Vitiligo and melanoma

The fine balance between autoimmunity and immune escape

Willemsen, M.

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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.

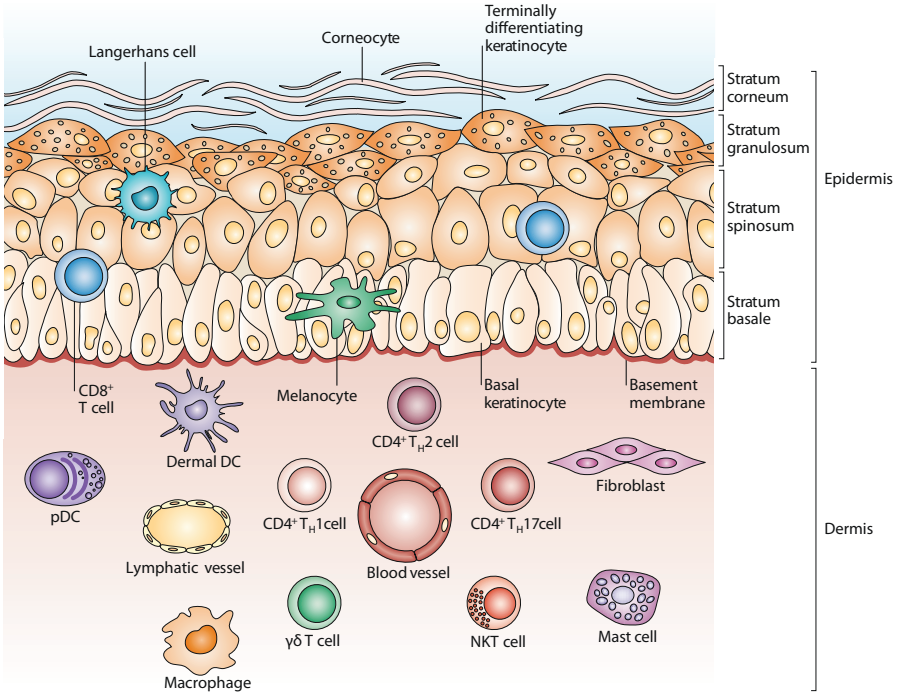

Figure 1. Skin anatomy and skin immune cells. The human skin consist of two layers: the epidermis and the dermis. The epidermis is mainly composed of keratinocytes, but also contains melanocytes, which produce pigment (melanin), Langerhans cells (type of dendritic cells) and T cells. The dermis constitutes 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].

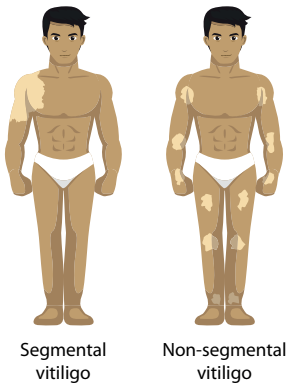

Figure 2. Types of vitiligo. Segmental vitiligo (left) is characterized by a unilateral distribution of the white patches, whereas in non-segmental vitiligo (right) depigmentation often appears symmetrical on both sides of the body.

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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