acquired on a Leica DM microscope using Leica software (Leica Biosystems). In order to make sections ready for the next staining round, coverslips were removed after warming up the mounting medium in demi water at 50°C, followed by washing the section in cold running tap water and demi water. Finally, antibodies and dye were stripped with β-mercaptoethanol-

containing stripping buffer pH 7.5 (2% SDS/Tris-HCl, 0.7% β-mercaptoethanol) for 30 min at 50°C. Sections were washed in cold running tap water and TBST, before being incubated with the next primary antibody. Lastly, sections were stained with hematoxylin (Klinipath/VWR International) and mounted for review.

Multiplex immunofluorescence

Multiplex immunofluorescence staining was performed with the Opal 7-color fluorescence immunohistochemistry kit (Akoya Biosciences, Marlborough, MA), according to the manufacturer's protocol, except for the repetitive heat-mediated antibody-stripping treatments. In short, after deparaffinization, rehydration, and blocking endogenous peroxidase, HIER was performed for 5 min. at 95°C in Tris-EDTA pH 9.0 buffer in a Lab Vision™ PT Module™ (Thermo Fisher Scientific). Sections were washed in TBST and blocked with blocking/antibody diluent for 10 min., before being incubated with primary antibody for 60 min. Then, sections were incubated with polymer HRP Ms + Rb for 10 min. followed by incubation with an Opal fluorophore (Opal480, Opal520, Opal570, Opal620, Opal690 or Opal780) for 10 min. Bound primary and secondary antibodies were then eluted with HIER treatment (as aforementioned) or with β-mercaptoethanol-containing stripping buffer (defined as above) for 30 min. in a water bath at 50°C. After washing in cold running tap water, demi water and TBST, the process of staining and antibody removal was repeated using a different Opal fluorophore. Finally, after staining with the sixth Opal fluorophore, tissue specimens were stained with DAPI for 5 min and mounted in ProLong Diamond Antifade Mountant (ThermoFisher Scientific).

Imaging

Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences) was used for multispectral imaging at 20x magnification. Thereafter, whole slide images were loaded into InForm image analysis software (Akoya Bioscience).

Results

Repetitive heating is deleterious to the morphology of tissues with low cell density

In some multiplex immunohistochemistry protocols for detection of multiple markers within a single tissue section, such as the Opal method, HIER is used to remove antibodies after each staining step. Repetitive high-temperature heating of tissue sections may lead to tissue damage. We investigated in tissues of different origin the effect of multiple HIER treatments on morphology, using Tris-EDTA pH 9.0 or Citrate pH 6.0 as retrieval buffer. We found that all FFPE whole tissue sections survived the obligatory initial HIER treatment, which is needed to enable binding of primary antibodies (**Figure 1, Supplementary Figure 1A-C**). However, additional HIER treatments led to considerable tissue damage (such as partial detachment and delamination) in sections from skin, lung and kidney, whereas cell-dense tissues, such as tonsil, melanoma and colon, remained fully intact, even after 6 treatments (**Figure 1, Supplementary Figure 1A-C**). As compared to the whole tissue sections, the small-sized (only 0.6 mm in diameter) circular tissue sections in the TMA appeared to be even more vulnerable for repetitive HIER exposures, regardless of the cell density. As expected, heating-related damage was found in all human skin (2/2), lung (2/2) and kidney (3/3) sections in the TMA, but in addition, significant tissue damage was observed in 51% of the cell-dense tissues (19/37) as well (**Supplementary Figure 1D**). In most cases, parts of the tissue section had detached from the glass or, if not completely detached, the tissue was folded.

As stripping buffer can be used as alternative way to remove antibodies, we questioned what effect this treatment would have on the tissue morphology. To investigate this, tissue sections were subjected to the obligatory single HIER treatment first, followed by repetitive exposures to β-mercaptoethanol-containing stripping buffer. We found that all types of tissue maintained their morphology with stripping buffer, even after 5 rounds of treatment (**Figure 1, Supplementary Figure 1**). In the small specimens in the TMA, we observed that the tissue morphology was maintained in the majority (88%) of tissues after multiple rounds of β-mercaptoethanol-containing stripping buffer, as compared to the tissue in the multiple-

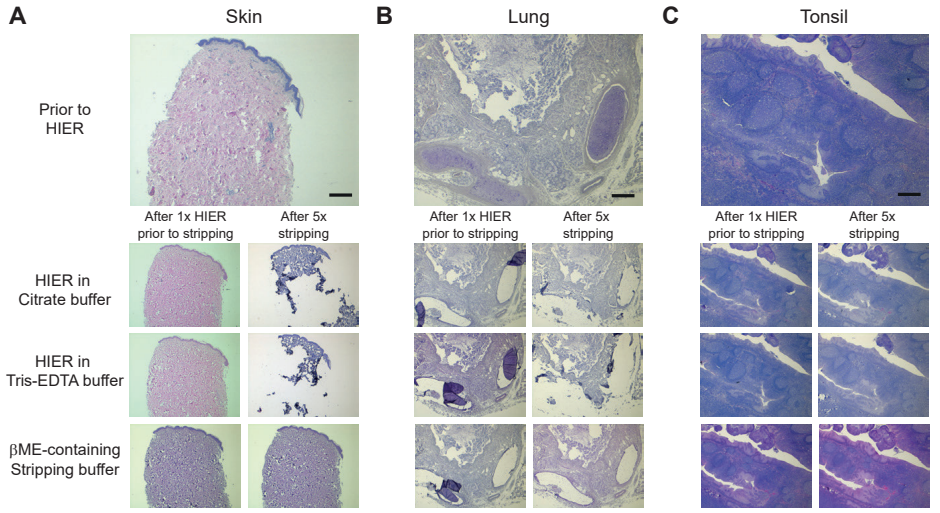

Figure 1. Cell-poor tissue is sensitive to repetitive high-temperature heating, while morphology is well conserved after multiple treatments with stripping buffer. On top is shown, hematoxylin and eosin staining of human skin (A), lung (B), and tonsil (C) tissue prior to heat-induced epitope retrieval (HIER). At the bottom is shown, treatment of skin (A), lung (B), and tonsil (C) tissue after 1 treatment (left) and 5 subsequent treatments (right) with HIER in citrate buffer, HIER in Tris-EDTA buffer, or β-mercaptoethanol-containing stripping buffer. Bars equal 1 mm.

heating treated TMA sample (**Supplementary Figure 1D**), hence underlining that exposure to stripping buffer is a relatively mild treatment, even for vulnerable tissues such as present in TMA.

These data indicate that application of stripping buffer is preferred over HIER for repetitive antibody removal in multiplex immunohistochemistry, in particular for cell-poor tissues such as skin, lung and kidney, in order to retain tissue spatial and morphological context.

Sensitivity of epitopes to the stripping buffer

We showed that stripping buffer does not affect tissue morphology and could be a better way to remove antibodies, compared to HIER. As complete removal of primary and secondary antibodies in between the staining steps is essential in multiplex immunostaining to avoid cross reactivity, this prompted us to demonstrate the efficacy of β-mercaptoethanol-containing stripping buffer to successfully removing all immunoglobulins. To this end we

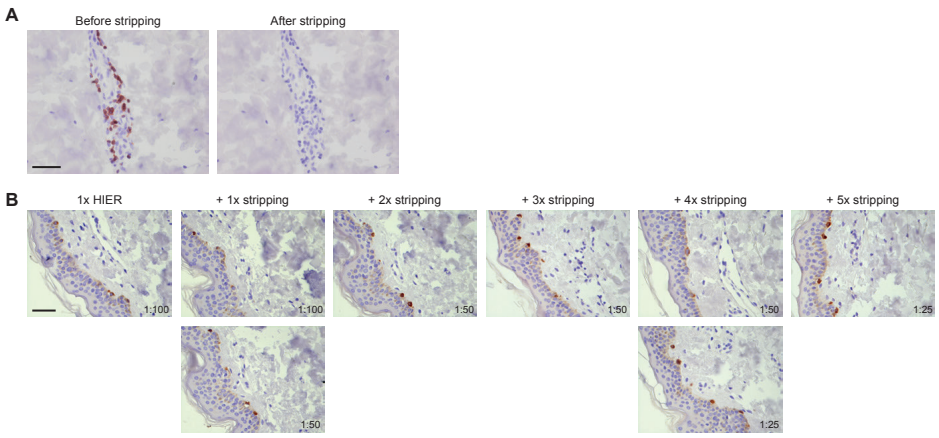

Figure 2. Sensitivity of epitopes for repetitive treatment with stripping buffer. (A) CD3-stained human skin tissue (left) was subjected to 1 round of treatment with β-mercaptoethanol-containing stripping buffer followed by incubation with poly-HRP anti-rabbit and NovaRED visualization (right). Lack of staining after treatment with staining buffer, indicated successful removal of primary and secondary antibodies. (B) Melan-A staining on human skin tissue after 1 round of HIER treatment with Tris-EDTA buffer, followed by indicated rounds of treatment with stripping buffer. Antibody dilutions are indicated. Bars equal 100 µm.

exposed a CD3-stained human skin section (**Figure 2A**, left) to the stripping buffer, and subsequently, the same section was incubated with poly-HRP secondary antibody and NovaRED for visualization (**Figure 2A**, right). Lack of staining indicated that originally bound primary and secondary antibodies (and also the non-permanent NovaRED chromogen) had been successfully erased by the stripping buffer (**Figure 2A**). This control to check whether complete antibody removal occurred was included for all antibodies in all our experiments.

However, we cannot exclude that multiple rounds of stripping buffer may be detrimental to any target epitope and hamper epitope detection in later staining cycles. To test this, we performed immunohistochemical staining on adjacent sections that have been subjected to the obligatory single HIER treatment followed by a differential number of treatments with stripping buffer. We observed for all antibodies in our panel that exposure to repetitive stripping cycles resulted in loss in staining intensity at a different rate and already starting after the first stripping cycle (**Figure 2B**). In all cases the staining intensity could be restored by using a higher concentration of

antibody (**Figure 2B**). The required increase of antibody concentration to regain optimal staining did vary among antibodies, indicating that epitopes have differential sensitivity to multiple rounds of stripping buffer. In general, after the 5th stripping round, the antibodies were applied 4-6 times more concentrated in order to maintain the staining quality. Based on the sensitivity of the different epitopes for the stripping buffer, we determined the optimal sequence of epitope detection in multiplex staining (**Supplementary Table 1**).

Multiplex immunofluorescence staining

We demonstrated that antibody removal by β-mercaptoethanol-containing stripping buffer is superior over HIER treatment in case of tissues with low cell density, and in addition, we assessed the sensitivity of our target epitopes to this stripping buffer, providing a vital clue about the optimal order of epitope detection by our panel of primary antibodies. Next, we implemented the application of stripping buffer in the Opal method and aimed to study if our adjustment would lead to improved multiplex immunostaining of cell-poor tissue, using healthy human skin as typical representative. Opal fluorophores were paired to antibodies based on brightness of the fluorophores, antigen abundance, co-localization of markers and skin-tissue autofluorescence. Preliminary tests showed that covalently deposited Opal fluorophores were not removed by the stripping buffer (data not shown). Direct comparison of the standard Opal protocol (including repetitive HIER) with our adjusted protocol (stripping buffer instead of HIER), revealed that application of stripping buffer markedly improved the quality of the Opal immunofluorescence staining technique, as a substantial higher number of differently-colored cells could be visualized (**Figure 3A** versus **3B**), while maintaining the spatial and morphological context. Concomitantly, cells detached during repetitive HIER treatment and subsequently randomly stuck to the slide, while this did not occur with our adjusted protocol. As a result of the damage caused by HIER treatment, cells couldn't be imaged in focus. Fluorophore intensity was unaffected by the stripping buffer. Signal intensity and exposure times were comparable between both protocols, when stained with similar antibody concentrations. Collectively, our data suggest that application of stripping buffer instead of HIER for antibody removal in the Opal protocol result in improved quality of this septuple immunofluorescence staining technique.

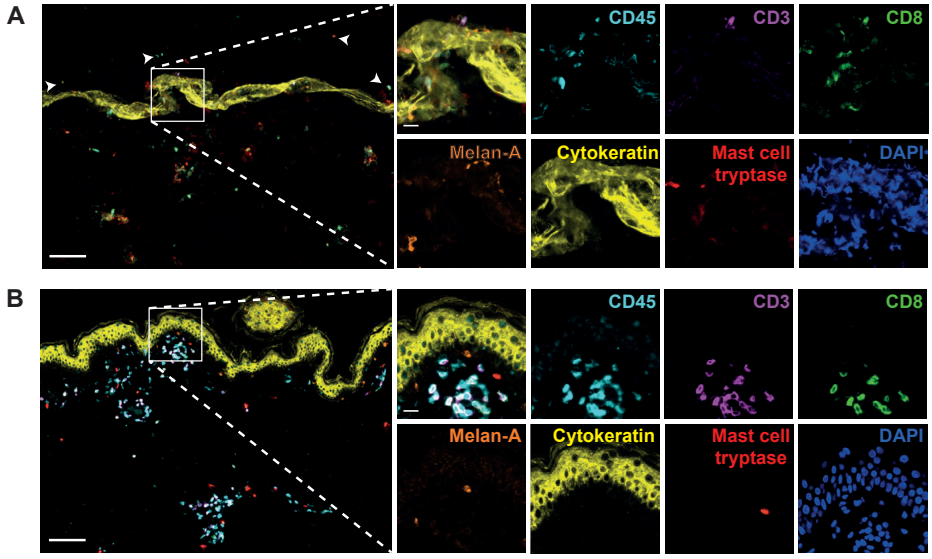

Figure 3. Application of stripping buffer instead of HIER results in improved quality of the septuple immunofluorescence staining technique. Opal multiplex immunofluorescence staining of human skin using heat-induced epitope retrieval (HIER) with Tris-EDTA buffer (A) or β-mercaptoethanol-containing stripping buffer (B) for antibody removal. Representative immunofluorescence images showing CD45 (in cyan), CD3 (in purple), CD8 (in green), Melan-A (in orange), cytokeratin (in yellow), mast cell tryptase (in red) and DAPI (in blue). Arrow heads indicate cells that detached during HIER treatment and subsequently randomly stuck to the slide. Bars equal 100 µm.

Discussion

Techniques to perform multiplex immunofluorescence staining in a single tissue section take advantage of the possibility to remove bound primary and secondary antibodies after each completed staining round leaving the color on the target epitope untouched, hence enabling the performance of multiple cycles of staining and immunoglobulin stripping. In this study we investigated the suitability of repetitive HIER exposures to perform antibody stripping –as used in the standard Opal multiplex immunofluorescence method– and found that this treatment is disastrous for sections with low cell density, such as sections from skin, lung and kidney, resulting in detachment of a large part of the tissue. In contrast, cell dense specimens

could resist iterative high-temperature heating. We have no explanation for this differential effect of HIER on cell-poor versus cell-rich tissues.

Release of primary and secondary antibodies from immunohistological stained sections can be achieved by stripping buffers (4, 5). To solve the problem of HIER-induced damage during antibody stripping in sections with low cell density, we tested the repetitive use of β-mercaptoethanol-containing stripping buffer and found that this treatment maintained the tissue morphology quite well. However, multiple exposures of tissue sections to stripping buffer is not without harm, as we found that the staining intensity decreased after each treatment with this buffer, suggesting that the stripping buffer had a detrimental effect on the epitopes. This loss of staining intensity could be overcome by using higher concentrations of antibody. The observation that stripping-buffer exposure can cause reduction in staining intensity in subsequent staining is in line with our earlier study (7). Cattoretti and colleagues refer this loss of detection to as re-masking of the epitopes, and suggested that this problem can be solved by adding disaccharides throughout the process, and avoiding glycerol/gelatin mounting media, which negatively affect reproducibility of epitope detection (12-14).

Nowadays, many laboratories prefer the use of autostainers over manual staining. Currently, these are mostly applied for single immunohistochemical stainings using 3,3'-Diaminobenzidine (DAB) chromogen. As it is possible to equip these (semi-)automated instruments with devices needed for epitope retrieval steps, e.g. HIER, it should not be a problem to customize autostainers for the use of β-mercaptoethanol-containing stripping buffer as antibody-removal application, which is simply a matter of adjusting time, temperature and type of buffer, provided good extraction due to the toxicity of β-mercaptoethanol. Concomitantly, predesigned Opal multiplex panels are optimized already for use on autostainers. Therefore, we believe our current multiplex immunofluorescence protocol can be implemented in an automated platform. Nevertheless, it would still be necessary to perform appropriate assay development steps manually, e.g. antibody optimization, antibody-Opal fluorophore pairings etc., which is also needed for the conventional Opal workflow.

In conclusion, we propose to use β-mercaptoethanol-containing stripping buffer, instead of HIER, to erase antibodies in the Opal protocol for simultaneous detection of multiple biomarkers. This adaptation avoids

heat-induced tissue damage, in particular in vulnerable tissue sections with low cell density, thereby enabling the identification of multiple biomarkers, while retaining spatial and morphological context.

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Competing Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contributions

MW and GK performed experiments, microscopy, and data analysis, and drafted the manuscript; MWB and RML participated in manuscript revision; MBMT critically revised the work and edited the manuscript. All authors have read and approved the final manuscript.

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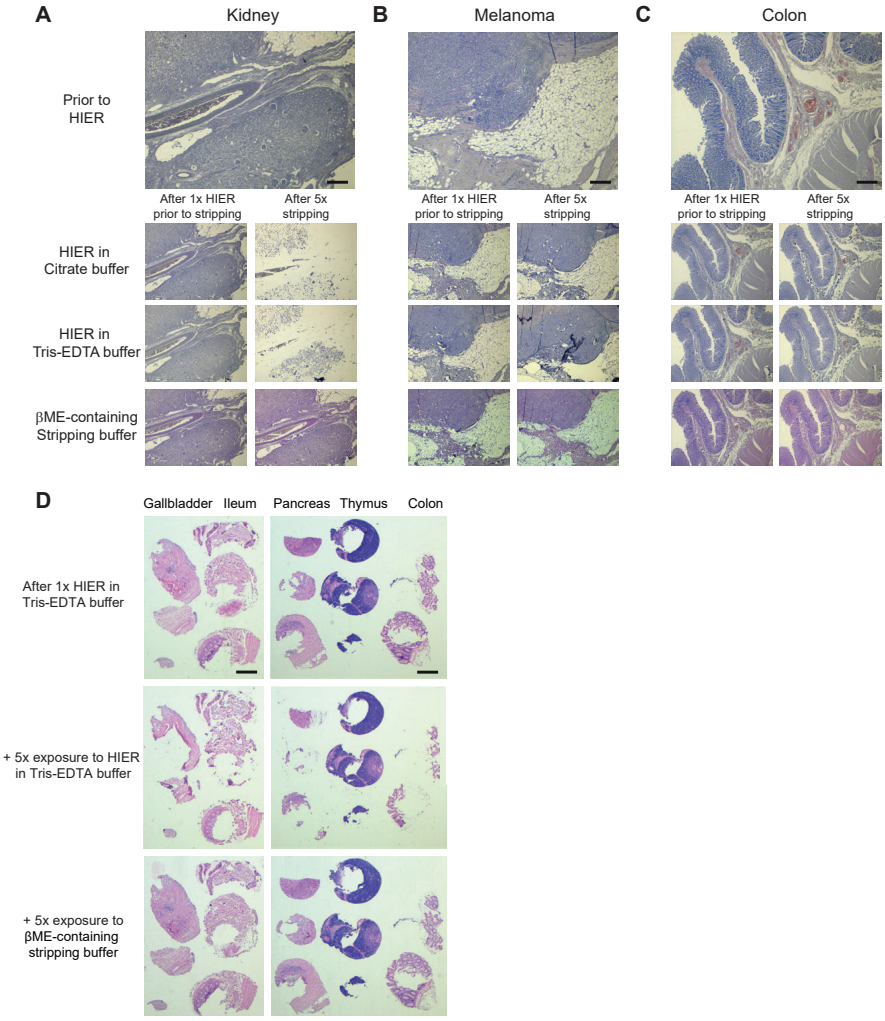
Supplemental material



Supplementary figure 1. Repetitive high-temperature heating leads to tissue damage, while tissues maintain their morphology after multiple rounds of treatment with stripping buffer. (A-C) On top is shown, hematoxylin and eosin staining of human kidney (A), melanoma (B), and colon (C) tissue prior to heat-induced epitope retrieval (HIER). At the bottom is shown, treatment of kidney (A), melanoma (B), and colon (C) tissue after 1 treatment (left) and 5 subsequent treatments (right) with either HIER in citrate buffer, HIER in Tris-EDTA buffer, or β-mercaptoethanol-containing stripping buffer. (D) Hematoxylin and eosin staining of gallbladder, ileum, pancreas, thymus and intestine tissue after 1 treatment (top), followed by 5 subsequent treatments with HIER in Tris-EDTA buffer (middle) or β-mercaptoethanol-containing stripping buffer (bottom). Bars equal 1 mm.

Supplementary table 1. Sequence of epitope detection in multiplex immunofluorescence staining.

Cell types to be detected	Target (Antibody clone)	Secondary antibody enzyme-conjugate	Opal fluorochrome
Melanocytes	Melan-A (A103)	Poly-HRP Ms + Rb	Opal 620
Keratinocytes	pan Cytokeratin (C11)	Poly-HRP Ms + Rb	Opal 570
T cell subset	CD8 (C8/144B)	Poly-HRP Ms + Rb	Opal 520
Mast cells	Mast cell tryptase (AA1)	Poly-HRP Ms + Rb	Opal 690
Leukocytes	CD45 (2B11+PD7/26)	Poly-HRP Ms + Rb	Opal 480
T cells	CD3 (polyclonal)	Poly-HRP Ms + Rb	Opal 780
Nuclear cells	DAPI		

Abbreviations: Ms, mouse; Rb, rabbit; HRP, horseradish peroxidase.

Chapter 9

Analyzing resistance of AXL- and/or MITF-expressing melanoma cells to immunotherapy

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Unpublished

Abstract

Tumor heterogeneity is commonly seen in melanoma patients and a hurdle to effective therapy as illustrated by the "mixed responses" frequently seen in immunotherapy-treated patients. Previously, AXL^+ cells were identified to be highly resistant to targeted therapy, whereas more differentiated $MITF^+$ cells responded well to RAF and MEK inhibitors. This study aimed to identify if AXL^+ melanoma cells show intrinsic resistance to immunotherapy, as seen for targeted treatment, and study if these cells are more resistant than $MITF^+$ melanoma subpopulations. Concomitantly, we aimed to validate melanoma subpopulations at protein level and correlate melanoma heterogeneity to immunological pressure. We analyzed the presence of melanoma cell populations in metastatic tissues, by single-cell RNA sequencing and *in situ* multiplex immunofluorescence, and the phenotypic changes after immunomodulating antibodies or autologous tumor lysate-loaded dendritic cell vaccination. Our data demonstrate large inter patient variability and variable therapy-induced changes that were independent of the type of therapy. Treatment with anti-CTLA-4 and dendritic cell vaccines did not exclusively select for AXL^- cells, as seen for targeted treatment. In addition, immunotherapy-induced changes in the abundance of AXL^+ $MITF^-$ cells did not correlate with improved survival. Nonetheless, $MITF^+$ tumor cells, showed a weak inverse correlation with $CD8^+$ T cells, suggesting these are more immunogenic and more easily recognized by effector T cells upon therapy. Our study highlights that AXL^+ cells seem not as resistant to immunotherapy as to targeted treatment. However, immunoreactivity is increased towards MITF-expressing tumor cells, that are therefore likely to respond better to immunotherapy.

Introduction

Melanoma is one of the most aggressive cancers with a high risk of invading lymphoid organs and spreading systemically. In the advanced stage, melanoma is difficult to treat and often develops resistance to most available forms of therapy. Although melanoma patients generally respond well to immunotherapy, there is still a fraction of patients that does not benefit from this[1]. Moreover, during immunotherapy, many metastatic melanoma patients experience "mixed response", with some tumor lesions regressing

and other ones progressing[2]. This resistance to therapy is, at least in part, due to tumor heterogeneity.

Heterogeneity involves the presence of cells with different phenotypic and molecular features within a tumor (intralesional) or between tumors (interlesional) in a patient. As a result, metastatic lesions arise from different subpopulations within tumors. Resistance to immunotherapy might result from selection for antigen-negative tumor cells[3-6] or tumor cells with stemness properties that have a phenotype different from their differentiated counterpart[7,8]. Earlier studies demonstrated that all tumors harbor melanoma cells from two distinct transcriptional cell states based on the expression of AXL receptor tyrosine kinase and MITF transcription factor[9]. Melanoma cells classified as AXL^{high} were associated with resistance to targeted therapy by RAF and MEK inhibitors and became enriched upon treatment[9-11]. Resistance to targeted therapy or cancer vaccines comprising few antigens can result from selection of tumor cells lacking the target or upregulation of rescuing molecules (e.g. multidrug resistance proteins), whereas immunomodulating antibodies (e.g. anti-CTLA-4, anti-PD-1) and whole tumor vaccines might be less affected by this type of resistance. However, immunotherapy itself can cause tumor heterogeneity, as it has been shown that tumor-specific cytotoxic T cells induce dedifferentiation of melanoma cells, which thereby acquire “stem cell-like” properties[12]. Tumor cells with stemness features are less represented in most melanoma patients and are less immunogenic, being hardly targeted by specific immunity[13]. This illustrates that due to tumor heterogeneity melanoma cells can evade immune destruction and indicates the need to understand melanoma heterogeneity in relation to immune evasion.

This research project therefore aimed to reveal if AXL-expressing melanoma cells are intrinsically resistant to current immunotherapies, as is the case for targeted treatment, and if these cells are more resistant than $MITF^{+}$ melanoma subpopulations. In addition, we aimed to validate these melanoma subsets at the protein level and study if melanoma heterogeneity could be influenced by immunological pressure from tumor infiltrating lymphocytes. To this end, we identified phenotypically diverse tumor cell subpopulations based on differential expression of AXL and MITF in metastatic melanoma tissues by single-cell RNA sequencing and multiplex immunofluorescence *in situ* imaging, and evaluated the phenotypic

changes upon immunotherapy by either autologous tumor lysate loaded dendritic cell vaccination or ipilimumab.

Methods

Patient material

Fragments of resected melanoma metastases (n=5) and formalin-fixed paraffin embedded (FFPE) metastatic tissue sections (n=18) pre and post immunotherapy (autologous tumor lysate-loaded dendritic cell (DC) vaccination or ipilimumab) from metastatic melanoma patients were collected by the department of Immunotherapy, Cell Therapy and Biobank Unit at the IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori" (Meldola, Italy). Resected tumor material was processed for single-cell RNA sequencing purposes. Multiple 4 µm FFPE sections were cut for hematoxylin and eosin (H&E) and multiplex immunohistochemical staining. The study was approved by the CEROM Ethics Committee (approval n° 2639/2019 I.5/63 of 13/03/2019) and was conducted in accordance with the principles laid down in the 1964 Declaration of Helsinki. Written informed consent was obtained from all participants. Detailed patient characteristics are listed in **Table 1**.

Tissue handling

Resected tumor material was mechanically dissociated using scalpels in RPMI (Lonza Group Ltd, Basel, Switzerland) on ice. Tumor pieces were disaggregated and pipetted up and down using pipettes of descending sizes (25 ml, 10 ml and 5 ml) until a single-cell suspension was obtained. The tumor cell suspension was then filtered using 100 µm nylon strainer (Corning, Glendale, Arizona) and residual cell clumps were discarded. The suspension was immediately placed on ice and centrifuged at 470 g at 4°C for 5 minutes. Subsequently, the supernatant was discarded and the cell pellet was resuspended in PBS with 2% FCS. A small aliquot was then placed on ice for the FACS analysis and the remaining cell suspension was immediately vitally frozen and stored in liquid nitrogen until use.

Cell sorting

Single-cell suspensions were stained with APC-H7-conjugated mouse anti-human CD45 (clone 2D1, BD Biosciences, Franklin Lakes, NJ) and Calcein AM (Life Technologies, Carlsbad, CA). Firstly, doublets were excluded from the analyses. Then, viable, non-immune cells (Calcein^{high} CD45^{-}) cells were sorted into two Precise WTA Single Cell Encoding 96-well plated (BD Biosciences) pre-chilled to 4°C. Next, the plates were sealed, vortex, centrifuged at 1000 g at 4°C for 1 minute, immediately placed on dry ice and transferred for storage at -80°C per manufacturer's recommendations.

Whole transcriptome amplification and library preparations

Whole transcriptome amplification (WTA) and library preparations were performed using the Precise WTA single cell kit (BD Biosciences), following the manufacturer's instructions. The 96 samples of a multiwall plate were pooled together and cleaned with two 0.8x DNA SPRIs (Beckman Coulter, Brea, CA). Library quality was assessed with a high sensitivity DNA chip (Agilent, Santa Clara, CA) and quantified with a high sensitivity dsDNA Qubit Kit (Life Technologies, Carlsbad, CA). Samples were sequenced on an Illumina Hiseq4000 instrument using 2x100 bp paired-end reads.

Single-cell RNA sequencing and bioinformatics analysis

Sequencing data were uploaded into the Seven Bridges-hosted pipeline, generating molecular index corrected counts for each gene per cell. Seurat R package were used for further analysis[14]. Genes were retained if MI_detection field is equal to "pass" and unique molecular identifiers are at least 5 in at least 5 cells. On the other hand, cells were filtered out according to the following conditions: raw read count is greater than 200,000; there are more than 1,000 expressed genes; the percentage of mitochondrial genes is less than 30%.

Based on the expression of both AXL and MITF signatures[15], tumor cells were categorized in 4 phenotypic groups: AXL^{high} and MITF^{high}; AXL^{high} and MITF^{low}; AXL^{low} and MITF^{high}; AXL^{low} and MITF^{low}. Then, we performed differential gene expression analysis between cells belonging to each one of these phenotypic groups against cells of the other phenotypic groups, and extracted the top 10 most and the top 10 least expressed genes from each

Table 1. Patient characteristics and samples included in this study

Sample ID	Age/ sex	Mutation status	Previous treatment	Treatment	Site of resection		Following treatment	BOR (RECIST)/ Duration (months)	Overall survival	Objective response	Analysis
					Pre-treatment	Post-treatment					
ITEM_v1	60/M	-	None	DC vax	Adrenal gland	Adrenal gland	None	SD/6	46,5	Progressing	mIF
ITEM_v2	45/M	V600E	None	DC vax	Lymph node	Lymph node	CT, high dose IL-2	CR/8	0	Progressing	mIF
ITEM_v3	36/F	V600E	BioCT	DC vax	Omentum	Stomach	Surgery, RT	SD/50	108+	Stable	mIF
ITEM_v4	64/M	V600E	BioCT	DC vax	Lymph node	Subcutis	Surgery	PR/68	87	Stable	mIF
ITEM_v5	68/M	V600E	BioCT	DC vax	Lung	Skin	Low-dose IL-2	SD/9	62	Progressing	mIF
ITEM_v5ipi	69/M	V600E	Low-dose IL-2	Ipilimumab	Skin	Subcutis	None	PD	37	Progressing	mIF
ITEM_v6	35/F	WT	CT, ipilimumab	DC vax	Peritoneum	Jejunum	RT	SD/4	16	Progressing	mIF
ITEM_v7	31/M	V600E	None	DC vax	Skin	Skin	Ipilimumab, vemurafenib, BioCT, pembrolizumab	PD	19	Progressing	mIF
ITEM_v8	79/M	WT	None	Ipilimumab	Skin	Subcutis	CT	PD	10,5	Progressing	mIF
ITEM_v9	44/M	-	BioCT	Ipilimumab	Skin	Subcutis	CT	PD	155+	Progressing	mIF
ITEM_v10	45/M	-	None	Ipilimumab	Lymph node	Lymph node	DC vax	PR/17	25,5	Progressing	mIF
ITEM_v12	58/F	V600E	CT	Ipilimumab	Skin	Lymph node	None	0	1	Progressing	mIF
ITEM_v13	73/F	WT	CT	Ipilimumab	Subcutis	Lung	None	0	3,6	Progressing	mIF
ITEM_v14	65/M	V600E	None	Ipilimumab	Skin	Skin	None	NED/152+	169+	Stable	mIF

P0153	-	-	-	-	-	-	-	-	-	-	scRNAseq
P0196	53/M	WT	None	Ipilimumab	N/A	Subcutis	DC vax, nivolumab, temozolomide	-/22	63	Progressing	mIF + scRNAseq
P0256	66/F	WT	None	Adjuvant nivolumab	Subcutis	N/A	None	NED/6	17	-	mIF + scRNAseq
P0258	41/M	WT	Ipilimumab	Pembrolizumab	Subcutis	N/A	High dose IL-2	PD	3	-	mIF + scRNAseq
P0262	45/F	V600E	None	Adjuvant nivolumab	Lymph node	N/A	None	NED/13	13	-	mIF + scRNAseq

Note: Patients' age is expressed in years, overall survival is expressed in months. WT, wild type; DC vax, dendritic cell vaccination; BioCT, cytokine (IL-2 and/or IFN-α) + chemotherapy; CT, chemotherapy, RT, radiotherapy; BOR, best overall response; SD, stable disease; CR, complete response; PR, partial response; PD, progressive disease; NED, no evidence of disease;
mIF, multiplex immunofluorescence; scRNAseq, single cell RNA sequencing; -, missing data.

comparison. Finally, stacked bar charts were generated for each sample showing the proportions of cells per phenotypic group.

Multiplex immunofluorescence

Multiplex immunofluorescence staining was performed according to manufacturers' instructions (except for antibody removal) using the OPAL 7-color immunohistochemistry kit (Akoya Biosciences, Marlborough, MA). Antibodies used included mouse anti-Melan-A (clone A103, Dako/Agilent, Santa Clara, CA), mouse anti-melanosome (clone HMB-45, Dako/Agilent), mouse anti-tyrosinase (clone T311, Dako/Agilent), mouse anti-MITF (clone D5, Dako/Agilent), mouse anti-CD8 (clone C8/144B, Dako/Agilent), mouse anti-CD45 (clone 2B11+PD7/26, Dako/Agilent), rabbit anti-PRAME (clone EPR20330, Abcam, Cambridge, UK) and rabbit anti-AXL (clone EPR19880, Abcam). Antibody removal was done by β-mercaptoethanol-containing stripping buffer pH 7.5 (2% SDS/Tris-HCl, 0.7% β-mercaptoethanol) for 30 minutes at 50°C. Slides were mounted with ProLong Diamond Antifade Mountant (ThermoFisher Scientific, Waltham, MA).

Imaging

Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences) was used for multispectral imaging at 20X. Thereafter, whole slide images were loaded into Phenochart Whole Slide Viewer and InForm image analysis software (both Akoya Biosciences) for unmixing. Component images of 10 high-power fields (5 from the border and 5 from the center of the tumor) were analyzed for phenotyping melanoma subsets and T cell infiltration in QuPath software (open source software).[16]

Results

Single-cell RNA sequencing identifies distinct transcriptional signatures

Previous studies have shown that AXL$^+$ tumors, in contrast to MITF$^+$ tumors, are more resistant to MAPK pathway inhibitors[9-11]. To date, it remains to be elucidated which melanoma cell phenotypes are intrinsically more

resistant to immunotherapy, indicating the rationale to study phenotypic heterogeneity in immunotherapy-treated melanoma patients. To study transcriptional diversity and changes herein upon immunotherapy, we measured transcriptomes from individual melanoma cells isolated pre and post immunotherapy treatment (**Figure 1A**).

Despite limited numbers of cells sequenced (**Table 2**), our single-cell RNA sequencing (scRNAseq) data confirmed the presence of four distinct melanoma transcriptional signatures identified by the expression of AXL and MITF[9] (**Figure 1B**). *In silico* analysis on the existing scRNAseq dataset used by Tirosh *et al*. (2016)[9] revealed similar and additional differentially expressed genes between the 4 subpopulations (**Figure 1C**). Among the top 10 most differentially expressed genes as identified from our scRNAseq analysis, 61 % was found to overlap with those identified by the existing database (**Figure 1C**). Some of the most discriminative markers that were identified by Tirosh *et al*. (2016), among which PRAME, TYR, PMEL and MLANA, were filtered out from our scRNAseq analysis because of reduced quality. Nonetheless, those identified in our dataset were largely confirmed during the analysis of the Tirosh *et al*. (2016) data (**Figure 1C**).

The AXL^+ $MITF^-$ cluster was associated with expression of markers of epithelial to mesenchymal transition, invasion and metastasis (**Figure 1B**). The AXL^- $MITF^+$ subpopulation was enriched for markers of melanocyte differentiation and pigmentation such as PMEL, TYR and GPR143 (**Figure 1B**). Cells expressing both AXL and MITF exhibited high levels of markers involved in e.g. antigen presentation, including CD74 and HLA-B (**Figure 1B**). Finally, cells lacking expression of AXL and MITF were characterized by expression of various ribosomal proteins (**Figure 1B**), which might reflect Myc activation[17,18].

Melanoma composition shows marked inter patient heterogeneity that proceeds during therapy

To obtain information about the abundance of the various melanoma phenotypes and to validate aforementioned subsets at the protein level, we performed multiplex immunofluorescence staining for the most discriminative markers of the AXL^+ $MITF^-$, AXL^+ $MITF^+$, AXL^- $MITF^+$, and AXL^- $MITF^-$ subpopulations. Besides AXL and MITF, PRAME, MLANA, TYR and PMEL were included as these were identified as differentially expressed from *in silico* analysis of the Tirosh *et al*. (2016) dataset (**Figure 1C**). Including

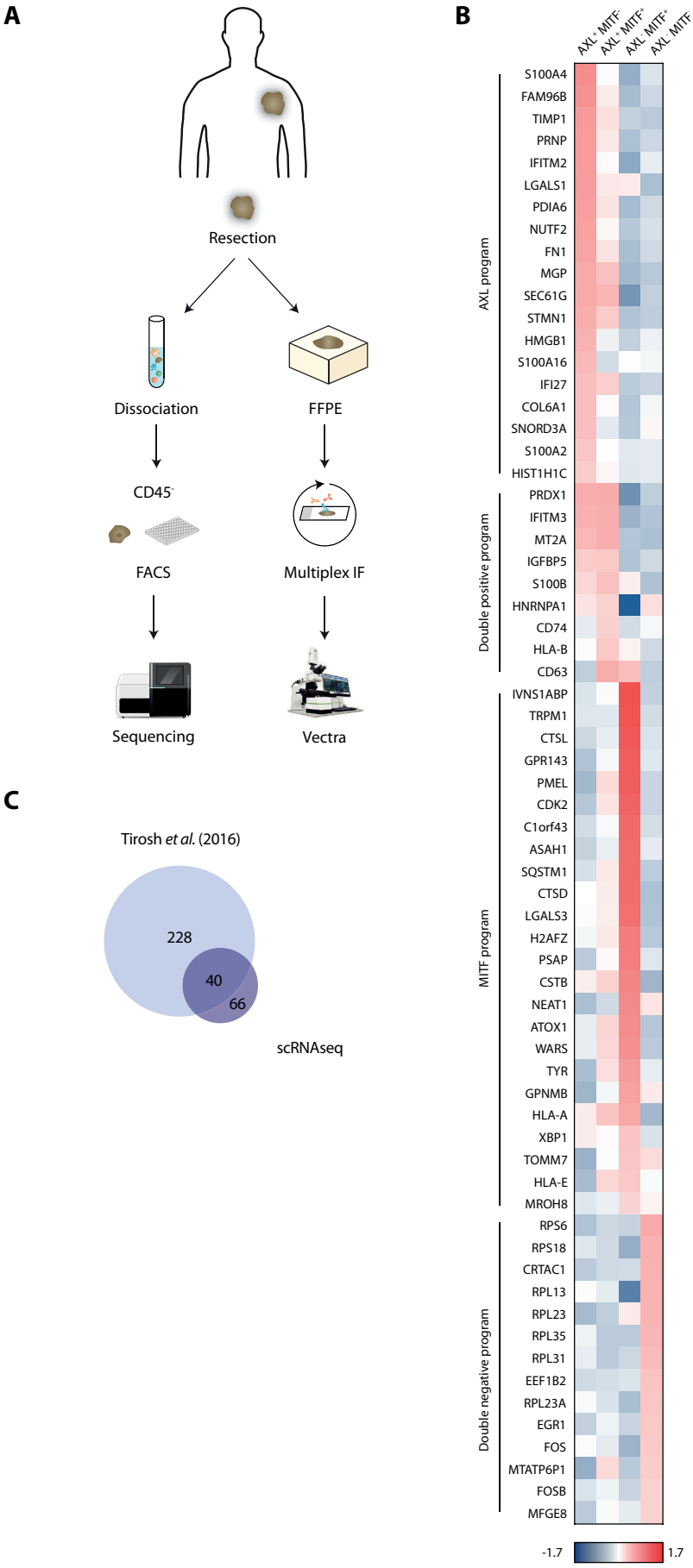

A
Resection
Dissociation
FFPE
CD45-
FACS
Multiplex IF
Sequencing
Vectra
B
AXL+ MITF-
AXL+ MITF+
AXL- MITF+
AXL- MITF-
S100A4
FAM96B
TIMP1
PRNP
IFITM2
LGALS1
PDIA6
NUTF2
FN1
MGP
SEC61G
STMN1
HMGB1
S100A16
IFI27
COL6A1
SNORD3A
S100A2
HIST1H1C
AXL program
PRDX1
IFITM3
MT2A
IGFBP5
S100B
HNRNPA1
CD74
HLA-B
CD63
Double positive program
IVNS1ABP
TRPM1
CTSL
GPR143
PMEL
CDK2
C1orf43
ASAH1
SQSTM1
CTSD
LGALS3
H2AFZ
PSAP
CSTB
NEAT1
ATOX1
WARS
TYR
GPNMB
HLA-A
XBP1
TOMM7
HLA-E
MROH8
MITF program
RPS6
RPS18
CRTAC1
RPL13
RPL23
RPL35
RPL31
EEF1B2
RPL23A
EGR1
FOS
MTATP6P1
FOSB
MFGE8
Double negative program
-1.7
1.7
C
Tirosh et al. (2016)
228
40
66
scRNAseq

Figure 1. Single-cell RNA-sequencing identified multiple transcriptional states. (A) Overview of workflow. FFPE, formalin fixed paraffin embedded; IF, immunofluorescence; FACS, fluorescence-activated cell sorting. (B) Heatmap showing the top 10 most differential expressed genes (population averages) for AXL^+ $MITF^-$, AXL^+ $MITF^+$, AXL^- $MITF^+$ and AXL^- $MITF^-$ melanoma subpopulations. (C) Venn diagram showing the overlap between the top most differential expressed genes for every subpopulation from the Tirosh *et al*. (2016) dataset and the top 10 most differential expressed genes from our scRNAseq analysis.

melanocyte differentiation antigens would help distinguish the more differentiated cells from dedifferentiated cells. On the other hand, including PRAME would give information on the potential to metastasize. These markers could further subdivide the melanoma subpopulations. Yet for this study we merely focused on aforementioned phenotypes. Concomitantly, CD45 and CD8 were included to decipher immune infiltration as an indication of the level of immunological "pressure" within the tumor tissue. It was feasible to detect the selected panel of markers by multiplex immunofluorescence (**Figure 2A**). In addition, the frequencies of different groups as defined by scRNAseq were compared to those defined by multiplex immunofluorescence. However, the low cell numbers in the scRNAseq analysis precluded quantitative comparison of the proportions of the phenotypic subpopulations.

To assess the clinical relevance of these subtypes in resistance to immunotherapy, we investigated paired biopsy specimens of patients undergoing DC vaccination or ipilimumab treatment by multiplex immunofluorescence (**Figure 2B**). Representative images pre- and on treatment biopsies from 4 patients are shown. These images were selected to illustrate the co-occurrence of the four distinct phenotypic groups. To explore if AXL^+ or less differentiated subpopulations also respond less well to immunotherapy, we quantified the abundance of every subpopulation pre- and post-therapy for every individual patient (**Figure 2C**). This data indicates large interpatient variability among melanoma patients. The presence of melanoma subpopulations prior to therapy also varied widely between patients. In pre-therapy specimens of 8 out of 14 patients (patient 1, 6, 2, 8, 14, 9, 12 and 10), more than 30% of the melanoma cells belonged to the AXL^+ $MITF^-$ subpopulation (**Figure 2C**). In the other 6 patients, most tumor cells prior to therapy displayed $MITF^+$ phenotypes, with or without

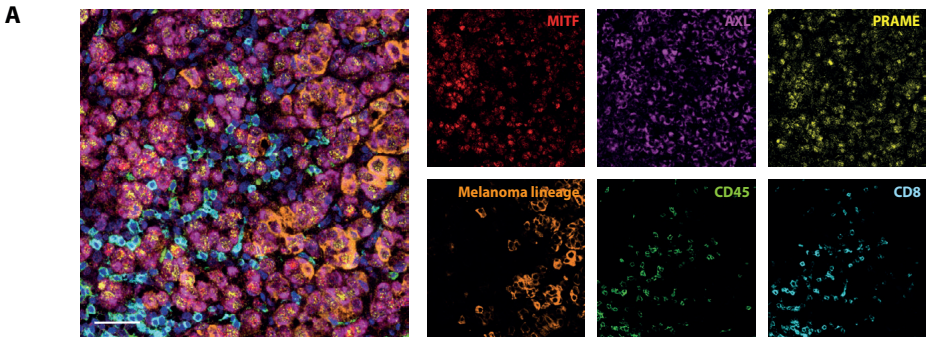
Table 2. Number of sequenced cells classified to each cell type in each tumor

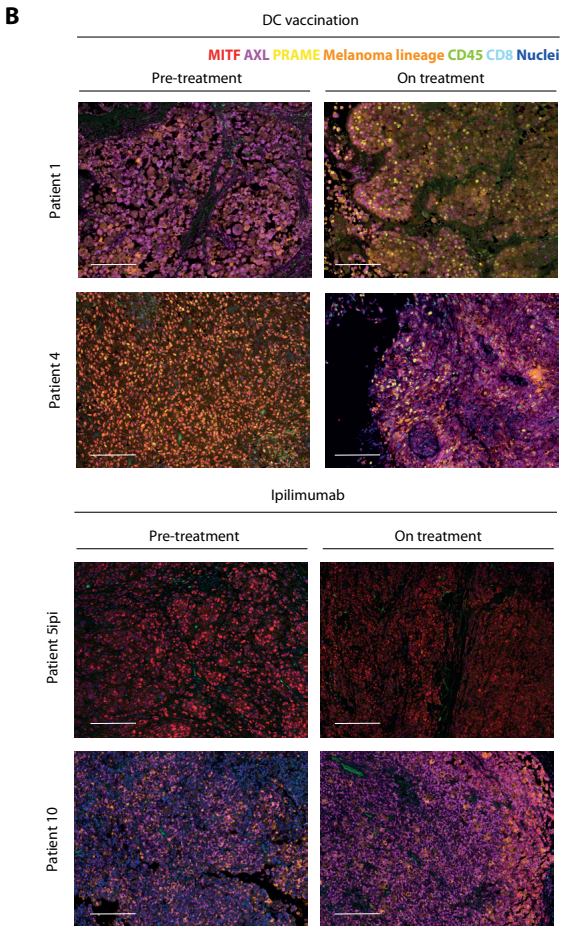
Tumor	AXL^{high} $MITF^{low}$	AXL^{high} $MITF^{high}$	AXL^{low} $MITF^{high}$	AXL^{low} $MITF^{low}$	Total
P0153	12	54	10	24	100
P0196	0	1	41	2	44
P0256	68	39	0	36	143
P0258	0	1	3	83	87
P0262	0	15	1	5	21

AXL expression, whereas the AXL^+ $MITF^-$ phenotype was less present (**Figure 2C**). After therapy, 57% of the patients still had significant proportions of AXL^+ cells (patient 1, 2, 7, 8, 9, 10, 12 and 14), while in the other patients MITF-expressing cells, and in some patients AXL^- $MITF^-$ cells (patient 3 and 6), were more dominant post-therapy.

To investigate if immunotherapy coincides with an increase/decrease of a specific phenotypic group, we assessed the significance of therapy-induced changes. None of the four phenotypes were significantly more/less present in all patients after treatment with immunotherapy (**Figure 2D**). Furthermore, the changes in melanoma phenotypes did not differ significantly between patients treated with DC vaccination or ipilimumab (**Figure 2E**), indicating that therapy-induced changes were independent of the type of therapy. These data demonstrate that the large variety in phenotypes and changes upon therapy in the entire patient group may obscure changes in subgroups of patients. Interestingly, AXL^+ $MITF^-$ cells were decreasing post-therapy in 6 out of 14 patients (42%), with a decrease of >30% in 3 patients (patient 1, 6 and 8) and a more limited decrease (<10%) in the remaining 3 patients (patient 3, 5ipi and 14) (**Figure 2F**).

Concluding, whereas dedifferentiated AXL^+ cells are considered to be highly resistant to targeted therapy, our data suggests that AXL^+ cells may be more sensitive to immunotherapy than to targeted treatment. Nonetheless, AXL^+ cells, although to a lesser extent, may show somewhat increased resistance to immunomodulating antibodies and whole tumor vaccines as compared to AXL^- subpopulations.


A
MITF
AXL
PRAME
Melanoma lineage
CD45
CD8


B
DC vaccination
MITF AXL PRAME Melanoma lineage CD45 CD8 Nuclei
Pre-treatment
On treatment
Patient 1
Patient 4
Ipilimumab
Pre-treatment
On treatment
Patient 5ipi
Patient 10

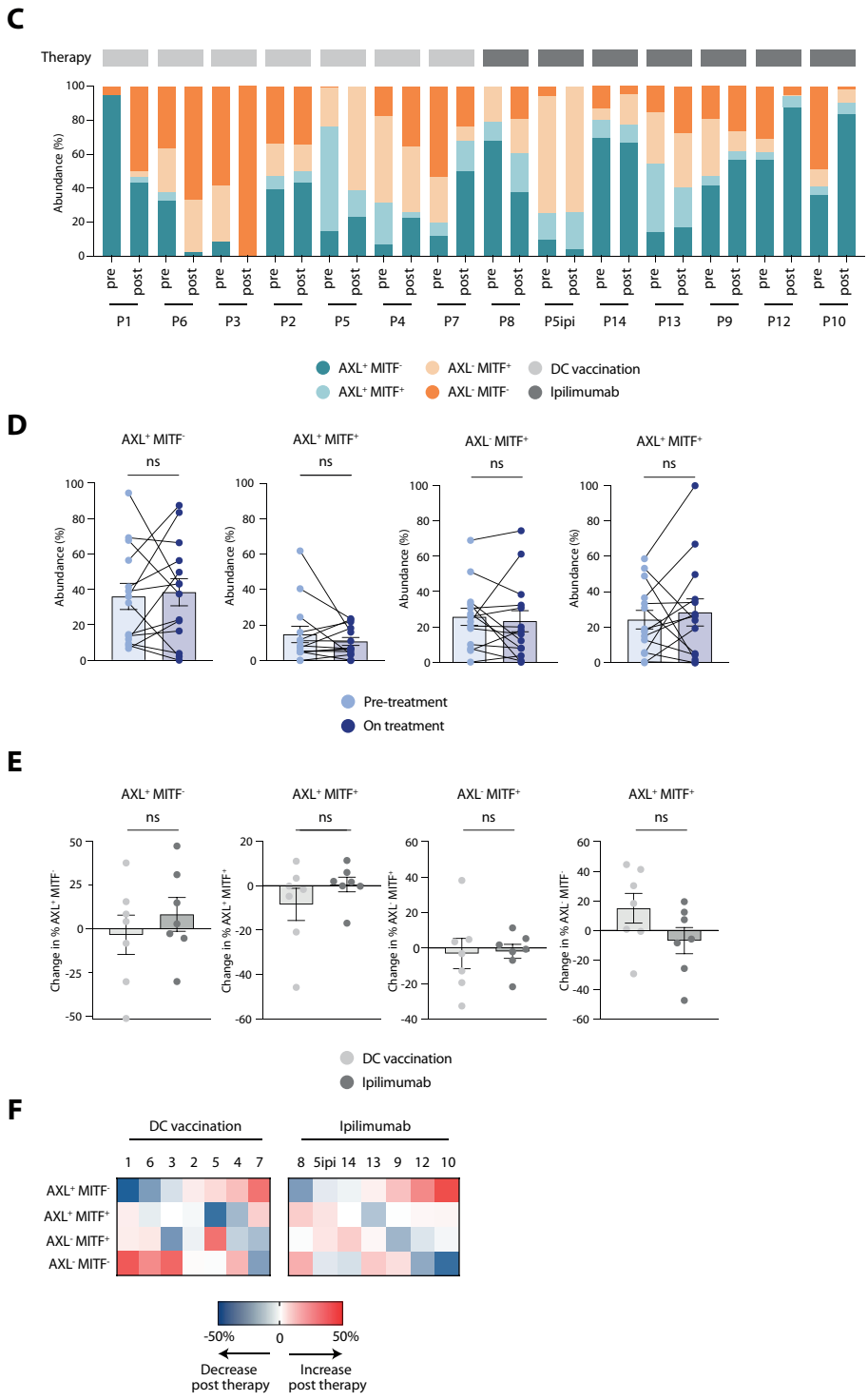

C
Therapy
Abundance (%)
pre
post
P1
P6
P3
P2
P5
P4
P7
P8
P5ipi
P14
P13
P9
P12
P10
AXL+ MITF-
AXL+ MITF+
AXL- MITF+
AXL- MITF-
DC vaccination
Ipilimumab
D
ns
Pre-treatment
On treatment
E
Change in % AXL+ MITF-
Change in % AXL+ MITF+
Change in % AXL- MITF+
Change in % AXL- MITF-
F
DC vaccination
Ipilimumab
1 6 3 2 5 4 7
8 5ipi 14 13 9 12 10
-50%
0
50%
Decrease post therapy
Increase post therapy

Figure 2. Phenotypic heterogeneity exhibits marked interpatient variability. (A) Multiplex immunofluorescence staining for MITF (red), AXL (purple), PRAME (yellow), melanoma lineage, comprising of TYR, PMEL and MLANA (orange), CD45 (cyan), CD8 (green) and nuclei (blue). Scale bar, 25 µm. (B) Multiplex immunofluorescence staining on melanoma metastases from patients treated with DC vaccination (patient 1 and 4) or ipilimumab (patient 5ipi and 10): pre-treatment (left) and on treatment (right). The color of each of the markers is indicated. Scale bar, 50 µm. (C) Stacked bar chart showing the relative presence of different melanoma cell subpopulations in the melanoma tissues prior to and after therapy and the type of therapy given (DC vaccination or ipilimumab) for each individual patient. (D) Bar charts showing the percentage of AXL^+ $MITF^-$, AXL^+ $MITF^+$, AXL^- $MITF^+$ and AXL^- $MITF^-$ cells pre-treatment and on treatment. (E) Bar charts showing the change in percentage of AXL^+ $MITF^-$, AXL^+ $MITF^+$, AXL^- $MITF^+$ and AXL^- $MITF^-$ cells upon treatment with DC vaccination or ipilimumab. (F) Heatmap showing the increase or decrease post-therapy of melanoma phenotypic subpopulations for each individual patient treated with DC vaccination or ipilimumab. Paired samples are shown connected by black lines. Paired t test or Wilcoxon matched-pairs signed rank test significant as indicated; ns, not significant. Mean ± SEM.

Melanoma heterogeneity in relation to immunological pressure

Melanoma cell heterogeneity can be influenced by the presence of infiltrating immune cells, and resulting immunological pressure. Multiplex immunofluorescence revealed that the proportion of none of the melanoma cells phenotypes significantly correlated with the density of leucocyte infiltration, as identified by CD45 expression (data not shown). As high infiltration of $CD8^+$ T cells (intratumoral, stromal or invasive marginal), but not $CD45^+$ cells, are predictive of treatment outcomes in patients with immunotherapy across different cancers[19], we specifically examined the presence of $CD8^+$ $CD45^+$ cells in these tumors in our multiplex immunofluorescence data (**Figure 3A-D**).Abundance of AXL^+ $MITF^+$ melanoma cells weakly inversely correlated with the presence of $CD8^+$ lymphocytes (R^2=0.15, p=0.04), meaning that the more $CD8^+$ T cells were present in the tumor tissue, the fewer melanoma cells with this phenotype were present and vice versa (**Figure 3B**). This could mean that AXL^+ $MITF^+$ cells are more effectively eliminated by infiltrating T cells than the other remaining melanoma cell phenotypes. This may imply that AXL^+ $MITF^+$ cells are more immunogenic and that these cells are therefore more easily targeted by DC vaccination or ipilimumab treatment. Consistent with this, scRNAseq analysis showed that the AXL^+ $MITF^+$ transcriptional state is

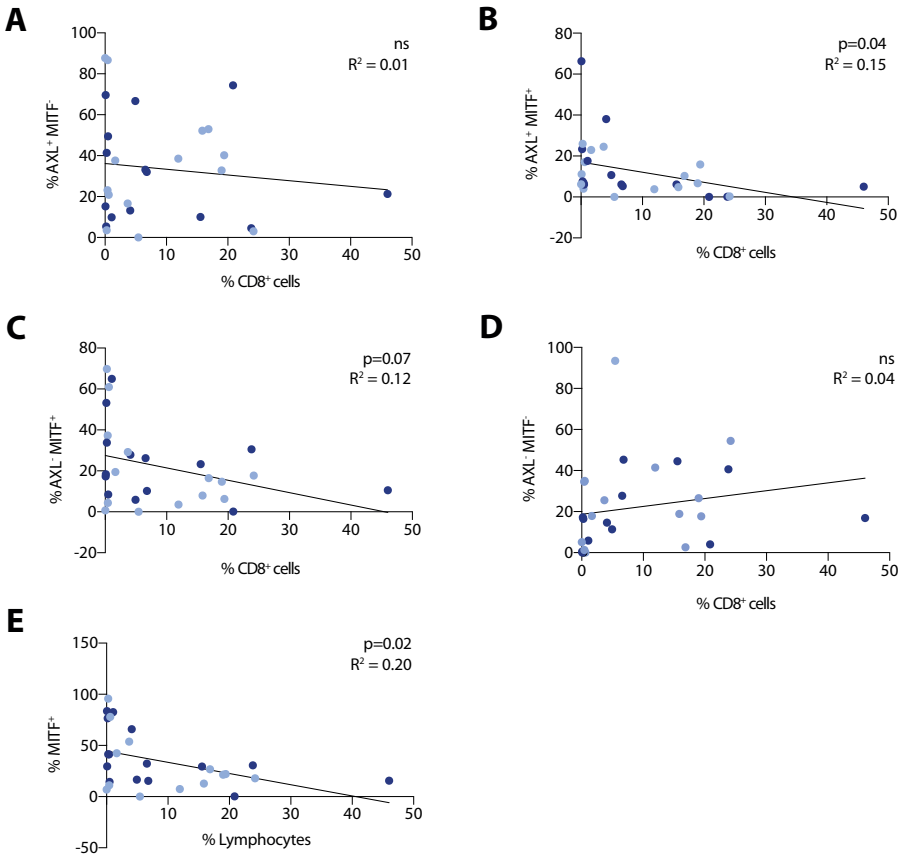


Figure 3. Melanoma heterogeneity and the correlation with immunological pressure. (A-E) Linear regression analysis of the relative abundance of $CD8^+$ T cells within a tumor and the relative abundance of AXL^+ $MITF^-$ (A), AXL^+ $MITF^+$ (B), AXL^- $MITF^+$ (C), AXL^- $MITF^-$ (D) and $MITF^+$ tumor cells (E). Pre-therapy samples are indicated in purple, post-therapy samples are indicated in lilac.

in antigen presentation (**Figure 1B**). In addition, the majority of tumor tissue samples that show a higher percentage of $CD8^+$ cells and lower abundance of AXL^+ $MITF^+$ melanoma cells were taken post treatment (**Figure 3B**, lilac dots). Looking at post-therapy samples only, this inverse correlation was slightly stronger, but not statistically significant (data not shown). This indicates that DC vaccination and ipilimumab treatment may have led to enhanced immunoreactivity to these cells. Although not significant, AXL^- $MITF^+$ cells

may also have an inverse correlation with the presence of intratumoral $CD8^+$ T cells (R^2=0.12, p=0.07) (**Figure 3C**). We therefore hypothesize this increased immunogenicity is largely because of MITF and target gene expression (among which are melanocyte differentiation antigens). Indeed, $MITF^+$ cells were inversely correlated with $CD8^+$ T cells, even more significant than AXL^+ $MITF^+$ cells solely (R^2=0.12, p=0.02) (**Figure 3E**).

Altogether, these data suggests that intratumoral $CD8^+$ T cell abundance is negatively associated with MITF-expressing melanoma cells, which in turn seem more immunogenic than the more dedifferentiated AXL^+ melanoma cells.

Immunotherapy-induced changes in AXL^+ $MITF^-$ cells do not correlate with improved survival

As mentioned before, earlier studies demonstrated that the expression of the AXL program was associated with drug resistance to RAF and MEK inhibition[9-11], meaning that this could negatively affect survival of patients with melanoma. We therefore analyzed whether immunotherapy-induced changes in AXL^+ cells correlated with patient survival in our cohort. The median survival of patients included in this study was 31.25 months (**Figure 4A**). Even though not significantly different, the patients treated with DC vaccination had a higher median overall survival of 46.5 months than the patients who received ipilimumab treatment (25.5 months) (**Figure 4B**). As the effect of survival was not statistically significant between the two treatment options, probably because of limited patient numbers, we performed the survival analyses of the patients from both groups together.

We next analyzed whether a decrease in AXL^+ $MITF^-$ cells led to improved survival of patients. Patients displaying a decrease in AXL^+ $MITF^-$ cells post-therapy (which was seen in patients 1, 3, 5ipi, 6, 8 and 14) did not have an improved survival, as compared to patients with an increase in AXL^+ $MITF^-$ cells (**Figure 4C**). Similarly, patients with a high proportion of AXL^+MITF^- cells prior to therapy (patient 1, 6, 2, 8, 14, 9, 12 and 10) did not show worse survival than patients that had <30% of AXL^+ $MITF^-$ cells before therapy (**Figure 4D**). Altogether this suggests that whereas AXL^+ melanoma cells may be less sensitive to immunotherapy than $MITF^+$ melanoma cells, this is not reflected in the survival of our cohort.

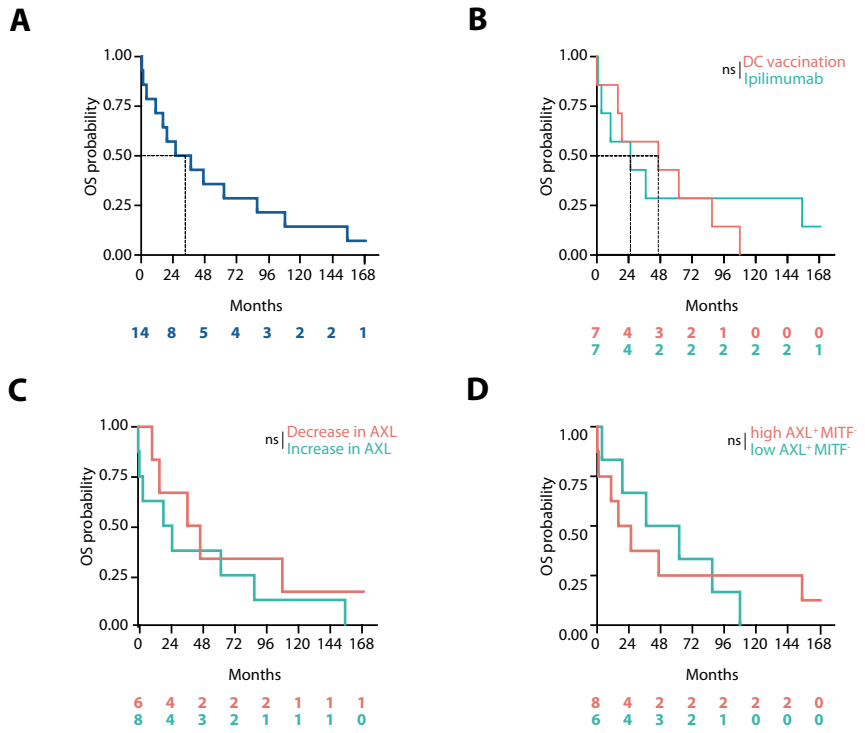


Figure 4. Immunotherapy-induced changes in AXL$^+$ MITF$^-$ cells do not correlate with improved survival. (A-D) Kaplan-Meier analysis showing the overall survival (OS) of metastatic melanoma patients (A), differences in OS between patients receiving DC vaccination (red) or ipilimumab (blue) (B), between patients showing a decrease (red) or increase (blue) in AXL$^+$ MITF$^-$ cells (C) and between patients having a high (red) or low (blue) percentage of AXL$^+$ MITF$^-$ cells prior to therapy (D). A log-rank test was applied to assess significance.

Discussion

Emerging data show that AXL$^+$ tumor cells contribute to increased resistance to targeted therapy, among which MAPK inhibitors[9-11]. More recently, these cells were shown to promote immune evasion[11,18] and are therefore thought to lead to increased resistance to immunotherapy as well. These studies demonstrated distinct transcriptional melanoma states based on scRNAseq data. We show that tumor heterogeneity can be detected by multiplex immunofluorescence *in situ* imaging of AXL and MITF protein expression, thereby translating transcriptional signatures into phenotypically distinct

subpopulations. Moreover, our results show large interpatient variability and variable therapy-induced changes that were independent of the type of therapy. Most interestingly, we did not observe selective resistance of AXL$^+$ cells to immunotherapy from our study. However, MITF-expressing cells were more immunogenic and negatively correlated with CD8$^+$ T cells.

Even though AXL$^+$ cells are believed to show intrinsic resistance to not only targeted treatment[9-11], but immunotherapy as well[18,20], our data demonstrate no generally increased resistance of AXL$^+$ cells to immunotherapy. Immunotherapy of melanoma even showed a marked decrease in AXL$^+$ MITF$^-$ melanoma cells in 3 out of 14 patients. A few years ago, Jerby-Arnon *et al*. (2018) used scRNAseq analysis to identify a melanoma resistance program that was associated with increased T cell exclusion and immune evasion after immune checkpoint inhibition[18]. They defined the most resistant cells to have a low expression of B2M, CD58, HLA-A, MLANA, SOX10, SRP54, TAP2 and TAPBP. Those resistant cells had significantly higher immune resistance scores and were enriched with cycling cells. These slow-cycling cells had been shown to express a MITF$^-$ dedifferentiated state, which includes AXL expression[11,21,22]. This immune exclusion program did include repression of genes involved in antigen processing and presentation. This could mean that MITF$^+$ subsets probably do not contribute to T cell exclusion, as genes involved in antigen presentation were enriched in these cells and among the top most differentially expressed genes as compared to MITF$^-$ cells. AXL$^+$ tumors thus seemed to lead immune exclusion and resistance to immune checkpoint blockade[18]. Even though we do not observe this clear intrinsic resistance of AXL$^+$ tumor cells in our cohort of patients, we only included 7 patients that were treated with ipilimumab, of which 4 did show a (slight) increase in AXL$^+$ MITF$^-$ cells and 1 remained more or less unchanged. These limited numbers of patients studied could therefore mean we may have missed this AXL resistance.

To date, resistance of AXL$^+$ MITF$^-$ cells to dendritic cell vaccination has remained unstudied. Our data demonstrate that 3 out of 7 patients that were treated with dendritic cell vaccination show a decrease in AXL$^+$ MITF$^-$ cells. Although not significantly different from ipilimumab treatment, this effect seemed slightly more prominent in dendritic cell vaccinated patients. This could mean that vaccination with autologous tumor-lysate dendritic cells will prime CD8$^+$ T cells to a wide range of tumor antigens, potentially

including those expressed by dedifferentiated cells, whereas ipilimumab treatment will reinvorgate the pre-existing T cell response, that might not have been directed towards those AXL^+ cells.

Data on tumor heterogeneity in melanoma and resistance to various immunotherapies is emerging, yet it remains critical to characterize these cells in more detail. Our results neither confirm nor rule out a possible increased resistance of AXL^+ $MITF^-$ melanoma to immunotherapy for melanoma. Further investigation on melanoma heterogeneity in relation to therapy resistance is therefore needed, specifically on whole tumor cell dendritic cell vaccinated melanoma patients.

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Chapter 10

General discussion

Autoimmunity and tumor immunity are often linked, as exemplified by the association between vitiligo and melanoma. Research on the role of the immune system in vitiligo and melanoma has grown exponentially in recent years. Partly because of this, the diseases processes and the way in which both diseases can be treated most efficiently are becoming increasingly clear. Nowadays, immunotherapy is considered the most promising therapeutic option for patients with melanoma. Clear objective clinical responses are seen, however there is still a part of the patients that does not benefit from immunotherapy. In vitiligo, both segmental and non-segmental, current treatment modalities also show limited clinical efficacy and vitiligo patients may benefit from more specific immunosuppressive drugs. Novel insights into pathogenic mechanisms and immune evasion/modulation will be essential for the next generation of therapies for vitiligo and melanoma patients. The main goal of this thesis therefore was to investigate immune dysregulation in skin autoimmunity and cancer. Specifically, in **part 1** of this thesis systemic and local immune activation is described. First systemic immune activation in the context of vitiligo is described (**chapter 2**), followed by a literature review and study on tissue-resident T cells in both vitiligo and melanoma (**chapter 3** and **4**). **Part 2** focusses on melanocytes and melanoma cells and whether and, if so, how these cells can contribute to immune evasion. In short, we found that melanocytes in vitiligo are inadequate in maintaining peripheral tolerance through PD-1/PD-L1 signaling (**chapter 6** and **7**). Alternatively, melanoma cells show marked phenotypic heterogeneity that persist during immunotherapy (**chapter 8**). These findings illustrate the potency of immunomodulatory drugs in the treatment of vitiligo, but also highlight the need for improving anti-cancer therapeutics. How do the findings from this thesis fit in the current autoimmunity and tumor immunology landscape? Below the context of these findings and their implications are discussed individually, including future areas of discovery.

Discovering novel therapeutic targets to improve vitiligo treatment

Perspectives on the role of humoral immunity human vitiligo

In this thesis, we have attempted to elucidate the immunopathogenesis of human vitiligo. Vitiligo, despite its relatively high prevalence, remains a disease with limited therapeutic options for patients. Current treatment modalities have overall limited efficacy and therefore it remains critical to identify new therapeutic strategies to treat patients.

Recent studies have yielded a strong mechanistic understanding of this disease. It is widely appreciated that $CD8^+$ T cells are both necessary and sufficient to mediate human vitiligo. $CD8^+$ T cells are consistently found at the edge of actively depigmenting skin of vitiligo patients, where $CD8^+$ T cells engage melanocytes and promote disease progression through the local production of IFN-γ. Concomitantly, IFN-γ-induced chemokines are then secreted from surrounding keratinocytes to further recruit T cells to the skin through a positive-feedback loop. In **chapter 2**, we formally demonstrated the systemic immunological differences between segmental and non-segmental vitiligo. Non-segmental vitiligo patients were found to have elevated serum titers of melanocyte-reactive antibodies, that were absent in segmental vitiligo patient and healthy control sera. How does this finding relate to our current understanding of immunopathogenesis of vitiligo? In the past, it has been shown that these autoantibodies can kill human melanocytes in culture and that these are more common in active disease[1]. Similarly, transplanting human skin onto nude mice, that subsequently got injected with purified IgG antibodies from patients with vitiligo, led to a marked decrease in the number of melanocytes, which was not observed when control IgG was injected[2]. Nonetheless, antibody-induced melanocyte damage was only mildly induced. This makes sense, as the cytotoxicity of antibodies against melanocytes in these assays assume these are directed against surface antigens, rather than against cytoplasmic antigens. Yet, research has shown that the vast majority of antibody responses in vitiligo are directed against intracellular antigens[3]. As antibody-induced cell death depends on the recognition of membrane-surface antigens, which are a minority in vitiligo patients, it is plausible to speculate that melanocyte-

specific antibodies are not a primary driver of vitiligo pathogenesis. The contribution of anti-melanocyte antibodies to vitiligo pathogenesis therefore remains largely unknown.

Instead of promoting antibody-dependent cellular cytotoxicity, humoral immunity can potentially improve the cellular autoimmune response. In the past, it has been shown that circulating antigen-specific antibodies are associated with improved cross-presentation, as a result from enhanced antigen capture by antigen-presenting cells[4]. Importantly, this led to improved $CD8^+$ T cell activation, demonstrating that humoral immunity can aid the initiation of systemic cellular immunity. Based on these observations, it is tempting to speculate that the presence of autoantibodies is associated with the robustness of the $CD8^+$ T cell response, although it remains to be unraveled if this is true for melanocyte-specific antibodies that are found in vitiligo. If this holds true, we hypothesize that antibodies diffuse into the skin to take up antigens from melanocytes undergoing apoptosis. Since immunofluorescence staining demonstrated the presence of human IgG antibodies across the epidermis in mice that were injected intravenously with IgG from vitiligo patients[2], we assume these antibodies can migrate into the skin and lead to improved antigen uptake in the skin as well.

Early studies showed that the number of HLA-A2 melanocyte-specific $CD8^+$ T cells in the blood of vitiligo patients correlated with disease severity, but its association with humoral melanocyte immunity has to be further investigated. Likewise, autoantibody titers correlate with disease activity[5,6] and the extent of the disease[7], although the presence of melanocyte-specific antibodies does not seem to be predictive of recent disease activity[8]. In this way, the autoimmune antibody response is suggested to be an enhancer of the disease, rather than an initial driver of disease that might affect the extent and duration of the disease. Concomitantly, increased opsonization of target antigens could provide a rationale for systemic spread of vitiligo over time, as seen in non-segmental vitiligo. Absence of melanocyte-reactive antibodies in sera of patients with segmental vitiligo, which generally stabilizes quickly and barely progresses systemically during life, is illustrative of this possible role.

Concluding, the involvement of antibodies directed to melanocytes in vitiligo pathogenesis has not yet been proven, but suggests a role in enhancing $CD8^+$ T cell activation. As a consequence, this might affect the extent and systemic progression of disease.

Immune checkpoints as therapeutic targets in skin autoimmunity

Novel and rational treatment approaches will be needed to increase response rates in patients with vitiligo. Despite systemic immune activation in non-segmental vitiligo, as demonstrated in **chapter 2**, local immune-based cytotoxic destruction of melanocytes by (resident) $CD8^+$ T cells in the basal layer seems most destructive. Therefore, local therapeutic intervention would be most effective.

Immune checkpoints, including programmed cell death 1 (PD-1) are commonly studied in human cancers. PD-1 is a key player in immune regulation. Upon infection, PD-1 gets expressed by effector T cells after signaling through the T cell receptor[9]. Upon ligation to its ligand programmed cell death ligand 1 (PD-L1), the T cell is repressed in its activation and proliferation. In this way, PD-1/PD-L1 plays a pivotal role in central and peripheral tolerance[10]. Work over the past years has demonstrated that immune checkpoint signaling is not only a way to control tolerance against normal tissue under inflammatory conditions. Dysregulation of immune checkpoints is commonly involved in tumor immune evasion and a variety of autoimmune diseases[11]. Whereas impaired PD-1/PD-L1 function has been demonstrated in amongst others type 1 diabetes and rheumatoid arthritis, vitiligo was left unstudied. To obtain insight in immune checkpoint signaling and its therapeutic potential in vitiligo, in **chapter 7**, we analyzed vitiligo melanocytes cultured with clinically relevant type I and II interferons. Exposure to IFN-γ led to increased PD-L1 expression in skin cells derived from healthy control individuals, but remained absent in melanocytes isolated from non-lesional vitiligo skin, indicating inherent differences from healthy donor cells.

There are several questions that remain to be answered regarding the role of immune checkpoint molecules in vitiligo pathogenesis and treatment. First of all, single-cell transcriptome and epigenetic analyses are needed to investigate whether the alterations in vitiligo melanocytes are due to genetic factors or because of aberrant methylation patterns. In the past, genome-wide association studies have identified various loci that contribute to vitiligo risk[12-15], yet none of these involve single-nucleotide polymorphisms in the *PD-L1* (*CD274*) or related genes. The same is true for transcriptome analysis of whole skin biopsies[16]. This is consistent with our data, showing

that this impaired PD-L1 upregulation is only seen in melanocytes and not in other skin cells, including keratinocytes and fibroblasts. Therefore this will most likely not be detected by RNA analyses of whole skin biopsies, but by single-cell RNA sequencing analyses of skin biopsies or bulk RNA sequencing of isolated melanocytes only. Very recently, a seminal paper reported single-cell RNA sequencing analysis of skin biopsies taken from healthy control individuals and peri-lesional skin biopsies taken from vitiligo patients[17]. Analysis of melanocytes revealed two clusters, named M1 and M2, in which upregulated genes in the M1 cluster coincides with strong immune responses. Therefore, the authors identified patients in whom the melanocytes were predominantly composed of M1 cells, as having a more progressive disease, whereas those patients who mostly had melanocytes from the M2 cluster (which was also the case for healthy individuals) presented stable disease. While genes related to (IFN-γ-induced) PD-L1 expression or signaling remained unstudied, this dataset enables us to study potential intrinsic defects of specifically vitiligo patients' melanocytes. In addition, this study proved that fibroblasts can drive autoimmune activity by recruitment and activation of $CD8^+$ T cells via secretion of chemokines[17]. Dermal fibroblasts were shown to exhibit intrinsic differences in IFN-γ-induced chemokine expression. This emphasizes that single-cell RNA sequencing of vitiligo skin is preferred over bulk RNA sequencing of solely melanocytes, for this can reveal aberrances in e.g. mesenchymal subpopulations.

Future research will have to focus on the origin of the impaired induction of PD-L1 to elucidate how this pathway defect can most effectively be overcome. Complex mechanisms underlie PD-L1 expression, which is regulated not only at the genetic, but also at the transcriptional and post-transcriptional level[18]. Based on this and the observation that vitiligo is commonly associated with autoimmune comorbidities (specifically for non-segmental vitiligo)[19], it is tempting to speculate that aberrant PD-L1 regulation is not limited to epidermal melanocytes, but may comprise other cells as well. To elucidate this regulation, it would be of interest to collect single-cell RNA sequencing data of melanocytes, together with e.g. thyroid cells (a common comorbid autoimmune disorder amongst vitiligo patients) and maybe even neural crest cell-derived cells (which are also present in the thyroid), from which melanocytes develop during embryonic development[20], of both healthy individuals and vitiligo patients. This would clarify if aberrant

epigenetic regulation rather than genetic factors contributes to this absence of PD-L1 expression and if this is restricted to melanocytes only or affects other organs as well.

Secondly, better understanding of the aberrant PD-L1 signaling in patients with vitiligo may give clues on how this pathway can most effectively be targeted. Nevertheless, regardless of the initial cause, the observed absence of (IFN-γ-induced) PD-L1 expression by melanocytes of vitiligo patients do provide a rationale to test local immunomodulating agents to repress skin depigmentation. Intraperitoneal injection with PD-L1 fusion protein in Pmel-1 vitiligo mice reversed/suppressed depigmentation, because of recruitment of regulatory T cells to the skin and suppressed effector T cell abundance[21]. Although promising, none of the mice yielded a permanent repigmentation effect, indicating the need for combining therapies.

It is important to realize that local immune suppression may carry a potential risk for oncogenic transformation. Even though these adverse events are not reported in pre-clinical studies on manipulating PD-1/PD-L1 signaling so far[22-24], it is important to realize this might be because of too short follow-up time and short-term immunotherapy treatment. Similarly, skin carcinogenesis was not observed in PD-L1 fusion protein-treated vitiligo mice[21]. These mice were treated for 6 weeks and observed during an additional 9 week period. Even though macroscopic tumors might have been absent in these mice, it remains unclear if lesions were still clinically occult at this point. As vitiligo patients have a 3-fold less risk of developing melanoma[25,26], we hypothesize that agonistic PD-L1 therapy will increase the probability of melanoma development to the risk of healthy, control individuals. Nonetheless, prolonged agonistic PD-L1 treatment and observation of treated vitiligo mice should clarify if no significant side effects occur.

Besides local immunotherapy, narrow-band ultraviolet B (UV-B) phototherapy might be necessary to obtain more complete repigmentation. Before, UV-B therapy has been shown to induce PD-L1 expression in human melanoma cell lines via an NF-κB-dependent manner[27]. Likewise, UV-B treatment led to NF-κB activation in primary melanocytes, implying that PD-L1 upregulation is a conserved stress response to UV exposure, similar to interferon-induced expression, in human melanocytes and melanoma cells.

Whether PD-L1 is upregulated upon UV-B treatment in patients with vitiligo and, if so, if PD-L1 is expressed on melanocytes, is unknown. Moreover, it is interesting to elucidate if PD-L1 upregulation on epidermal melanocytes is an early biomarker for response to UV-B therapy, as repigmentation of the skin generally takes a few months.

Finally, our study of PD-L1focussed on non-segmental vitiligo only and did not involve segmental vitiligo patient material. Impaired cytokine-induced PD-L1 expression might apply to melanocytes in these patients as well. More specifically, as the absence of systemic immune activation might provide a rationale for disease stabilization in segmental vitiligo, so would a somatic mosaicism of melanocytes, involving the regulation of PD-L1 expression. The aforementioned results therefore provide a rationale to elaborate research on this pathway in segmental vitiligo.

These data, together with the data demonstrated in **chapter 2**, emphasize the potency of local immune suppression in the treatment of vitiligo. What would be the most effective approach remains to be further investigated.

Perspectives on the role of tissue-resident memory T cells and tumor heterogeneity in immunotherapy to melanoma

Currently, melanoma is considered one of the most immunogenic tumors[28], in which infiltration of T cells exhibiting a resident phenotypes correlates with a more favorable prognosis in human melanoma[29,30]. After reviewing seminal reports on skin-resident T cells in the context of melanoma in **chapter 3**, we conducted an explorative study to investigate the presence of T_{RM} cells in non- and pre-malignant melanocytic lesions as compared to melanoma in **chapter 4**. Our data showed that progression of healthy and pre-malignant melanocytes into overt cancers coincided with increased expression of T_{RM} cell markers (CD69 and CD103). However, T_{RM} cells were not abundantly present in premalignant tissues, suggesting that T cell infiltration and T_{RM} cell differentiation did not yet increase at the premalignant stage. To formally demonstrate that resident-memory like T cells have no functional significance in pre-malignant cancer surveillance, some major questions remain to be answered.

Firstly, even though a fraction of dysplastic nevi can rapidly turn into an overt melanoma, the majority of dysplastic nevi are stable and will not develop into a melanoma. Potentially, we have selected those nevi that would not have progressed into a melanoma in time. This suggests that the expression of T_{RM} cell markers could be increased in precancerous tissues, as compared to true non-malignant tissues after all. Analysis of longitudinal paired specimens of dysplastic nevi that did develop into melanoma is needed to confirm or disprove this hypothesis. Regardless of this, melanocyte senescence seems to be the most likely cause for preventing progression to melanoma rather than increased immunosurveillance by T_{RM} cells in true benign melanocytic nevi. This would be in line with hypothesis presented by Park *et al*. (2019) that suggest that $CD8^+$ T_{RM} cells can induce a cancer immune equilibrium by maintaining melanoma cells in a dormant, but viable state[31]. This explains how occult melanomas can be controlled for up to decades in human skin. In turn, immunological pressure from elimination or suppression of susceptible cancer cells by these T_{RM} cells may lead to immunoediting, selecting those mutated cancer cells with less immunogenic features that are not easily recognized by the immune system, which eventually leads to cancer escape and tumor outgrowth[31]. This could refer to the more dedifferentiated AXL^+ tumor cells as identified in **chapter 9**.

In **chapter 9** we aimed to visualize *in situ* melanoma heterogeneity prior to and upon ipilimumab treatment or autologous tumor-lysate loaded dendritic cell vaccination. We found large interpatient variability and variable therapy-induced changes that were independent of the type of therapy. Whereas AXL^+ tumor cells show increased resistance to MAPK pathway inhibitors, these cells do not seem to be as resistant to immunotherapy from our study. However, we do not formally demonstrate whether this is surely not the case. These cells were identified to promote immune evasion by different studies[32,33] and MITF-expressing cells are believed to be more immunogenic[32]. Similarly, reports in breast and head and neck cancer indicated that AXL might fulfill an active role in immune suppression[34,35]. There may be various reasons why we don't observe this resistance as clearly in our study. The most important one is that we included dendritic cell vaccination, which remained unstudied in aforementioned studies. In addition, we included limited numbers of patients and only 5 out of 14 patients specimens were taken from the same site of resection. Therefore, it is still possible that AXL-

expressing cells are the more resistant tumor cells in our study. If AXL$^+$ cells are truly more resistant, this suggests that intratumoral T_{RM} cells increase tumor heterogeneity and abundance of cycling, dedifferentiated AXL$^+$ MITF$^-$ cells. In **chapter 9** we did not look specifically at CD69$^+$ CD8$^+$ T cells. However, we did observe a trend that AXL$^+$ MITF$^+$ cells, and supposedly AXL$^-$ MITF$^+$ cells, were inversely correlated with CD8$^+$ T cells. In depth *in situ* visualization of both T_{RM} cells as well as melanoma heterogeneity would give information if there is a correlation between T_{RM} cells and a specific melanoma phenotype.

Another major question that remains unanswered involves the T cell receptor repertoire of T_{RM} cells among these skin tissues. We demonstrated that the proportion of CD103$^+$ CD8$^+$ T cells is significantly higher in benign melanocytic nevi, as compared to healthy skin. Hypothetically, these CD103$^+$ T_{RM} cells might be specific for melanocyte differentiation antigens and consequently (cancerous) melanocytes undergo dedifferentiation. This could explain why AXL$^+$ cells may be more resistant to, at least some, immunotherapies,. While immune checkpoint inhibition will unleash the negative feedback on T cell activation, thereby reinvigorating the pre-existing T cell responses, autologous tumor-lysate dendritic cell vaccination will prime CD8$^+$ T cells to a wider range tumor antigens, potentially including those expressed by dedifferentiated cells. According to this theory, intralesional T_{RM} cells that are specific for AXL$^+$ cells are less present prior to therapy and unleashing inhibitory signaling will have limited effect. Tumors that show unresponsiveness of AXL-expressing cells upon immune checkpoint inhibition, may then benefit from additional AXL targeting. This was recently shown by a study proving that targeting AXL effectively targets tumors that demonstrate insensitivity to immunotherapy or tumor-specific T cells[36]. In addition, treatment with an antibody-drug conjugate targeting AXL, promoted the induction of memory-like CD8$^+$ T cells. Combining this drug with tumor-specific T cells proved superior in a xenograft model of melanoma that was initially insensitive to immune checkpoint inhibition. In addition, bemcentinib, a small molecule AXL inhibitor, in combination with pembrolizumab is currently being tested in patients with metastatic melanoma in a randomized phase Ib/II study (NCT02872259).

Summarizing, our data demonstrate large variability among patients and variable changes in tumor heterogeneity upon treatment. We neither confirm nor rule out that AXL$^+$ cells are more resistant to both targeted and

immunomodulating therapies, but this needs further investigation. If so, combining immunotherapy with more specific targeting of factors specific for AXL$^+$ cells seems promising.

Concluding remarks and prospects for future investigation

This thesis provides novel insights into immune dysregulation, aberrant vitiligo melanocytes and tumor heterogeneity, however, much remains to be explored in order to optimally treat human vitiligo and melanoma patients.

Essential to the development of novel therapies for human vitiligo, at least for non-segmental vitiligo, are immune suppressive approaches. As these patients often experience progressive disease, in which 1) the CD8$^+$ T cell response might be enhanced by a humoral autoimmune response and 2) melanocytes show impaired PD-L1 expression, immunosuppressive therapy could on the one hand inhibit destructive CD8$^+$ T cells or on the other hand strengthen melanocytes' protection against the T cell attack. Potentially, these effects may even act synergistically. Depending on the presence of melanocyte stem cells within the pigment cell reservoir, combination treatment, such as immunosuppressive therapy and UV-B therapy, could be needed to obtain complete repigmentation.

Current and future research will aim at unravelling the effect of melanocyte-reactive humoral immunity on the autoreactive T cell response and its correlation to disease activity and/or progression. Concurrently, the cause of impaired peripheral tolerance induction by melanocytes and its role in segmental vitiligo deserves more attention. This should lead to better understanding of immune activation and regulation in autoimmune diseases, in particular vitiligo, and could improve therapeutic options for many patients that experience autoimmunity.

While major advances have been made in the treatment of metastatic melanoma by the introduction of immunotherapy, improvements are still needed as many patients experience relapse of the disease. Data on melanoma heterogeneity and tumor-resident memory T cells are emerging. Considering inflammation-induced dedifferentiation, possibly from intratumoral T_{RM} cells, and the presence of less immunogenic AXL$^+$ melanoma cells that are hardly recognized, immunotherapeutic modalities

should not only aim at reactivating the present anti-tumor response. Targeting AXL$^+$ cells either with targeted therapy or dendritic cell vaccines loaded with lysate from AXL$^+$ cells, could increase the response rates of patients responding to immunotherapy. Future research should therefore aim at improving CD8$^+$ T cell responses towards immunotherapy-resistant melanoma cells. This is not only relevant for the treatment of melanoma, but may also improve treatment outcomes in other human cancers, thereby more broadly impacting on cancer immunotherapy in general.

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Chapter 11

Summary and conclusions

Nederlandse samenvatting voor niet ingewijden

Summary and conclusions

Work over the past years has increased our understanding on the role of the immune system in human vitiligo and melanoma. Yet, pathogenic mechanisms of vitiligo are still incompletely studied and immune evasion still occurs among immunotherapy-treated melanoma patients. The work described in this thesis therefore aimed to on the one hand investigate the role of the immune system in melanoma and vitiligo, and on the other hand study whether and, if so, how melanocytes and melanoma cells contribute to immune evasion.

Systemic immune activation in human vitiligo

Part 1 of this thesis presents systemic and local immune activation in skin pigment cell disorders. Whereas non-segmental vitiligo shows an unpredictable disease course, segmental vitiligo typically stabilizes a few months after onset, suggesting distinct pathophysiology pathways may be involved, that could clarify differences in clinical presentation and disease course. In **chapter 2**, we show that in contrast to non-segmental vitiligo, no increased systemic immunity is found in patients with segmental vitiligo. Most interestingly, antibody responses against melanocyte antigens were present only in non-segmental vitiligo patients, suggesting less involvement of B cell immunity in segmental vitiligo. Our data indicate that segmental vitiligo pathogenesis is associated with a localized cytotoxic reaction targeting epidermal melanocytes, while in non-segmental vitiligo also systemic immune activation is observed.

Skin-resident memory T cells in pigment cell disorders

Besides systemic autoimmunity, local immune activation is believed to be involved in skin depigmentation. For some lesions recur at the same skin sites as previous lesions it is suggested that resident T cells are involved. In **chapter 3**, we review seminal reports on skin-resident T cells and their role in the context of vitiligo and melanoma, as well as their potential as therapeutic targets in both diseases. Autoreactive T_{RM} are abundantly present in lesional vitiligo skin and blocking the generation, maintenance and coordination of T_{RM} cells efficiently inhibits melanocyte destruction in vitiligo mouse models. This indicates that targeting these cells might be effective as a durable

treatment strategy for vitiligo. Conversely, vaccine-induced T_{RM} cells can effectively suppress tumor growth in mouse models of melanoma.

Even though current data suggest an important protective role for T_{RM} cells in melanoma, little is known about the abundance of T_{RM} cells in non- and pre-cancerous tissues. In **chapter 4** we demonstrate that progression of healthy skin into melanoma coincides with increased expression of T_{RM} cell markers. Nonetheless, T_{RM} cells were not abundantly present in premalignant tissues, which suggests that T cell infiltration and T_{RM} cell differentiation do not occur yet at the pre-malignant stage.

Isolation of melanocytes from human skin

In **Part 2** of this thesis we sought to characterize melanocytes and melanoma cells that may contribute to immune evasion. To enable melanocyte research it is important to isolate these cells properly. In **chapter 5**, we describe a new isolation protocol for instant isolation of highly-purified primary human melanocytes by taking advantage of their cell surface expression of CD117. Purified melanocytes expressed the melanocyte differentiation antigen Melan-A, showed a multi-dendritic phenotype *in vitro* and produced melanin upon stimulation. This protocol enabled to isolate melanocytes within several hours and, therefore, the freshly-isolated human melanocytes can be considered as proper equivalents of melanocytes *in vivo*.

Impaired PD-L1 signaling by human vitiligo melanocytes

Immune checkpoints are commonly dysregulated in human cancer, but also in a variety of autoimmune diseases. However, immune checkpoint signaling remained incompletely studied in the context of vitiligo. **Chapter 6** is a critical review of the current knowledge on the PD-1/PD-L1 axis in vitiligo as a new therapeutic target for vitiligo therapy. PD-1/PD-L1 is suggested to be involved in loss of peripheral tolerance in human vitiligo. $PD\text{-}1^{+}$ cells were found to be present in (peri)lesional vitiligo skin and increased in the blood of patients with vitiligo. Additionally, i*n vivo* PD-L1 protein therapy reversed depigmentation in murine vitiligo, indicating targeting this pathway might induce effective long-term melanocyte-specific tolerance in human vitiligo. In **chapter 7**, we therefore investigated the role of PD-1/PD-L1 signaling in melanocyte destruction in vitiligo and how interferons can influence this

signaling route. PD-1$^+$ T cells were abundantly present in vitiligo skin, whereas its ligand PD-L1 was barely expressed. Exposure to IFN-γ, a key cytokine in vitiligo pathogenesis, led to increased PD-L1 expression in human primary melanocytes, fibroblasts and keratinocytes. Most interestingly, melanocytes derived from non-lesional vitiligo skin showed no PD-L1 upregulation upon IFN-γ, even though other skin cells did. Our data indicate that melanocytes do not confer protection against T cell attack during vitiligo pathogenesis by PD-L1 upregulation and illustrates the potential of manipulating PD-1/PD-L1 signaling as a therapeutic option for human vitiligo.

Melanoma heterogeneity and resistance to immunotherapy

Besides aberrant PD-1/PD-L1 signaling, melanoma cells have other properties to evade immune destruction, among which tumor heterogeneity. To be able to visualize melanoma heterogeneity *in situ*, it is important to have good quality multiplex immunofluorescence staining. **Chapter 8** describes a method to improve the quality of multiplex immunofluorescence stainings. We demonstrated that heating-related damage can be overcome by repetitive exposure to β-mercaptoethanol-containing stripping buffer. This markedly improved the quality of the multiplex immunofluorescence staining.

In **chapter 9** we aimed to study *in situ* melanoma heterogeneity prior to and upon immunotherapy treatment, to reveal those that are insufficiently targeted by current immunotherapies. Using multiplex immunofluorescence, we found large interpatient variability and variable immunotherapy-induced changes. In addition, whereas AXL$^+$ tumor cells showed increased resistance to targeted therapy in the past, these cells did not seem to be as resistant to immunotherapy from our study. Nonetheless, AXL$^+$ MITF$^-$, and presumably also AXL$^-$ MITF$^+$ cells, were inversely correlated with CD8$^+$ T cells, and are therefore more immunogenic than the more dedifferentiated AXL$^+$ melanoma cells.

Future prospects

Research on the role of the immune system in vitiligo and melanoma has grown exponentially in recent years. Because of this, it is becoming increasingly clear how these diseases can be can be treated most efficiently.

In **chapter 10** we integrate the findings of our studies in the light of current literature. In summary, the studies presented in this thesis provide a rationale to study the possibilities of immunosuppressive therapy in the treatment of vitiligo. In addition, future research should aim at improving $CD8^+$ T cell responses towards immunotherapy-resistance melanoma cells.

Nederlandse samenvatting voor niet ingewijden

Onze huid speelt een cruciale rol in de afweerreactie tegen ziekteverwekkers door de aanwezigheid van verschillende immuuncellen. Onderzoek van de afgelopen jaren heeft ons inzicht in de rol van het immuunsysteem in vitiligo en melanoom vergroot.

Vitiligo is een auto-immuunziekte waarbij immuuncellen de lichaamseigen cellen aanvallen. In het geval van vitiligo gaat het om de pigmentcellen in de huid, ook wel melanocyten genoemd. Als gevolg hiervan ontstaan er pigmentloze, witte vlekken. Hoewel er veel kennis is opgedaan over welke immuuncellen vitiligo veroorzaken en hoe, toch zijn er nog onbeantwoorde vragen omtrent het ziekteproces.

Melanoom, een agressieve vorm van huidkanker, ontstaat uit melanocyten en is relatief ongevoelig voor de huidige behandelopties. Immuuntherapie is een recent ontwikkelde behandeling die het immuunsysteem stimuleert om de kankercellen te herkennen en te vernietigen. Kankercellen zijn echter in staat het immuunsysteem te misleiden of te remmen, waardoor de kankercellen niet worden vernietigd. Hoewel er door immuuntherapie vooruitgang is geboekt, zijn er nog steeds patiënten waarbij de kankercellen het immuunsysteem tegenwerken.

Het werk in dit proefschrift had enerzijds als doel de betrokkenheid van het immuunsysteem bij vitiligo en melanoom verder te onderzoeken. Anderzijds had ons onderzoek als doel te onderzoeken of, en zo ja, hoe melanocyten en melanoomcellen in staat zijn het immuunsysteem te misleiden.

Segmentale en non-segmentale vitiligo

Er bestaan twee typen vitiligo: segmentale en non-segmentale vitiligo. Non-segmentale vitiligo komt voor bij circa 80% van de patiënten met vitiligo. Bij deze patiënten komen de witte vlekken verspreid over het lichaam voor, vaak in een symmetrisch patroon, en breiden zich met de jaren verder uit. Bij segmentale vitiligo verdwijnen de pigmentcellen in een specifiek huidgebied, aan één zijde van het lichaam. Deze variant stabiliseert binnen verloop van tijd, waarna er geen verdere depigmentatie meer plaatsvindt. Dit verschil in ziekteverloop suggereert dat er mogelijk verschillende immuuncellen bij betrokken zijn. In **hoofdstuk 2** laten we zien dat er bij

segmentale vitiligo enkel sprake is van een lokale afweerreactie tegen melanocyten. Daarnaast bleek dat er bij non-segmentale vitiligo patiënten naast de lokale auto-immuunreactie, ook antistoffen tegen melanocyten aanwezig zijn in het bloed. Dit laat zien dat er verschillende processen betrokken zijn bij segmentale en non-segmentale vitiligo.

De rol van weefselresidente T geheugencellen in vitiligo en melanoom

In **hoofdstuk 3** beschrijven we de rol van immuuncellen in de huid in vitiligo en melanoom, evenals het potentieel als therapeutisch aangrijpingspunt. Weefselresidente T geheugencellen (afgekort als T_{RM} cellen), een specifieke type immuun cel, zijn overvloedig aanwezig in witte, vitiligo huid. Deze cellen herkennen de melanocyten als lichaamsvreemd en vernietigen deze vervolgens. Onderzoek in muizen laat zien dat het remmen van T_{RM} cellen kan leiden tot repigmentatie van de huid. T_{RM} cellen kunnen daarom een mogelijk nieuw aangrijpingspunt zijn voor de behandeling van vitiligo. Omgekeerd kunnen T_{RM} cellen de groei van kankercellen in melanoom onderdrukken. Patiënten waarbij de tumoren relatief veel T_{RM} cellen bevatten, hebben over het algemeen een betere overlevingskans.

Hoewel eerdere onderzoeken laten zien dat T_{RM} cellen een beschermende rol vervullen tegen de groei van melanoomcellen, bleef nog onduidelijk of deze cellen mogelijk ook een rol spelen in voorstadia van melanoom (bijv. een onrustige moedervlek). In **hoofdstuk 4** onderzoeken we de aanwezigheid van T_{RM} cellen in verschillende weefsels, waaronder gezonde huid, moedervlekken en melanoom. T_{RM} cellen bleken verhoogd aanwezig in melanoom, maar waren niet overvloedig aanwezig in moedervlekken en gezonde huid.

Isolatie van melanocyten uit de huid

Om melanocyten in meer detail te kunnen bestuderen, is het essentieel deze cellen uit de huid te isoleren. In **hoofdstuk 5** beschrijven we een handleiding om in een kort tijdsbestek melanocyten uit de huid te isoleren. Eerdere handleidingen hanteerden een tijdrovende procedure, waarbij een potentieel risico bestaat dat melanocyten qua uiterlijk vertoon en functie veranderingen ondergaan. De procedure beschreven in dit hoofdstuk is

gericht op onmiddellijke isolatie van melanocyten. Na isolatie vertoonden de melanocyten ook het uiterlijk en de functionele capaciteiten van melanocyten.

De rem op het immuunsysteem in vitiligo

Een afweerreactie wordt normaliter geremd door aanwezigheid van het PD-L1 eiwit. PD-L1 beschermt cellen tegen de vernietigende werking van een afweerreactie. In een normale situatie is dit nodig om onder andere auto-immuunziekten en afstoting van het ongeboren kind in een zwangerschap te voorkomen. Echter, kankercellen zijn in staat het immuunsysteem te misleiden door ook het PD-L1 eiwit bij zich te dragen. Hierdoor wordt de afweer tegen de kankercellen onderdrukt. PD-L1 is dan ook veel bestudeerd in kankeronderzoek. Echter, er is nog weinig onderzoek gedaan naar de rem door PD-L1 in auto-immuniteit van de huid. **Hoofdstuk 6** geeft een overzicht van de huidige kennis van het PD-L1 eiwit en schetst een hypothese over de mogelijke betrokkenheid van PD-L1 in vitiligo. Daaropvolgend onderzoeken we in **hoofdstuk 7** of, en zo ja, hoe PD-L1 bijdraagt aan de auto-immuunreactie tegen melanocyten. PD-L1 was niet aanwezig op melanocyten in huid tijdens de actieve auto-immuunreactie. Ook na het toevoegen van signaalstoffen die er normaal voor zorgen dat een cel PD-L1 bij zich draagt, bleken melanocyten van vitiligo patiënten hier niet toe in staat. Dit laat zien dan melanocyten van vitiligo patiënten minder goed beschermd zijn tegen de aanval door immuuncellen dan melanocyten van mensen zonder vitiligo.

Resistentie van melanoomcellen tegen immuuntherapie

Zoals eerder benoemd, gebruiken melanoomcellen onder andere het PD-L1 eiwit om een immuunrespons tegen de kankercellen te remmen. Daarnaast kunnen melanoomcellen op andere wijze het immuunsysteem misleiden. Zo kunnen melanoomcellen verschillende uiterlijke gedaanten aannemen, waarvan sommige niet altijd even goed worden herkend door immuuncellen. Om melanoomcellen in detail te kunnen bestuderen, is het essentieel deze cellen in tumormateriaal te kunnen identificeren. In **hoofdstuk 8** presenteren we een protocol om cellen in weefsels te kunnen visualiseren. Bij eerdere handleidingen liep het weefsel veel schade op,

waardoor het visualiseren van cellen lastig bleek. Het protocol beschreven in dit hoofdstuk laat zien dat cellen geïdentificeerd kunnen worden, zonder dat er weefselschade optreedt, wat de kwaliteit ten goede komt. Vervolgens gebruiken we deze handleiding in **hoofdstuk 9** om verschillende uiterlijke gedaanten van melanoomcellen te identificeren. Hiervoor gebruikten we tumormateriaal voor en na behandeling met immuuntherapie, om zo te achterhalen welke melanoomcellen niet door het immuunsysteem worden aangevallen. Al bleek er geen populatie van melanoomcellen volledig resistent tegen immuuntherapie, melanoomcellen die het eiwit AXL bij zich droegen oogden iets ongevoeliger voor de behandeling. Daarnaast leken melanoomcellen die MITF bij zich droegen beter te worden herkend en aangevallen door immuuncellen.

Toekomstperspectief

Onderzoek naar de rol van het immuunsysteem in vitiligo en melanoom is de afgelopen jaren exponentieel toegenomen. Mede hierdoor wordt het ziekteproces en de manier waarop beide ziektebeelden het meest efficiënt kunnen worden behandeld steeds duidelijker. In **hoofdstuk 10** integreren we de bevindingen van onze onderzoeken in de huidige stand van zaken. Samengevat geven de onderzoeken van dit proefschrift aanleiding tot aanvullend onderzoek naar de mogelijkheden van immuuntherapie in de behandeling van vitiligo. Daarnaast tonen we aan dat melanoomcellen verschillende uiterlijke gedaanten aannemen en immuuntherapie voorkeur lijkt te hebben een bepaald uiterlijk aan te vallen. Het beter karakteriseren van die kankercellen die door de huidige therapieën onvolledig worden aangevallen, geeft de mogelijkheid immuuntherapie voor melanoom in de toekomst te verbeteren.

A

Appendices

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List of publications

PhD portfolio

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List of publications

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Willemsen M, Linkutė R, Luiten RM*, Matos TR*. Skin-resident memory T cells as a potential new therapeutic target in vitiligo and melanoma. *Pigment Cell and Melanoma Research* 2019; 32(5): 612-622.
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Willemsen M, Krebbers G, Bekkenk MW, Teunissen MBM*, Luiten RM*. Improvement of Opal multiplex immunofluorescence workflow for human tissue sections. *Journal of Histochemistry and Cytochemistry* 2021; 69(5): 339–346.
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Other publications

Hoekstra E, Das AM, **Willemsen M**, Swets M, Kuppen PJ, van der Woude CJ, Bruno MJ, Shah JP, Ten Hagen TL, Chisholm JD, Kerr WG, Peppelenbosch MP, Fuhler GM. *Oncotarget* 2016; 7(45): 73525-73540.

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Tio D, **Willemsen M**, Krebbers G, Kasiem FR, Hoekzema R, van Doorn R, Bekkenk MW, Luiten RM. Differential expression of cancer testis antigens on lentigo maligna and lentigo maligna melanoma. *American Journal of Dermatopathology* 2020; 42(8): 625-627.

PhD portfolio

Name PhD student:	Marcella Willemsen
PhD period:	September 2016 – February 2021
Name promotor:	Prof. dr. R.M. Luiten
Name co-promotor:	Dr. M.W. Bekkenk, M.D.

1. PhD training	Year	ECTS
Courses		
Laboratory Safety	2015	0.4
The AMC World of Science	2016	0.7
Laboratory Animal Science (art. 9)	2016	3.0
In the footsteps of Antoni van Leeuwenhoek/Basic Microscopy	2017	1.5
Radiation protection	2017	1.7
Electronic Basic Course Legislation and Organization for Clinical Researchers (eBROK)	2018	1.0
Workshops		
How to be(come) a successful grant applicant (Onderzoeksschool Oncologie Amsterdam)	2018	0.05
Conferences and meetings		
Tumour Infiltrating Myeloid Cell Compartment symposium, Leiden, The Netherlands	2016	0.25
Annual Dutch Society for Experimental Dermatology meeting, Lunteren, The Netherlands	2017	0.5
1st and 2nd Cancer Center Amsterdam (CCA) retreat, Noordwijkerhout, the Netherlands	2017, 2018	1.0
Annual Dutch Tumor Immunology meeting, Breukelen, The Netherlands	2017	0.5
3rd CRI-CIMT-EATI-AACR International Cancer Immunotherapy Conference, Mainz, Germany	2017	1.0
Annual Dutch Society for Immunology Lunteren Symposium, Lunteren, The Netherlands	2018	0.5
Presentations		
Annual graduate student retreat OOA, Renesse, The Netherlands *Poster presentation*	2017	1.0
Annual Dutch Tumor Immunology meeting, Breukelen, The Netherlands *Laptop presentation*	2018-2019	1.0

5th European Congress of Immunology, Amsterdam, The Netherlands *Poster presentation*	2018	1.0
21th European Society of Pigment Cell Research, Rennes, France *Flash talk and poster presentation*	2018	1.0
Annual PhD retreat Amsterdam Infection & Immunity Institute (AI&II), Heemskerk, The Netherlands *Oral presentation*	2018	1.0
Annual Dutch Society for Experimental Dermatology meeting, Lunteren, The Netherlands *Poster presentation*	2019	0.5
22nd European Society of Pigment Cell Research, Brussels, Belgium *Poster presentation*	2019	1.0
Annual Dutch Society for Immunology meeting, Noordwijkerhout, The Netherlands *Poster presentation*	2019	0.5
Annual Dutch Society for Experimental Dermatology meeting, Lunteren, The Netherlands *Oral and poster presentation*	2020	1.0
4th Cancer Center Amsterdam (CCA) retreat, Noordwijkerhout, The Netherlands *Oral presentation*	2020	0.5
35th Society for Immunotherapy of Cancer Meeting, virtual event *Poster presentation*	2020	1.0
Annual Dutch Society for Immunology meeting, virtual event *Poster presentation*	2020	0.5
6th European Congress of Immunology, virtual event *Poster presentation*	2021	1.0
European Society of Pigment Cell Research online meeting, virtual event *Oral presentation*	2021	0.5
2021 Vitiligo International Symposium, virtual event *Oral presentation and invited speaker*	2021	1.0
Other		
PhD committee CCA, Cancer Immunology theme	2016-2019	3.0

2. Teaching	**Year**	**ECTS**
Lecturing		
Lecturing Clinical Cell Biology course, Master Biomedical Sciences University of Amsterdam	2018-2020	0.3
Supervising		
R. Linkuté, master thesis, Biomedical Sciences	2017	1.0
F.R. Kasiem, master research internship, Medicine	2017-2018	2.0
A. Louis, master research internship, Biomedical Sciences	2018-2019	2.6

V.A.L. Konijn, bachelor research internship, Biomedical Sciences	2019	1.6

3. Parameters of esteem	**Year**
Grants	
Travel grant, 22nd European Society of Pigment Cell Research, Brussels, Belgium	2019

4. Publications	**Year**
Publications about my research	
Weefselresidente T-geheugencellen in vitiligo en melanoom (Nederlands Tijdschrift voor Dermatologie en Venerologie)	2020
Soms breidt vitiligo uit, soms niet. Waarom? (Spotlight)	2021
Dwarsliggende eiwitten (Spotlight)	2022
Editorial activities	
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Curriculum Vitae

Marcella Willemsen werd geboren op 4 juni 1993 te Sliedrecht. Na het behalen van haar VWO diploma Natuur en Gezondheid aan De Lage Waard te Papendrecht, startte zij in 2011 met de bachelor Gezondheid en Leven aan de Vrije Universiteit te Amsterdam. Tijdens haar bachelor liep zij stage op de afdeling Maag-, Darm- en Leverziekten van het Erasmus Medisch Centrum onder begeleiding van dr. Gwenny Fuhler met de focus op de rol van fosfatasen in colorectale kanker. In 2014 haalde zij haar bachelor diploma (Bachelor of Science, BSc) en in hetzelfde jaar begon zij de master Oncology, wederom aan de Vrije Universiteit te Amsterdam. Tijdens haar master legde zij de focus op immunologie en oncologie met bijbehorende stages en scriptie. Haar eerste stage volgde zij op de afdeling Dermatologie van het Amsterdam Universitair Medische Centra, locatie AMC, in de groep van prof. dr. Rosalie Luiten. Tijdens deze stage bestudeerde zij de betrokkenheid van aangeboren lymfoïde cellen bij immuuntherapie van melanoom. Voor de daaropvolgende stage vertrok zij begin 2016 naar Southampton (Engeland) om onderzoek te doen naar de herkenning van het CD1c eiwit door γδ T cellen en de rol hiervan in kanker. Dit deed zij in de groep van prof. dr. Tim Elliott op de afdeling Klinische en Experimentele Wetenschappen aan de Universiteit van Southampton. In 2016 behaalde zij haar master diploma (Master of Science, MSc) en vrijwel direct daarna begon ze als onderzoeker in opleiding (OIO of PhD student) op de afdeling Dermatologie van het Amsterdam Universitair Medische Centra, locatie AMC, opnieuw onder supervisie van prof. dr. Rosalie Luiten en daarnaast ook prof. dr. Marcel Bekkenk. Gedurende haar promotieonderzoek deed zij onderzoek naar vitiligo en melanoom, wat resulteerde in dit proefschrift. Vanaf mei 2021 is zij werkzaam als postdoc op de afdeling Longziekten van het Erasmus Medisch Centrum bij prof. dr. Joachim Aerts.