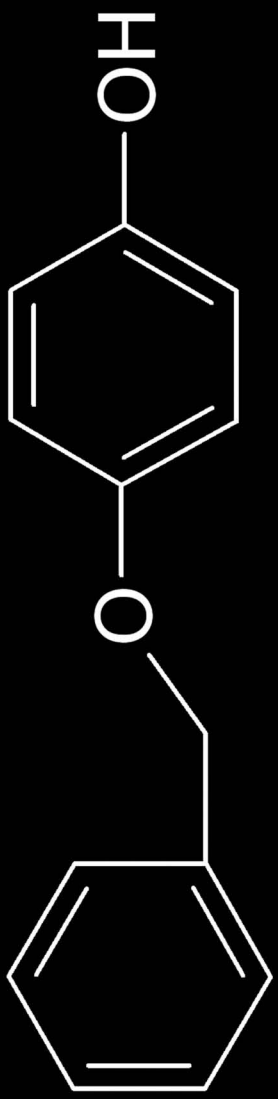
# Vitiligo pathogenesis and immunotherapy of melanoma




Jasper G. van den Boorn

# VITILIGO PATHOGENESIS AND IMMUNOTHERAPY OF MELANOMA

Jasper G. van den Boorn

Voor Pap en Mam

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# CHAPTER 1

GENERAL INTRODUCTION

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## IMMUNOLOGY AS A SCIENCE

As a relatively young field of science, immunology was initiated around 1796 when Edward Jenner established that vaccination with bovine cowpox protected people from subsequent infection with smallpox. Jenner likely became interested in this "preventive infection" by the attempts of the Dutch cattle-trader Geert Reinders to stop the spread of cowpox among local cattle in 1768[1]. Reinders observed that animals surviving cowpox produced offspring that were temporarily protected from cowpox infection (by *maternal inherited immunity*). When he deliberately infected these young animals with virulent cowpox they developed mild symptoms, and were protected from successive infection. Repeated infections mediated long lasting and complete protection (*booster immunizations*), and thereby Reinders successfully carried out the first anti-viral immunizations[2]. In turn, Jenner knew that cattle-workers infected with cowpox experienced only mild symptoms and subsequently seemed protected from human smallpox. This led him to combine *variolation*, the skin inoculation with low-virulence smallpox in a risky attempt to induce protection, with the use of cowpox as the inoculum. Jenner tested this approach on an acquaintance, who became ill with cowpox for several days and was thereafter protected from smallpox infection[3].

After this first successful human immunization, he named his approach *vaccination* after the origin of the cowpox virus (Latin: *vacca*-cow). Later discoveries by Pasteur in the 1870's further propagated the use of vaccination[4]. Since then, immunology has evolved into a broad and productive field of biomedical science, especially since the 1950s when organ transplantation became more common and interest shifted to include serology and basic immunology besides microbiology[5]. The central aim has always remained to discover how the body's own defences can be stimulated to better clear infectious pathogens, kill malignant cells, or suppress self-destructive immunity.

## INNATE AND ADAPTIVE IMMUNE CELLS

By the cowpox inoculation, Jenner successfully breached one of the body's first lines of defence: the physical barrier of the skin. Being the largest organ of the human body it has several essential functions. Its impermeability, mainly mediated by the outermost *stratum corneum*, protects the body from invading pathogens, noxious agents and dehydration. Furthermore, the resident pigment cells (melanocytes) aid in the shielding against harmful ultraviolet-radiation. Importantly, together with the internal mucosa, the skin employs an intricate network of immune cells, the skin immune system (SIS) and thereby mediates the initial response against many invading agents[6].

The first immune cells to react to infection are those of the innate system. They employ a set of cross-species evolutionarily conserved receptors on their surface, called pattern-recognition receptors (PRRs). These PRRs recognize conserved structures widely expressed across families of microorganisms, called pathogen-associated molecular patterns (PAMPs)[7, 8]. PRRs enable rapid recognition of pathogens by the innate immune cells, facilitating their timely clearance. However, innate cells only

display standardized response patterns and they lack the capability to form so-called "immunological memory". This memory is essential for the immune response to evolve upon persistent or repetitive infection with similar pathogen and is the hallmark of the adaptive immune system. The latter responds to very specific parts of the pathogen (antigens), which are not necessarily conserved in evolution. The adaptive immune cells are the CD4+ and CD8+ T cells, and B lymphocytes. Prior to carrying out their full function, adaptive immune cells need precise activation by their innate counterparts.

The T cells from the adaptive immune system are activated by recognizing specific antigens on antigen presenting cells (APC), typically the dendritic cells (DCs). Discovered initially in 1868 by Paul Langerhans[9], Steinman *et al.* in 1973 characterized DCs as stellar cells[10] with distinct immunostimulatory properties[11]. Since then the understanding of DC function has advanced, and many functional subsets in the skin and mucosa have been identified[12, 13]. By their immune stimulatory properties, DCs have become central to an array of immunotherapeutic regimens[14, 15]. Together with DCs, other innate immune cells are also able to act as effective APC, namely macrophages[16, 17] and basophils[18-20]. Important for the work described in this dissertation, DCs are the essential APC initiating CD8+ T cell immunity against skin-derived antigen, as demonstrated by different DC antigen-targeting and *in vivo* depletion studies[21, 22].

Immature DCs reside in the peripheral tissues, preferentially at sites near the exterior. Upon antigen uptake and subsequent cellular activation, by PRR for example, the DC migrates to a nearby lymph node where it acquires a stimulatory phenotype. Here, it expresses T cell co-stimulatory molecules such as CD83 and CD86 together with high levels of surface MHC class-I and –II molecules loaded with processed antigenic peptides. Furthermore, it attracts naïve T cells by producing the chemokine CCL18[23]. Principally, DCs present ingested lysosome-degraded antigenic material to CD4+ T cells in MHC class-II, while proteasomal degradation products are presented to CD8+ T cells in MHC class-I. Antigen for proteasomal breakdown can be acquired biosynthetically, for example by direct infections of DCs, or from ingested extracellular antigen via the so-called cross-presentation process[24]. The latter is an important mechanism in the priming of CD8+ T cells against antigens from virus-infected- or malignant cells. Figure 1 schematically shows the initiation routes of adaptive immunity.

## AUTOIMMUNITY: FRIEND OR FOE?

Upon entering the lymph node, a DC encounters the circulating peripheral T cell pool. Several mechanisms ensure that these T cells are not functionally autoreactive. Central thymic selection mechanisms shape the reactivity pattern of the peripheral T cells in their developmental phase, by deleting those thymocytes that bind their T cell receptor (TCR) to autoantigen-MHC complexes with either a too low- or too high affinity[25]. However, the fact that a single TCR can cross-react with a substantial number of antigen-MHC complexes, and one antigen-MHC complex can be recognized by an array of TCR[25-28], demonstrates that the deletion of autoreactive T cells is no qualitative all-or-none process. Hence, more mechanisms predispose T cell tolerance

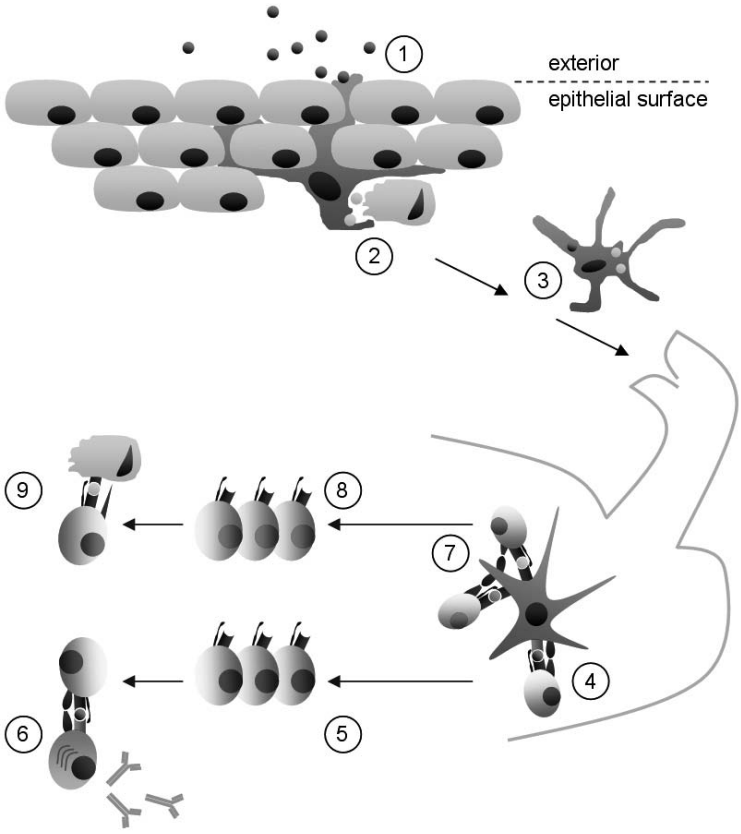


**Figure 1. Initiation of the adaptive immune response.** Antigen presenting cells such as dendritic cells (DCs) can engulf extracellular antigenic matter, such as microbial compounds (1) or debris from stressed- or dying cells (2). Upon activation, the DCs migrate to the local lymph node via afferent lymph vessels (3). In the lymph node these DCs acquire a highly stimulatory phenotype, and process the acquired antigens into peptides to be presented in their surface MHC class-I or -II. DCs can activate CD4+ T cells by presenting extracellular-derived antigen in their surface MHC-class-II molecules (4). The activated CD4+ T cells will subsequently proliferate (5), and are then able to mature local B cells displaying the CD4+ T cell's cognate antigen in their surface MHC class-II. These mature B cells can differentiate into antibody-producing plasma cells, secreting monoclonal antibodies directed against the antigen initially displayed (6). Alternatively, DCs can activate CD8+ T cells by presenting intracellular-derived antigen, or extracellular-derived antigen via cross-presentation, in their surface MHC class-I (7). Following initial proliferation (8), activated CD8+ T cells will enter the circulation via efferent lymph vessels. In the peripheral tissues, these T cells can kill nucleated cells on which they recognize their specific antigenic peptide in the context of surface-expressed MHC class-I (9).

to autoantigens, namely the sensitivity of the T cell for triggering by its antigen (termed avidity). This is in part dependent on TCR affinity and -expression. Selection on the basis of avidity deletes those T cells readily activated by autoantigen-MHC complexes, while it saves those with low- or intermediate avidity[29-31]. Importantly, T cells are centrally selected on the basis of interactions that would not induce peripheral T cell

activation, generating a safe margin against instantaneous activation in the tissues[32]. Autoreactive T cells can still escape central selection, for example by actively lowering their avidity through tuning, which can involve their TCR-, CD4 and CD8 expression levels[33-35] or the phosphorylation status of TCR-signalling cascades[36]. Alternatively, the TCR's antigen cross-reactivity also facilitates the escape of autoreactive T cells to the periphery. Basically, TCR cross-reactivity helps memory T cells to cross-react to slightly mutated epitopes from the same pathogen, overcoming the direct need for a primary response against a dynamic or related pathogen[37]. However, this cross-reactivity can enable T cells which were not thymically deleted as they had low avidity to their major autoantigen, to display high cross-reactive avidity to peripheral autoantigens. Thereby, up to 50% of TCR can possibly cross-react with autoantigen at dangerous levels[38]. Therefore, it is essential to keep peripheral T cells under control, which is accomplished by peripheral tuning[39]. In the periphery, resting APC such as DCs, present autoantigen to circulating T cells at low, tolerizing levels. Moreover, the level of autoantigen presented directly regulates the activation threshold of autoantigen-reactive T cells[40, 41]. Together central selection and peripheral tuning maintain a T cell population which is tolerant to the normal levels of autoantigen presented in the periphery, while still being highly responsive to exogenous agents.

Reactivity of the peripheral T cell pool being under fine control, subsequent disruptions in the levels of autoantigen-presentation can result in autoimmunity. These disruptions may relate to the central thymic level. For example defects in the autoimmune regulatory element (AIRE), a protein optimizing autoantigen presentation in the thymus, can result in massive thymic escape of high avidity autoreactive T cells mediating primary immunodeficiency syndromes such as the Omenn syndrome[42, 43]. Otherwise, distortion may also take place in the periphery and can be related to microbial infection. Pathogens may carry antigens structurally resembling self-antigens. By the sudden infection-mediated elevated presentation of this antigen, combined with PAMPs released by the pathogen, autoimmunity can start via T cell antigen cross-reactivity. This process, known as molecular mimicry, is suspected to be active in atherosclerosis and diabetes[44, 45]. Additionally, tissue damage caused by microbial infection can liberate self-antigens, thereby elevating local presence of autoantigen on APC. The possible presence of infection-related PAMPs provokes activation of APCs and initiation of an autoreactive T cell response. This bystander-effect is assumed to initiate cardiomyopathy and multiple sclerosis[46-48]. When the infection eventually subsides, the T cell response may have optimized via avidity maturation; either via optimization of cellular antigen sensitivity[49-51], or selective expansion of higher-avidity clones[52, 53], this allows autoimmunity to proceed independently.

Autoimmunity can also be caused by non-microbial insults. For example, reactive oxygen species (ROS) and xenobiotics can alter antigen processing and gene expression[54] and may increase the level of autoantigen being processed, via autophagy for instance[55]. The augmented processing and presentation of autoantigen can subsequently trigger autoreactive T cells. Additionally, these insults can mediate the expression of new

splice-variants and antigen isoforms. Since these are structurally different from the native antigen expressed in the thymus, they basically represent exogenous stimuli to the T cell pool, and provoke a primary response. Moreover, ROS and xenobiotics can post-translationally modify cellular proteins and form neo-antigens through oxidation of amino acids[56] or formation of haptens[57]. These types of antigen can trigger cross-reactive T cells or provoke primary responses to the newly formed antigen structure.

Regularly causing morbidity, loss of organ function and even death, autoimmune syndromes illustrate the relentless energy the immune system can invest in destroying the body's own tissue. This energy may also be exploited beneficially since it can be used to attack autologous malignant tissue. To this end, most of the above mentioned initiating mechanisms are in some form employed in cancer immunotherapy regimens.

## THE IMMUNE RESPONSE AGAINST CANCER: REMOVING THE NEEDLE FROM THE HAYSTACK

The potential of cancer immunotherapy was demonstrated in 1891 by William Coley, who injected the tumor of a patient with inoperable cancer with a streptococcal preparation (known as Coley's toxins), and subsequently observed the tumor regress alongside a florid infection[58]. Since then, immunological knowledge has advanced to develop more specific forms of anti-tumor immune-activation. The difficulty with cancer immunotherapy is to restrain the desired autoimmune activation to maintain specificity towards the malignant tissue, without allowing uncontrollable autoimmunity. Nevertheless, tumorigenesis might not go unnoticed by the immune system. In 1957 Burnet proposed the principle of immune surveillance[59], which suggests that frequently the immune system clears microscopic sub-clinical tumors before they grow unimpeded. This is likely for virus-induced tumors[15] since these cancers regularly express viral antigens able to initiate protective immunity, and also for some non-viral tumors displaying native immunogenicity such as melanoma and renal cell cancer. Besides this direct clearance, tumors can also become "dormant", kept in an equilibrium state between growth and attack by the adaptive immune system[60, 61]. This situation may last for decades and seems to be applicable to non-viral chemically-induced tumors. It is supported by the finding that immuno-suppressed individuals show a significantly higher cancer incidence[62, 63]. Nonetheless, the overall incidence of cancer illustrates that the immune system is unable to keep all tumors under control. A tumor may escape adaptive immunity in several ways. To make itself invisible to the adaptive immune system it can downregulate antigen processing pathways[64] or surface MHC expression[65], minimizing the chances for T cell recognition. Furthermore, the tumor can actively recruit myeloid-derived suppressor cells[66] or induce regulatory T cells[67] in order to actively suppress anti-tumor immunity. The efficiency by which a tumor can suppress T cell reactivity is strikingly illustrated by the observation that in vitiligo patients melanocyte antigen-specific T cells progressively destroy melanocytes, while T cells with identical specificities found in melanoma-nodules display a deteriorated functional phenotype[68].

Many strategies have been developed to persuade the immune system to attack tumor cells. There are passive immunization strategies, such as infusing tumor-reactive antibodies[69], or the adoptive transfer of autologous tumor-infiltrating lymphocytes following myeloablative patient pre-conditioning[70, 71]. Although the latter has shown objective responses, it requires elaborate patient-specific *in vitro* cell cultures and puts a high physical burden on the patient. Less strenuous for the patient is the use of DCs as an active immunization method. Loaded with-[72] or expressing[73] tumor-antigens, they are injected either directly into lymph-nodes or intradermally to enhance tumor antigen presentation and activate antigen-specific T cells *in vivo*. Results have been modest, but progress is being made[74]. A multitude of other active immunizations has been developed, which basically aim at mimicking the bystander effect seen in autoimmunity. Therefore, they employ autoantigens in the context of one or several PAMPs, such as Toll-like receptor ligands. For instance, vaccinations can consist of irradiated tumor cells[75], tumor cell-derived vesicles (exosomes)[76], or tumor-specific antigenic peptides[77] sometimes directly linked to a PAMP[78]. Nonetheless, while most vaccination approaches induce well-measurable immune responses, overall these are not associated with significant increases in patient survival[79-81]. Possibly because tumor immune resistance is already high, mere vaccination stimuli could be insufficient.

The death of tumor cells can be immunogenic in itself. Especially apoptosis mediated by chemotherapeutics or irradiation can elevate tumor cell immunogenicity, by inducing surface expression of calreticulin and release of high mobility group box-1 protein (HMGB1)[82]. Since calreticulin promotes tumor cell engulfment by DCs[83], and HMGB1 acts on TLR4 to activate it[84], they together enhance tumor antigen presentation by DCs. Furthermore, irradiation modulates the peptide repertoire and antigen presentation machinery in tumor cells, creating altered- and neo-antigens promoting anti-tumor reactivity[85]. These findings emphasize that tumor cells can be manipulated to co-operate in an active immunization and emphasize the attractiveness of combining immunotherapy with tumoricidal approaches, such as adoptive transfer or vaccination with chemotherapy[86], or combined use of compounds with both innate immune-stimulatory and tumoricidal properties[87]. The outcome of immunotherapy will be better when the tumor itself already carries a certain level of immunogenicity. One of the most immunogenic tumor types is melanoma, which also is one of the most deadly when it reaches the metastatic stage.

## VITILIGO AND MELANOMA

Melanoma is a malignancy derived from the pigment cell, the melanocyte, and its incidence shows a steady increase over the last decades[88]. The immunogenicity of malignant melanoma is illustrated by the spontaneous regression of the primary lesion in about 9% of patients with metastatic disease[89]. Furthermore, tumor-infiltration by melanoma antigen-specific T cells results in an improved prognosis[90]. While in localized melanoma surgical therapy is often curative, metastatic disease is highly resistant to current treatments such as chemotherapy and irradiation[90]. This is caused by the

anti-oxidant properties of melanin, which antagonizes the reactive oxygen species these treatments typically generate in mediating their cytotoxic effect[91]. Consequently, overall survival from metastatic melanoma is poor. For these reasons melanoma patients could benefit greatly from immunotherapy and many such regimens, as stated above, have been developed. Nonetheless, these do not significantly improve patient survival above the level observed with standard regimens[81].

Due to its immunogenicity, autoimmune side-effects are regularly seen during melanoma immunotherapy. These are regarded as a hopeful prognostic sign[92, 93]. Typically, vitiligo-like depigmentation is observed. Vitiligo is a disease clearly different from melanoma as it is defined by the progressive loss of melanocytes from the skin, resulting in depigmented skin maculae. Strong evidence suggests active CD8+ T cell involvement in vitiligo. For example, these T cells have been found clustering near apoptotic melanocytes in vitiligo perilesional skin[94]. Furthermore, melanocyte antigen-specific skin-homing T cells have been found circulating in vitiligo patients[95] and additionally these cells have been isolated from incipient vitiligo lesions in vitiligo patients[96]. Moreover, melanocyte antigen-specific T cells adoptively transferred to melanoma patients as immunotherapy have been recovered from therapy-related depigmented skin lesions[97]. Nevertheless, disagreement still exists concerning the pathogenesis of vitiligo. Theories encompass autoimmune- and genetic mechanisms, and reactive oxygen species mediating melanocyte destruction[98-101]. Importantly, proof of active T cell-mediated autoimmunity in vitiligo would solidify the basis for T cell-based melanoma immunotherapies.

## MONOBENZONE-INDUCED SKIN DEPIGMENTATION

Since vitiligo development during melanoma therapy relates to a better prognosis, active vitiligo induction in melanoma patients could offer a shortcut to successful immunotherapy. Certain chemical compounds are known to induce leukoderma upon skin exposure. In 1939 Oliver *et al.*[102] discovered monobenzone, present in the rubber gloves of leather tannery workers, to induce rapidly progressive skin depigmentation. This depigmentation was indistinguishable from vitiligo since it spread to non-exposed sites, suggesting a systemic reaction. Since then, additional phenol-derivatives have been found to induce local- or progressive forms of leukoderma[103-105]. Even so, monobenzone remains the most potent depigmenting agent[103], and therefore has been used in a cream formulation for completely depigmenting vitiligo universalis patients[106]. Monobenzone has a specific and inactivating interaction with the key enzyme in pigment synthesis, tyrosinase[103, 107], present in the melanosome organelle of the melanocyte. Demonstrating its dependence upon melanocytes is the observation that monobenzone, when applied to vitiligo skin, will selectively provoke a profound skin irritation in pigmented areas of skin[108]. Moreover, monobenzone is known to induce vitiligo in healthy individuals who initially use it to lighten their skin tone[109-111], and acts as a skin sensitizer[111-113]. Although the mechanism behind monobenzone-induced vitiligo has remained unknown, compounds closely resembling it have been shown to activate systemic immunological

responses. Interestingly, the effects actuated by these agents appear dependent on the enzymatic conversion of the native compound into a reactive quinone product, by the enzyme tyrosinase[114, 115]. For example, benzoquinone, the conversion product of the monomethyl ether of hydroquinone, has been demonstrated to act as a potent skin sensitizer[116] by forming haptens to proteins[117, 118]. Furtermore, *p*-benzoquinone, the reaction product of hydroquinone, has been revealed to induce a T cell-dependent, hapten-specific B cell response *in vivo*[119]. Combined with the clinical observations on monobenzone usage, these characteristics suggest that monobenzone, by its selective interaction with melanocytes and its dependence on tyrosinase induces a systemic autoimmune reaction against pigment cells. This makes monobenzone-induced vitiligo very appropriate for exploration as a new immunotherapeutic approach for melanoma.

## AIMS AND SCOPE OF THIS THESIS

To investigate the feasibility of melanoma immunotherapy by the direct induction of vitiligo, this dissertation firstly aimed at defining how CD8+ T cells are involved in the progressive eradication of skin melanocytes, the hallmark of vitiligo. Thereafter, we advanced to characterize how monobenzone induces vitiligo, a mechanism which was obscure. To this end, we sought to unfold the molecular-, cellular- and immunological effects which occur when pigmented cells are exposed to monobenzone. Moreover, we aimed to develop a monobenzone-based immunotherapy regimen for melanoma in the B16-B6 model[120] of transplanted malignant murine melanoma.

The autoreactivity of T cells, which is central to many immunotherapy regimens, has long been clouded by the paradigm that a single T cell can only recognize one single antigen. As this implicates that auto-reactive T cells are deleted, an effective auto-reactive response would be virtually unattainable. By a study of the literature, **chapter 2** shows that instead T cells can recognize a multitude of antigens, and that the balance between self-tolerance and –recognition is a dynamic one, depending to a large extent on T cell avidity and –response tuning.

The first experimental chapter of this thesis examines the active role of CD8+ T cells in the progressive skin depigmentation typical for vitiligo. Thereby, **chapter 3** aimed to directly correlate melanocyte apoptosis in the vitiligo skin to local CD8+ T cell infiltration and the reactivity of these T cells towards melanocyte differentiation-antigens. Since vitiligo-associated T cells are known to display superior reactivity towards melanocyte differentiation antigens, compared to T cells with identical specificities found in melanoma patients, **chapter 4** discusses the possibilities of using these vitiligo patient-derived T cells or their T cell receptors for use in melanoma immunotherapy, by a review of the literature.

In the following experimental chapters we aimed to dissect the immunological repercussions of monobenzone-exposed pigmented cells. In **chapter 5** we first investigated the molecular effects of monobenzone on the tyrosinase enzyme and proceeded to identify the functional immunogenicity of monobenzone-exposed pigmented cells at the level of DC antigen cross-presentation to autologous T cells.

Moreover, this chapter aimed to resolve the immunological mechanisms by which monobenzone provokes CD8+ T cell-mediated autoimmunity to both monobenzone-exposed and –unexposed pigmented cells. **Chapter 6** then advances to address the impact of monobenzone on melanogenesis and cellular stress responses within the exposed pigmented cell itself. In particular, this chapter aimed at studying how monobenzone confers immunogenicity to pigmented cells. Finally, **chapter 7** is devoted to establishing an effective monobenzone-based melanoma immunotherapy regimen in the *in vivo* model of transplantable malignant murine melanoma. We here focused on combining the vitiligo-inducing effect of monobenzone with the immunostimulatory adjuvants imiquimod and CpG (MIC therapy) and characterizing the effectual anti-melanoma immune response.

A general discussion of this dissertation is provided in **chapter 8**, which integrates and discusses its findings in the context of the present literature.

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# CHAPTER 2

## T CELL AVIDITY AND TUNING: THE FLEXIBLE CONNECTION BETWEEN TOLERANCE AND AUTOIMMUNITY

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## ABSTRACT

Thymic T cell selection mechanisms generate a cross-reactive, self-MHC restricted peripheral T cell pool. Affinity and avidity are of profound influence on this selection and the generation of immunity. Auto-reactive T cells can escape thymic deletion by lowering their avidity, and retain this 'tuned' state in the periphery. Upon activation, tuned T cells can cause autoimmunity, while immunotherapeutic strategies may be hampered by existing T cell tolerance. The regulation of T cell avidity and tuning therefore determines the balance between tolerance and autoimmunity and should be taken into account in the design of therapeutic strategies aimed at T cell reactivity.

## INTRODUCTION

T cells are of profound importance to adaptive cellular immunity. These cells express a surface receptor of a given specificity, and circulate between peripheral lymphoid tissues and the bloodstream, until they encounter their specific antigen presented by an antigen-presenting cell (APC) in the context of a major histocompatibility complex (MHC). Upon antigen recognition and proper co-stimulation, naïve T cells are activated and differentiate into effector T cells mediating the removal of pathogen-infected cells from the body.

Generating a T cell mediated immune response involves events surrounding and following the recognition of antigen by the T cell receptor (TCR). Besides TCR affinity, T cell avidity (or antigen-triggering sensitivity) is essential in T cell activation. Multiple aspects and mechanisms determine T cell affinity and avidity and mediate the balance between T cell tolerance and the generation of immunity, and are therefore instrumental in the understanding of autoimmunity.

## T CELL RECEPTOR AFFINITY

An essential process in the adaptive immune response is the intimate contact between the T cell and APC. The TCR is of fundamental importance to this process. It interacts with the MHC expressed on the APC for example a dendritic cell (DC), -B cell or –macrophage, which have potent co-stimulatory and antigen-presenting properties when activated [1]. The TCR is able to recognize specific peptide antigen fragments presented to the T cell in the peptide-binding cleft of the MHC. An APC can present many different antigen fragments derived from numerous peptide sources. Basically, intracellular derived antigens are presented in MHC class-I to CD8 T cells and extracellular derived antigens in MHC class-II to CD4 T cells. TCRs are restricted to recognizing antigen as presented by one specific self-MHC molecule, termed MHC-restriction.

The TCR-MHC interaction is relatively weak, and several measures increase TCR affinity (or binding-energy) for the MHC-peptide complex. As to date, it is unclear if the peptide or the MHC molecule makes the major contribution to this affinity. The peptide contributes a smaller portion of the interacting surface than does the MHC molecule. Additionally it is known that the TCR complementarity determining regions (CDRs) undergo minor conformational changes upon MHC ligation to adapt to its surface. It could well be that TCR-MHC contacts play a permissive role, while TCR-peptide contacts provide the majority of the binding energy [2;3].

Antigen presentation via MHC class-I is not as strict as stated above. An alternative antigen presentation pathway exists in which APCs present extracellular derived antigen embedded in MHC class-I molecules to CD8 cells. This process is called cross-presentation [4], and is crucial in generating CD8 T cell mediated immunity against certain viral- or tumor antigens, and is important in the maintenance of self-tolerance [5]. Recent data shows that this cross-presentation takes place via direct transfer of proteasome substrates from the cell of origin, instead of antigen fragments being

internalized by the APC [6]. It has also been reported that specific virus- and tumor antigens may not be detected by CD8 T cells because of impaired cross-priming, an important finding for optimizing immunotherapy and vaccine design [7].

If thymic selection (further discussed below) aims at deleting those T cells that show too high affinity for auto-antigens, the auto-antigen specific T cell repertoire would be small and of limited reactivity, creating a functionally self-tolerant state. It appears that more factors than TCR affinity influence the T cell's sensitivity to antigenic triggering, having consequences for thymic selection and the generation of immunity.

## T CELL AVIDITY

The availability of the TCR, co-receptors and peptide-MHC complexes to interact with each other, determine T cell structural avidity as outlined in figure 1. Additionally, the sensitivity of a T cell to triggering by its antigen is termed 'functional avidity' or 'avidity', and is defined as the concentration of peptide able to induce 50% activation of the antigen-specific T cell pool. Basically this is a measure of passing activation thresholds and subsequent induction of signaling and biological function upon establishing a stable interaction with the APC [8-12].

Avidity relies on the availability of, and affinity by which the antigen is bound to the MHC molecule, combined with the affinity of the TCR for the MHC-peptide complex. An increase in one, or both, of these factors will increase the avidity of the interaction and thereby the T cell's sensitivity for activation. As a consequence, TCR affinity for the peptide-MHC complex, and the peptide to MHC affinity are compensatory. This means that the expression of a high-affinity TCR allows T cell activation by poorly MHC-binding peptides, and vice versa [13]. This phenomenon is of essential importance when designing adoptive T cell transfer therapies against a target antigen. The affinity by which the antigen is bound to the MHC complex directs the choice of T cell avidity, as a low-affinity bound antigen requires a high avidity T cell for a successful response. 'Mismatching' a T cell with antigen in such a case would result in improper stimulation and T cell unresponsiveness.

In addition to the TCR-MHC complex interaction additional factors influence avidity. For example, co-receptor ligation (CD4 or CD8) influences the duration of contact between T cell and APC. The CD8 co-receptor plays a distinct role in this process. T cells expressing high levels of CD8αβ heterodimeric co-receptors are of higher avidity than cells predominantly expressing CD8αα homodimers. This is likely caused by the αβ-dimer associating with the TCR more easily than does the αα-dimer [14]. Therefore presence, dimeric type and mobility (availability to co-localize) of co-receptors has direct effects on T cell avidity.

Besides co-receptors, different types of co-stimulatory and adhesion molecules also influence the T cell – APC interaction. The interaction half-lives of the molecular contacts themselves is in the order of seconds, whereas and T cell activation requires interaction in the order of hours. Therefore the formation of a stable interaction is required [8;10;15].

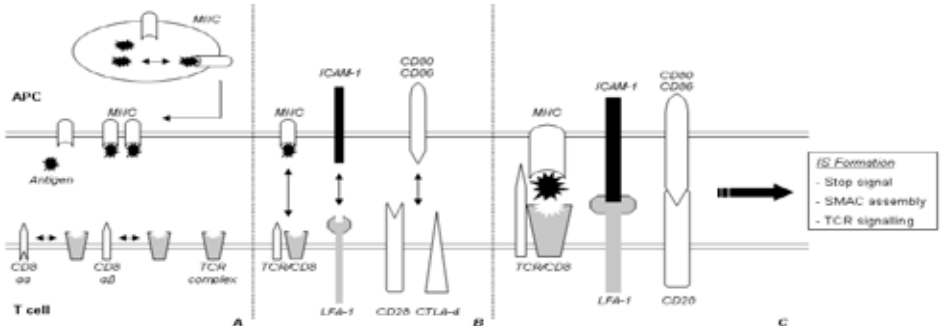


**Figure 1: Several factors influence structural T cell avidity.** ***A.*** Expression levels of TCR and MHC molecules, affinity of the peptide for the MHC molecule, and the affinity of the TCR for the peptide-MHC complex are basic factors of structural T cell avidity. Furthermore, different co-receptor subtypes (such as CD8-αα or -αβ) display different affinities for the TCR complex, additionally influencing antigen recognition efficiency. ***B.*** Besides availability of the MHC and TCR complexes, the expression levels of different co-stimulatory and adhesion molecules on the surface of the APC and T cell, and their ability to interact, are important determinants for proper activation and prolonged cell-cell contact. ***C.*** Recognition of the peptide-MHC complex by the TCR, combined with co-stimulatory signalling and adhesion-molecule interaction, will induce a transient stop signal for the migrating T cell. This allows for prolonged TCR signalling and the assembly of an immunological synapse (IS), and eventually the formation of a supramolecular activation cluster (SMAC). Subsequent biological function depends on the T cell's functional avidity, determined by the regulation of activation thresholds and induction of different signalling cascades, as will be discussed below.

This is provided by the formation of an immunological synapse (IS), which can materialize in several different manners depending on the circumstances of contact, extensively reviewed by Friedl *et al* [10] and Jacobelli *et al* [16].

The relevance of the IS to T cell avidity becomes clearly evident when taking into account the different signaling cascades emanating from IS-mediated interaction, as depicted in figure 2. In the initial phases of formation, the TCR interacts with the peptide-MHC complex. Together with interactions between lymphocyte associated antigen-1 (LFA-1 on the T cell) and intercellular adhesion molecule-1 (ICAM-1 on the APC) this leads to a transient stop signal to the migrating lymphocyte [17]. Immediately after this stop signal, IS assembly and initial signaling events will start. Continuous TCR signaling upon antigen recognition is essential for the maintenance and further maturation of the IS [11].

Upon initial TCR engagement the tyrosine residues in the immunoreceptor tyrosine-based activation motifs (ITAMs) of the TCR-associated ξ-chain dimer and CD3 complex (γ, δ, and ε subunits) become phosphorylated, and subject to binding by different molecules. This phosphorylation is mediated by two protein tyrosine kinases (PTKs), namely Lck (released from Csk inhibition by CD4 or CD8 co-ligation with the TCR) and to a lesser degree Fyn. The most important event following ITAM phosphorylation is the binding of ZAP-70 to two phosphorylated ITAMs. ZAP-70, being a PTK on its own subsequently gets phosphorylated as well by Lck, and is able to bind additional molecules [18;19]. High levels of co-receptor ligation will mediate

high levels of Csk release, resulting in a more rapid T cell activation. Co-receptors thereby have a dual role concerning T cell avidity: they prolong the contact between TCR and MHC complex, and aid ZAP-70 phosphorylation. T cell tuning (described below) appears to involve the phosphorylation levels of the TCR-ξ chain and ZAP-70 on their inhibitory residues, thereby directly influencing activation thresholds.



Importantly, phosphorylation events are equally dependent upon the enzymes that subsequently remove the phosphates from the residues, such as protein tyrosine phosphatases (PTPases). There are more PTPases controlling T cell activation than there are PTKs, nonetheless research has mainly focused on the PTKs. In normal cells tyrosine phosphorylation is rapidly reversible, ensuring tight signal regulation [18]. It follows that subtle changes in PTK / PTPase balance can have major impacts on phosphorylation levels, and hence T cell avidity, activation and proliferation.

Illustrating the importance of CD28 co-stimulation, is the molecule Ltk. CD28 ligation will activate Ltk, thereby phosphorylating phospholipase-Cγ1 (PLC-γ). This molecule interacts with the linker for activation of T cells (LAT), and PLC-γ is essential in propagating and amplifying the TCR signal, via cleavage of membrane phospholipid phosphatidylinositol biphosphate ($PIP_2$) into its components inositol triphosphate ($IP_3$) and diacylglycerol (DAG). Via complex signaling cascades involving protein kinase C (PKC) and Calcineurin, this leads to the activation and differentiation of the T cell [19]. Consequently, T cells expressing high levels of CD28 will exhibit a high avidity.

LAT influences cytoskeletal rearrangements, essential in the formation and continuation of the IS as it stabilizes the cellular interaction and subsequent signaling events. Involving the protein SLP-76, LAT influences downstream actin reorganization via the Rho-A signalling cascade, allowing the T cell to rearrange and 'spread out' its cytoskeleton to the site of contact with the APC [20;21]. Being downstream of TCR and CD28 ligation LAT expression is essential in T cell activation and avidity. LAT deficiency is known to cause impaired cellular interaction and disorganized IS formation [20].

When TCR signaling is sustained, these initial signaling events will lead up to the formation of a mature IS, characterized by the formation of a supramolecular activation cluster (SMAC) between the T cell and APC, setting the stage for full effector potential [10;15]. Additionally, the T cell can express other co-stimulatory molecules such as tumor necrosis factor receptor family members (TNFRs) like CD40, CD30, CD27 and 4-1BB having further influence on the outcome of interaction, reviewed by Croft [22].

All signaling cascades emerging from interactions at the cell surface ultimately lead to the activation or inhibition of transcription factors, which play essential roles in T cell activation and differentiation. During T cell selection and the generation of immunity, these cascades will direct a T cell to its fate. Variations on the level of signal regulation, and the expression level of signalling surface receptors, are indispensably linked to the regulation of T cell avidity. Defects or alterations can cause abnormal T cell activation. For instance it is known that polymorphisms in the CTLA-4 molecule are associated with vitiligo in humans [23] and thyroid autoimmunity and type-1

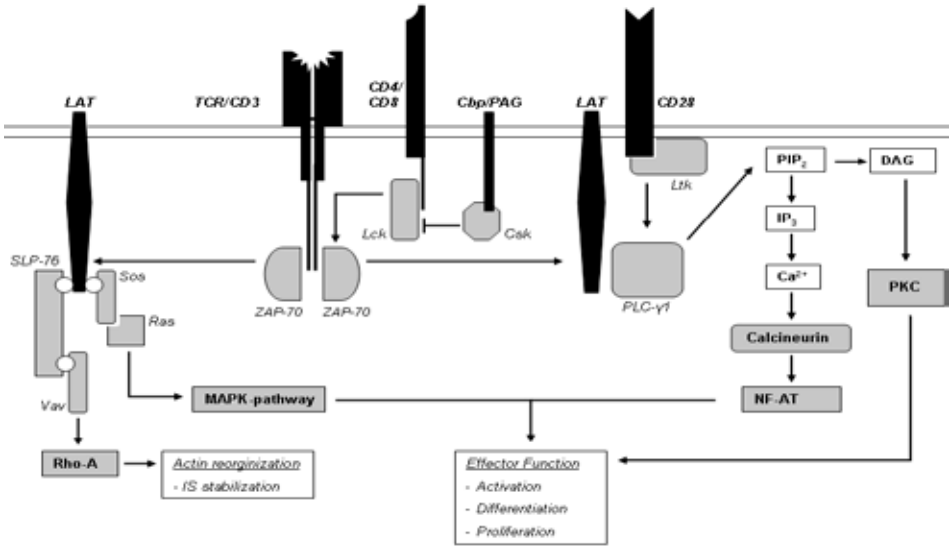


**Figure 2: Schematic representation of the signaling pathways involved in T cell activation.** Antigen recognition by the TCR and co-receptor (CD4 or CD8) ligation releases Lck from Csk inhibition, leading to phosphorylation of the TCR ξ-chains and recruitment of ZAP-70. ZAP-70 phosphorylates LAT, which ligates PLCγ-1 and thereby amplifies and sustains the activation signal. PLCγ-1 can activate different signaling cascades via cleavage of PIP2, such as the PKC and calcineurin pathways leading to cellular activation and differentiation. The function of PLCγ-1 is further enhanced by CD28 ligation, via interaction with Ltk. Additionally, LAT can interact with several linker molecules, leading to activation of the MAPK-pathway via activation of Ras, or the Rho A pathway leading to actin reorganization.

diabetes in mice [24]. Expression of HLA-DR4 is also associated with vitiligo, which may suboptimally present certain autoantigens during thymic selection facilitating escape of autoreactive T cells [25;26]. Additionally, several mutations in transcription factors are known to cause autoimmunity, for example the IL-2 promoter binding factor T-bet in Crohn's disease [27], and the SLC22A4 / RUNX1 polymorphism proposed in rheumatoid arthritis [28]. Other defects appear to have a more widespread effect, for instance polymorphisms in the FoxP3-gene induce autoimmune disease such as the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). Defective FoxP3 inevitably results in defective regulatory T cell (Treg) development, allowing unrestrained generation of immune responses [29;30]. Additionally, there are defects which are able to influence the avidity to autoantigens of the entire T cell pool. Defects in the autoimmune regulator (AIRE) can influence thymic selection and reactivity of the entire T cell pool, and like FoxP3 cause widespread autoimmunity.

## T CELL SELECTION

The generation of the peripheral T cell repertoire is dependent upon thymic selection, and selection thereby predisposes the diversity and availability of the T cell repertoire. Insight into central selection is important in understanding the basis of T-cell mediated autoimmunity. For a major part, T cell affinity and functional avidity determine a cell's fate during selection.



There are two main models describing T cell selection: the qualitative- or peptide model, and the quantitative- or avidity-based model. The present focus is on the role of peptides during selection, and preferentially the avidity-based model is used to explain the selection of T cells. The main issue in thymic selection is the nature of the selecting ligand; do qualitatively distinct ligands promote positive- or negative selection, or do specific TCR interactions promote intracellular signals to meet quantitative signaling thresholds for T cell survival [13;31]?

Immature T cells (thymocytes) express a broad range of TCR specificities and affinities, and the key in successful selection is to lose those T cells that recognize self-peptide-self-MHC complexes with either a too low- or too high affinity. This produces mature T cells recognizing self-MHC with moderate affinity, while not being reactive to self-peptides [13]. Importantly, the interactions leading to negative selection are much weaker than those required to induce T cell activation. In this way negatively selected T cells are deleted as a result of interactions that would not lead to activation in the periphery. Possibly this provides an important safety margin, allowing some fluctuations in MHC and self-peptide expression in the periphery [32].

The qualitative- or peptide model predicts that qualitatively different peptides presented in the thymus mediate selection. Thereby, the thymocyte is positively selected for when its TCR interacts with low- or intermediate affinity to the presented self-peptide (termed antagonist peptide). The cell is deleted through activation induced cell death, when it interacts with high affinity to this peptide-MHC complex (termed agonist peptide). Experiments using peptides having amino acid substitutions at residues known to contact the TCR when presented by an MHC molecule, known as altered peptide ligands (APL), have shown that any given peptide and its variants can form a gradient from antagonist to agonist. Furthermore, for any given TCR a gradient of ligands can be described in terms of agonist qualities [33]. Thus, peptides do not fall within absolute categories, and thymocyte selection probably requires more than these all-or-none interactions [31].

In contrast to the qualitative model, the avidity-based model proposes that low-avidity thymocyte interactions with self-peptide promotes positive selection, and if this recognition passes an avidity threshold the cell will undergo apoptosis [13]. A large spectrum of functional avidities in the thymocyte population would interfere with thymocyte fate. However, avidity-influencing factors are more or less constant suggesting that the key factors in thymocyte selection are TCR affinity, and thymocyte avidity. In contrast to the qualitative model, in which absolute TCR / MHC complex interactions govern the fate of a thymocyte, the avidity-based model integrates multiple

TCR / MHC complex interactions to form a gradient defining cell fate. For example, numerous weak TCR binding events or limited high-affinity interactions provide sufficient avidity to induce thymocyte survival. Additionally, high-affinity interactions will provide high avidity and cause deletion. This model allows for a concentration dependent overlap of positively and negatively selecting signals, as thymocyte signaling is an integrated process [31]. Convincing evidence for avidity-based selection comes from studies showing that low expression of a strong agonist peptide in the thymus mediates positive selection, while high expression of the same peptide causes clonal deletion of reactive thymocytes [34;35]. Recent data has again confirmed these results in favor of the avidity-based model, and that emerging cells are possibly auto-reactive [36].

Although the exact mechanisms of central T cell selection remain elusive, it has become clear that thymic presentation of peripheral antigens is essential in generating a self-tolerant T cell pool. For instance in mice, when positively selected thymocytes encounter medullary thymic epithelial cells (MTECs) functionally deficient of MHC molecules or B7 co-stimulatory receptors, all thymocytes will reach the periphery without negative selection [37;38]. These pathogenic T cells will subsequently cause extensive autoimmunity. Similar effects take place when there is a dysfunction of the AIRE protein. AIRE appears to optimize the presentation of peripheral antigens in the thymus. Deficiency of AIRE causes autoimmune polyendocrinopathy (APECED) characterized by defective negative selection of effector T cells [39]. AIRE deficiency does not inevitably mean deficiency of auto-antigen presentation by the thymus. In some cases the antigen is still presented while autoimmunity initiates. This suggests AIRE also influences thymic selection beyond the antigen expression level, likely by influencing chemokine expression or antigen processing and -presentation by MTECs [40]. Absence of AIRE lowers the MTEC's stimulatory state, resulting in weak avidity interactions for negative selection, allowing high avidity self-reactive cells to escape to the periphery. Likewise is the case for the myelin basic protein Ac1-9 antigen in EAE [41]. This auto-antigen on itself has extremely low affinity for the MHC molecule. Alternatively, an auto-antigen being absent from the thymus also causes escape from deletion, for example due to alternative splicing variants having differential expression of isoforms throughout the body, as is the case with the myelin proteolipid protein-antigen in EAE [42].

As the T cell repertoire is shaped by the numerous interactions that take place during thymocyte development, the thymus can establish susceptibility to- or protection from autoimmune disease. Inevitably, some T cells do escape deletion and these will encounter additional measures such as peripheral tuning in absence of danger signals. Even 'successfully selected' T cells may react to auto-antigens as antigen recognition is not all-or-none. Greatly enlarging the specificity of the T cell repertoire, and hence the auto-reactive T cell pool, the cross-reactivity of the TCR gives additional flexibility to T cell antigen recognition.

## TCR CROSSREACTIVITY

In the past decade new light has been shed on the specificity of T cell immunity. Although the TCR remains of given specificity, and is unable to undergo affinity maturation, it is apparent that there is considerable flexibility in TCR antigen recognition. Mathematical models have shown that the T cell pool is not large enough to give rise to immunity against all possible foreign antigens on the basis of the classical one-TCR-one-epitope model. It has been estimated that for sufficient protection, each T cell should be able to react to as much as $10^6$ structurally similar peptides. This is probably an over-estimate, nonetheless it illustrates that most (if not all) TCRs will react to more than one ligand including numerous self-antigens [43;44]. Not only are single T cell clones able to react to an array of different peptide-MHC complexes, more T cell clones are also able to react to one single peptide-MHC complex, indicating substantial cross-reactivity within the T cell pool [13;45]. Highly variable junctional residues add diversity to the flexible CDRs, able to adapt to the peptide-MHC surface. These processes highly contribute to TCR crossreactivity and diversity in the T cell pool [2].

Multiple studies have defined TCR crossreactivity with the use of APLs [46-48]. Currently an APL describes any peptide that has one or more residues modified from the wild-type peptide of interest [13]. APL studies have shed new light on TCR antigen recognition. Early studies of molecular mimicry involved a foreign peptide provoking an immune response crossreactive with a self-antigen, and this peptide often showed obvious sequence homology to a self-peptide [49]. Later studies indicated that only a few amino acid residues of a given peptide were required in TCR / peptide-MHC complex interaction. This insight practically supported the view that different antigens could stimulate the same T cell, as long as they showed similarity on these critical residues [50;51]. This cross-reactivity based upon minimal residue similarity furthermore underscores the importance of the CD4 and CD8 co-receptors in providing prolonged T cell / APC contact [45].

Similar studies have also demonstrated that different T cell subsets exhibit different levels of TCR cross-reactivity. TCRs of CD4 T cells show higher crossreactivity than do TCRs of CD8 T cells. This could be explained by the generally higher affinity of the CD8 TCR for the peptide-MHC complex compared to the CD4 TCR, possibly associated with the monomer-dimer state of the CD8 co-receptor resulting in a more stringent T cell activation threshold [52]. Additionally, the more constrained molecular interaction of peptide with MHC class-I molecules in comparison to class-II MHC molecules could be another cause of a less cross-reactive CD8 T cell pool.

Because T cells are able to respond to antigen over an antigen-concentration range, two effects should be considered concerning T cell affinity and avidity: First, T cells have quantitatively different responses to related antigens, meaning that T cells will react to low ligand density if their avidity is high, but only to a high ligand density if their avidity for that antigen is low. In addition, the TCR repertoire responding to a single antigen is variable. Those cells expressing high affinity TCRs will respond to low ligand densities while low affinity TCRs will react to high antigen concentrations

[13]. Taken together, this means that a single T cell is able to respond to different related antigens to which it has various affinities on the basis of TCR cross-reactivity. Functional avidity for those individual antigens will determine the response threshold to that antigen.

Next to optimizing T cell responses, crossreactivity can also prevent pathogen escape by mutation. Upon secondary antigenic challenge, T cells respond to antigens shared with the original pathogen (due to memory cell expansion). In repeated infection immunity is thereby optimized for specificity and speed against non-mutating pathogen antigens. This phenomenon is referred to as 'original antigenic sin'. However, regularly mutating pathogens such as influenza pose a problem. Repeated mutation can put the pathogen outside the view of T cell memory, now requiring a primary response for clearance. This is a disadvantage for the host, and TCR crossreactivity can help the memory cells to cross-react with related, mutated epitopes of the same pathogen. Or even between epitopes of related pathogens [53]. Less favorable aspect of cross-reactivity is so-called molecular-mimicry: T cells initially directed to a pathogen could develop a destructive response to cross-reactive auto-antigens [54]. However, this theory is under strong debate as convincing evidence lacks [55]. With rapidly mutating pathogens, cross-reactivity offers no solution. HIV for instance mutates so rapidly that a T cell response never is completely specific for the virus. As numerous viral subtypes exist in one patient, antigenic sin forces the induction of primary responses. Unable to keep pace with the virus, the immune system eventually fails through clonal exhaustion [9].

The crossreactivity of TCRs illustrates that T cells exhibit flexibility in antigen recognition, and one T cell can react to many ligands (or vice versa) including self-antigens. It has been estimated that up to 50% of TCRs are able to bind auto-antigen with dangerous efficiency [26]. Add to this the existence of dual $\alpha\beta TCR^+$ T cells displaying autoreactivity and escape from deletion [56-58], the question rises how this situation is controlled in so many of us. Recent research has shown that $CD4^+CD25^+$ Treg limit the risk of autoimmunity caused by cross-reactive TCRs [59]. As Treg cells impose their regulatory control at antigen levels lower than those required to activate effector cells, it is believed that Treg exert dominant control on auto-reactive cells with lower sensitivity while still allowing high avidity reactions to develop. In this setting it is known that high antigen levels and strong co-stimulation (as present in infection) are factors that can overrule Treg suppression, which possibly focuses on inhibiting CD4 helper cells [60-62]. Additionally, it has been shown that Treg home to the target organ of the auto-immune response, which coincides with recovery from autoimmunity [63].

Crossreactivity illustrates that besides individual deletion of autoreactive T cells in the thymus more mechanisms probably govern tolerance. It appears that upon activation T cells can undergo extensive functional maturation and -modification. These phenomena are of special importance, as it poses problems and solutions for autoimmunity.

## AVIDITY MATURATION AND SELECTIVE EXPANSION

During secondary antigen encounter approximately two- to fourfold increases in TCR affinity occur compared to initial antigen encounter. This probably happens via selective expansion of memory cells expressing the highest affinity TCR [64]. In contrast to B cell mediated antibody production, which shows considerable affinity maturation, there may be a functional 'ceiling' to T cell affinity. T cells expressing high-affinity TCRs may become ineffective if serial TCR-triggering and MHC sampling is impaired by extended TCR binding, or become unresponsive when activated without co-stimulation. If affinity maturation were the only way of fine-tuning an immune response, the T cell response would become limited by its own restraints. Therefore T cells have evolved avidity maturation as optimization method.

For example, CD8 T cell activation caused by acute viral infection can induce a 50- to 70-fold increase in the T cell's functional avidity without selection of a higher affinity TCR *in vivo* [65]. Maximal T cell avidity in this case, was reached 8 days after initial infection and was passed to memory T cells for the life of the host. These results are supported by *in vitro* data using T cell clones [66], and shows that T cells undergo profound functional maturation upon activation.

It is known that stimulation of low-avidity T cells requires co-ligation of CD8, however this is often dispensable in high-avidity cells [67;68]. T cells are very susceptible to CD8 co-ligation at the initiation of an antiviral response, while 8 days later following avidity maturation, this ligation is expendable [65]. Furthermore, no changes in TCR affinity or expression of adhesion- or co-stimulatory molecules were found, which could explain the enhanced antigen responsiveness. Instead, they identified a marked increase in Lck expression. This kinase is associated with the cytoplasmic domain of the CD4 and CD8 co-receptors, mediating ITAM phosphorylation on accessory chains of the TCR complex. Additionally, changes in the TCR-surrounding plasma membrane were found to aid increased avidity after T cell activation [69]. It appears that T cells will gradually reorganize and cholesterol-enrich their membrane lipid rafts upon activation, in which the TCR and many co-stimulatory molecules are embedded. This reorganization enhances TCR cross-linking and the formation of the SMAC, allowing T cells to bind multimeric MHC complexes with improved efficiency, enhancing intracellular signaling. This allows more efficient recognition of low-density peptide-MHC complexes.

Besides the avidity maturation observed on the single cell level, the T cell response as a whole can display progressive maturation. Refinement of the TCR repertoire takes place via selection of optimal avidity clones [64;70;71]. At the initiation of a primary response high avidity T cells, expressing high affinity TCRs, will dominate over low-affinity cells. During the response intermediate- and high affinity T cells have a proliferative advantage and the population will express an increasingly optimal TCR range of 'narrowing' affinity, thereby exhibiting avidity maturation. The loss of low-affinity T cells may be obvious in this process, nonetheless the high-affinity T cells expanding at the initiation of the response can become deleted when the response

progresses. Leaving room for intermediate-affinity cells to dominate, this is mediated by the increasing antigen dose being present during the course of infection [72]. This mechanism ensures the selective deletion of high avidity T cells from the reactive pool and guarantees a consistent optimization of the activation threshold. Constant selection mediates avidity maturation and maintains control over T cell reactivity. However, in some cases avidity maturation can cause the progression from local inflammation to autoimmune disease. This shows important in the progressive pathology of autoimmune diabetes. Local inflammation will cause an accumulation of T cells while avidity maturation subsequently drives the inflammation progressively to diabetes. As illustrated in non-obese diabetic (NOD) mice, timely treatment of pre-diabetic mice with relevant antigenic peptide will cause deletion of high avidity clones, slowing down the avidity maturation and inhibiting the progression from insulitis to diabetes [73;74].

This pathogenesis is probably important in the development of many autoimmunities originating from (chronic) inflammation, and can explain the progressive severity of many autoimmunities. Evidently, this phenomenon differs from molecular mimicry as the causative inflammation is not caused by a pathogen mediating auto-immunity via cross-reactivity. Instead, inflammation is likely caused by low-avidity auto-reactive T cells accumulating on site, gradually increasing in efficiency via avidity maturation. Certainly, the inflammation could very well be initiated by a pathogen or trauma. Causing a temporary rise in local auto-antigen-presentation via tissue-damage, facilitating the activation of the low-avidity T cells. Subsequently, as avidity maturation progresses the reactive T cell pool can react to the physiological auto-antigen levels present when the trauma has subsided, and the inflammation can spread.

## T CELL TUNING

Based on the avidity-based model of thymocyte selection, autoreactive T cells entering the periphery are of low avidity, and hence insensitive to the auto-antigen levels presented here. These T cells may still be reactive to foreign antigen to which they may exhibit sufficient cross-reactive avidity when co-stimulatory conditions are optimal. During thymic development T cells are able to desensitize (tune) their avidity, altering their survival and future reaction to antigens via modifying avidity-regulating mechanisms.

Although the phenomenon remains elusive, a classic example involves CD5. T cell-expressed CD5, can negatively influence TCR signaling and promote thymocyte positive selection [31;75]. Additionally, thymocytes are able to up- or down-regulate their CD4 or CD8 expression (and thus the recruitment of the Lck signaling molecule to the CD3 complex) thereby directly influencing their maturation by modulating their avidity [76;77]. Furthermore, tuning can also involve the phosphorylation state of the regulating signal cascades. Recently it has been shown that ZAP-70 gain-of-function transgenic mice, exhibiting enhanced basal phosphorylation of ZAP-70 and LAT, display reduced TCR and CD5 levels together with decreased cytokine production and activation. This hyporesponsive state could be explained by the reduced TCR and CD5

levels. Nonetheless it appeared that TCR-induced activation signals were also more rapidly down-regulated, indicating a role for increased dephosphorylation by PTPases. Furthermore this tuned state could be inverted by inhibiting TCR interactions with auto-antigen for several days. This indicates that peripheral tuning requires constant interaction of the TCR with the auto-antigen, and that constant exposure will tolerize T cells [78].

Additionally, studies using altered peptide ligands have shown an added phenomenon of tuning. T cells matured in the presence of endogenous peptide are able to respond to a weak-agonist APL of that antigen, as well as to the endogenous peptide itself. However, T cells matured in the presence of the APL lost the ability to respond to it, but retained their ability to react to the endogenous peptide. This is in line with reports that APLs which fail to induce T cell activation in the periphery can nevertheless be used as ligands in thymic positive selection. This tuning process by non-deleting self-antigens induces mature T cells that have high avidity for foreign antigens while having lost self-reactivity that was present in early stages of development [13;79].

Besides tuning in the thymus, mature T cells can also undergo tuning in the periphery, as proposed by the tunable activation-threshold model by Grossman and Paul [80]. Autoantigen presented by resting APCs in the periphery appears to be essential in maintaining T cell self-tolerance. Low-level presentation of autoantigen directs to a state of tolerance to those antigens. It has been shown that CD4 TCR transgenic T cells adoptively transferred into a lymphopaenic host expressing their specific antigen in the periphery, will undergo an initial limited expansion to 'populate' the host, where after these cells became increasingly insensitive to antigenic stimulation. Within 38 days the T cells required 30-fold greater amounts of antigen to become activated, with 90% reduction in cytokine production and a clear desensitization of proliferation while TCR levels remained normal. Surprisingly, transfer of these hyporesponsive cells into a second lymphopaenic host (not bearing the antigen), induced a gradual re-gain of T cell responsiveness and cytokine production with gradual expansion after 7 days. If this second host did express the antigen, the hyporesponsive cells underwent a slow repopulation expansion as seen in the original host, here after entering an even more profound hyporesponsive state [81;82]. These results form strong evidence that the auto-antigen tolerant state of T cells in the periphery is dependent upon constant auto-antigen presentation intensity. Disrupting the steady-state of auto-antigen presentation intensity will disturb the tuned equilibrium and set off a T cell response. It remains to be confirmed if the same mechanisms of tuning apply to T cells being activated by their ligand under immunizing conditions, subsequently undergoing avidity-maturation.

These means of tuning the immune response prove important in maintaining peripheral tolerance, and make it more difficult to induce an autoimmune response. In this way a 'reserve' of autoantigen-specific cells remains available when antigen levels suddenly rise. Tumors can cause a sudden rise in self-antigen presentation, and triggering tuned cells can induce potent anti-tumor immunity.

In order for autoimmunity to develop, it is likely that the tolerance of tuned T cells needs to be overcome. There are several mechanisms by which this could take place.

Most obvious would be a sudden rise in antigen levels, under conditions not favoring additional tuning. In this case infection can induce the necessary upregulation of co-stimulation on the local APCs while also providing auto-antigens by causing tissue damage [83]. Furthermore, this inflammatory environment could induce alterations in antigen-processing by the APC. New auto-antigens could also be presented as a result of changes in the cellular environment, inducing changes in protein processing or synthesis [84]. This could introduce antigens the T cell was not tuned for. In addition, there could also be a role for reduced expression of self-antigen in the periphery [81]. As constant expression of antigen will tune T cells, a prolonged decrease in the levels of antigen presented causes 're-sensitization' of these cells. Caused by a physiological disruption, a subsequent re-establishment of antigenic load could prove sufficient to induce activation of these now sensitized T cells. Nonetheless, all these changes in the surroundings of the T cell will likely induce further tuning, if there is no activated APC to supply essential activation signals. As suggested in the danger model, an APC needs to sense a threat in order to be activated and induce an immune response. As proposed by Matzinger [85] the factors controlling the generation of immunity are possibly the tissues themselves. Under healthy conditions tissues will maintain tolerance, under stress they will promote the generation of immunity. The endpoint being a stress-induced tissue-specific response, in all immune reactions the activated APC plays a key role. Combining an activated APC with the above mentioned mechanisms could result in tuned T cells becoming activated to react against auto-antigens. From there on, cross-reactivity, selective expansion and avidity maturation will ensure an optimal and progressive response. It appears that the same mechanisms could be considered to terminate the initiated response.

## IMPLICATIONS FOR AUTOIMMUNITY AND IMMUNOTHERAPY

The classic view of T cell selection proposes that the balance between auto-reactivity and tolerance is regulated by the individual exclusion of self-reactive cells from the circulation. This appears to be only partially the case, and recent findings have shed new light on mechanisms shaping the T cell repertoire. The regulation of T cell tolerance shows flexibility, providing a window of opportunity for the treatment of autoimmune disease or the induction of successful immunotherapy.

Vitiligo and melanoma are two conditions in which the state of the immune system is clearly opposed. In both conditions melanocyte antigen-specific cytotoxic T cells are present. Nevertheless, in vitiligo these cells mediate destruction of melanocytes and cause the characteristic depigmented lesions. In contrast, melanoma-infiltrating T cells will often not achieve any effective response. Recent research has shown that T cells in both pathologies are directed against identical melanocyte antigens, and exhibit the same activation status. However, melanoma-infiltrating T cells display a profound reduction in the affinity for their antigens. Additionally, T cells from vitiligo patients were capable of efficient IFN-γ production and TCR down-regulation in response to HLA-matched melanoma cells, while melanoma-derived T cells were not [86]. These

findings suggest that in melanoma the slow rise in peripheral antigen presentation of melanocyte antigens, resulting from progressive tumor necrosis, most likely tunes responsive T cells. Especially in the first phases of growth, a melanoma does not provide sufficient danger signals to induce proper APC activation. In later phases of progression and metastasis it is likely that the minor APC activation induced by local tissue damage is unable to overrule the tuned T cell activation thresholds. Moreover it is believed that antigen-shedding from progressing tumors activates natural Treg, thereby impeding development of efficient T cell responses [62].

Recent research has shown that vaccination of autologous melanoma cells transduced with GM-CSF can induce successful anti-melanoma immunity. This is illustrated by the long-term survival of 6 out of 14 patients, and the additional development of vitiligo in 2 of these patients. Remarkably, vitiligo and long term survival were only observed in patients experiencing non-evaluable disease [87]. This suggests that lowered presentation of melanocyte-antigens under tolerizing conditions in the periphery, could result in a re-sensitized and higher avidity T cell repertoire. This could ensure better vaccine efficacy, and illustrates that supplying proper activation signals to an antigen-sensitive T cell population can result in successful and specific immunotherapy. Besides the induction of immunity, future immunotherapy should also focus on breaking the tolerance of tuned T cells. As this tolerant state often impairs an efficient response, combining the two could drive tolerance over the edge. The most fit strategy here is probably the use of adoptive cell transfer (ACT), as reviewed by Overwijk [88]. The isolation and subsequent culture of T cells under re-sensitizing conditions could provide an expanded, antigen-sensitive T cell population to be transferred into the donor. Furthermore it allows for the genetic optimization of these cells before their use in therapy. In order to promote the activation of these cells, and cross-reactive residual T cells, drive avidity maturation and provide the necessary cytokines, the activation of local APCs via vaccination is absolutely essential. Vaccination will only be optimal though upon transfer of antigen-sensitive T cells and the use of strong adjuvans combined with sufficient immunogenic antigen to bypass the inhibition of natural Treg. Additionally, it has recently been shown that the success of adoptive transfer can depend on the presence of CD4 helper T cells providing IL-2, especially in the absence of Treg. When limited amounts of antigen-specific CD8 cells are transferred, sustained immunity is dependent upon this IL-2 and lost in the presence of Treg cells [62]. Moreover, exogenous IL-2 therapy failed in the presence of regulatory cells, illustrating that Tregs are able to suppress helper T cells and prevent auto-reactivity under the tested circumstances. Depletion of Treg could therefore boost immunotherapy by uninhibited T cell help during the activation and effector phase. Additionally, lymphodepletion of the host before adoptive transfer enhances subsequent autoimmunity [89], possibly by depleting the suppressive Treg pool. Novel immunotherapy designs should aim at combining the use of re-sensitized T cells with activation under non-inhibited conditions when pursuing an optimal effect.

On the contrary, the restraints in immunotherapy constitute the excess in T-cell mediated autoimmune disease. Therefore therapies should aim at re-establishing

tolerance via T cell tuning. For autoimmune diabetes it has been shown that treating pre-diabetic NOD mice with agonistic APL peptide of the causative antigen, protects mice from developing the disease. Unexpectedly, reactive T cells were not deleted in these mice due to overtriggering by the APL. Instead the cells exhibited a significantly less cytotoxic phenotype, while still infiltrating the islets in great numbers [73]. Indicative of T cell tuning this illustrates how autoimmunity could be prevented when the causative antigen is known. Use of APLs presented on peripheral immature APCs before disease onset, could prove useful in tuning reactive T cells and prevent disease. Besides this possible preventive strategy, stopping progressive autoimmune disease will probably require the influence of Treg as these are able to suppress auto-reactive T cells and mediate recovery from autoimmune disease [63]. Isolation and expansion of Treg, followed by an adoptive transfer of these cells could provide recovery from autoimmune disease. Importantly, Treg seem to influence CD4 T helper cells in their stimulatory function. In this context, recent data shows that antibody-mediated autoimmune responses can also be interrupted by Treg [90]. Because these cells appear to be powerful regulators of the humoral and adaptive immune responses, they could be useful targets in novel therapeutic approaches. Increasing Treg presence or function could thereby weaken co-stimulation by APCs and induce T cell tuning.

Taken together, future immunotherapeutic designs should exploit these insights into the regulation of T cell activity. These designs need to take into account the fact that the balance between tolerance and auto-immunity is flexible, and this will have implications for the course and outcome of therapy.

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# CHAPTER 3

## AUTOIMMUNE DESTRUCTION OF SKIN MELANOCYTES BY PERILESIONAL T CELLS FROM VITILIGO PATIENTS

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## ABSTRACT

In vitiligo, cytotoxic T cells infiltrating the perilesional margin are suspected to be involved in the pathogenesis of the disease. However, it remains to be elucidated whether these T cells are a cause or a consequence of the depigmentation process. T cells we obtained from perilesional skin biopsies were significantly enriched for melanocyte antigen recognition, as compared to healthy skin-infiltrating T cells, and were reactive to melanocyte antigen-specific stimulation. Using a skin explant model, we were able to dissect the *in situ* activities of perilesional T cells in the effector phase of depigmentation. We show that these T cells could infiltrate autologous normally pigmented skin explants and efficiently kill melanocytes within this microenvironment. Interestingly, melanocyte apoptosis was accompanied by supra-basal keratinocyte apoptosis. Perilesional T cells did however not induce apoptosis in lesional skin, which is devoid of melanocytes, indicating the melanocyte-specific cytotoxic activity of these cells. Melanocyte killing correlated to local infiltration of perilesional T cells. Our data show that perilesional cytotoxic T cells eradicate pigment cells, the characteristic hallmark of vitiligo, thereby providing evidence of T cells being able to mediate targeted autoimmune tissue destruction.

## INTRODUCTION

Vitiligo is a common skin disorder characterized by the progressive development of areas of skin devoid of melanocytes. Histological analysis of the perilesional margin surrounding the depigmented skin, reveals a lymphocytic infiltrate consisting of activated T cells (Badri *et al.*, 1993). It was found that these T cells were skin-homing, polarized towards type-1 effector function, and evidently cytotoxic while clustering near disappearing melanocytes (Wankowicz-Kalinska *et al.*, 2003; van den Wijngaard *et al.*, 2000). In addition, vitiligo patients often have melanocyte-specific antibodies in their blood (Cui and Bystryn, 1995), as well as circulating skin-homing melanocyte-specific cytotoxic T cells (Ogg *et al.*, 1998). Occasionally, leukoderma is observed following melanoma immunotherapy using tumor cell vaccination or adoptive T cell transfer therapy, and was found to be associated with a prolonged survival (Dudley *et al.*, 2002; Luiten *et al.*, 2005; Phan *et al.*, 2003; Yee *et al.*, 2000). During adoptive transfer therapy the infused melanocyte antigen-specific T cells were found to accumulate in the perilesional margin of the incipient skin depigmentation (Yee *et al.*, 2000). Moreover, repigmentation therapies such as UV-B irradiation and steroids have an immunosuppressive effect, further indicating an underlying autoimmune process. Nevertheless, a causative role for T cells in vitiligo has not been established, and therefore the question remains whether perilesional T cells are a cause or consequence of melanocyte destruction. The complex interactions during vitiligo pathogenesis are difficult to mimic *in vitro*. To closely examine the effector phase of vitiligo development within the skin microenvironment, our present study utilizes the skin explant model. Originally developed as a predictive test for graft-versus-host disease (GVHD) in patients receiving bone marrow transplantation, the model is based on the co-culture of donor lymphocytes with skin biopsies of the recipient (Vogelsang *et al.*, 1985). GVHD studies have shown that the skin explant model is well suited for studying the infiltration and effector function of T cells in the skin (Dickinson *et al.*, 1988; Dickinson *et al.*, 2002). By the skin explant technology, we examined the autoimmune T cell process underlying vitiligo vulgaris, and demonstrate that T cells isolated from perilesional skin cause melanocyte death upon infiltration of autologous pigmented skin. Our data underline the active role of T cells in the initiation and progression of vitiligo vulgaris.



## RESULTS

### Melanocyte antigen-specific T cells are present in perilesional vitiligo skin

T cells are often found infiltrating the perilesional margin of a vitiligo lesion, as illustrated in figure 1. We have isolated these T cells by *in vitro* culture of perilesional skin biopsies from progressive vitiligo lesions of 14 patients, and lesional skin biopsies of one patient (vit L). The emerging perilesional T cells were subsequently expanded using CD3/CD28 mAb stimulation. These T cell cultures were successful for all vitiligo patients, producing well-growing T cell populations. Cultures of skin biopsies from

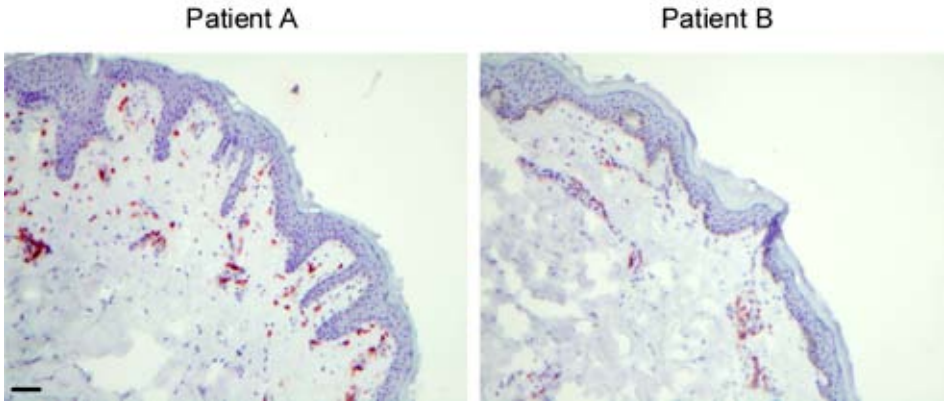


**Figure 1. Presence of T cells in the perilesional skin of progressive vitiligo lesions.** Immunohistochemical analysis of the perilesional skin of 5 patients revealed large $CD3^+$ T cell infiltrations. All patients experienced progressive depigmentation at the time of analysis. Photos show the anti-CD3 mAb (red) staining of two representative patients. Scale bar = 40 μm.

16 healthy donors produced only 12 growing T cell cultures, indicating that vitiligo-infiltrating T cells proliferate more readily upon *in vitro* stimulation than T cells residing in healthy skin. In order to gain insight into the ability of perilesional T cells to recognize melanocytes, we carried out flowcytometric analysis using HLA-A2/peptide tetramers for the melanocyte differentiation antigens tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$, MART-1$_{26\text{-}35}$ and the control antigen influenza virus$_{58\text{-}66}$ (flu$_{58\text{-}66}$). As this analysis was HLA-A2 restricted, 9 out of 14 vitiligo patients, patient vit L and 5 out of 12 healthy skin donors were suitable for analysis. As indicated in table 1, significantly increased levels of T cells recognizing melanocyte antigens were found in the perilesional T cell populations, as compared to healthy donor skin-residing T cells. The flu$_{58\text{-}66}$ data showed no significant difference, which is in line with the fact that T cells recognizing the influenza virus are normally not found in the skin. Additionally, when melanocyte antigen recognition by perilesional T cells was compared to recognition of these antigens by autologous PBMC, no significant increases were found. This indicates that presence of melanocyte antigen-specific T cells in the skin usually coincides with likewise increased levels of these specific cells in the blood. Importantly, recognition of the flu$_{58\text{-}66}$ control antigen differed significantly between perilesional T cells and PBMC, which confirms that flu$_{58\text{-}66}$-specific T cells can be present in the blood and absent in the skin. These results demonstrate that melanocyte antigen-specific T cell levels are evidently elevated in the vitiligo perilesional skin, and that this elevation coincides with an increased presence of these cells in the blood. Furthermore, only minor T cell reactivity was found against antigens that are normally absent in the skin.

**Table 1:** Antigen-specificity of perilesional vitiligo skin-infiltrating T cells as compared to healthy skin-residing T cells.

| | | | % tetramer-positive cells of the CD8+ T cell population* | | | | |
|---|---|---|---|---|---|---|---|
| **Patient ID** | **Tissue** | **HLA-typing#** | **A2/ MART-1** | **A2/ Tyrosinase** | **A2/ gp100(280)** | **A2/ gp100(209)** | **A2/flu** |
| **vit 1** | PL | A02 A68 B15 B44 Cw03 Cw07 | 33,73 | 1,38 | 1,66 | 4,23 | 0,14 |
| **vit 2** | PL | A01 A02 B15 B45 Cw06 Cw12 | 0,51 | 2,64 | 1,34 | 4,92 | 0,03 |
| **vit 4** | PL | A02 A11 B08 B15 Cw03 Cw07 | 0,42 | 0,50 | 0,35 | 2,74 | 0,00 |
| **vit 5** | PL | A02 A03 B07 B51 Cw07 Cw14 | 0,21 | 0,88 | 0,18 | nt | 0,00 |
| **vit 8** | PL | A02 A02 B15 B44 Cw03 Cw04 | 0,24 | 1,03 | 0,16 | 2,23 | 0,02 |
| **vit 16** | PL | A01 A02 B52 B57 Cw06 Cw12 | 1,96 | 3,12 | 3,78 | 2,32 | 0,01 |
| **vit 17** | PL | A02 A23 B39 B44 Cw04 Cw07 | 0,26 | 0,18 | 1,10 | 1,02 | 0,01 |
| **vit 19** | PL | A02 A03 B07 B44 Cw05 Cw07 | 3,59 | 1,60 | 3,12 | nt | 0,02 |
| **vit 20** | PL | A01 A02 B08 B35 Cw04 Cw07 | 0,04 | 0,09 | 0,12 | 0,53 | 0,01 |
| **vit L** | Lesional | HLA-A2 | 0,79 | 3,87 | 4,06 | nt | 0,00 |
| **vit 1** | PBMC | A02 A68 B15 B44 Cw03 Cw07 | 0,57 | 0,82 | 0,88 | 1,76 | 0,18 |
| **vit 2** | PBMC | A01 A02 B15 B45 Cw06 Cw12 | 0,55 | 1,47 | 1,02 | 2,91 | 0,72 |
| **vit 4** | PBMC | A02 A11 B08 B15 Cw03 Cw07 | 0,25 | 0,14 | 0,41 | 1,97 | 0,22 |
| **vit 8** | PBMC | A02 A02 B15 B44 Cw03 Cw04 | 0,09 | 0,03 | 0,12 | 0,45 | 0,02 |
| **vit 16** | PBMC | A01 A02 B52 B57 Cw06 Cw12 | 4,12 | 3,51 | 6,13 | 0,47 | 0,01 |
| **vit 17** | PBMC | A02 A23 B39 B44 Cw04 Cw07 | 0,26 | 0,52 | 0,47 | 5,56 | 1,05 |
| **vit 20** | PBMC | A01 A02 B08 B35 Cw04 Cw07 | 0,18 | 0,12 | 0,07 | 0,90 | 0,04 |
| **SKIN 1^** | normal skin | HLA-A2 | 0,12 | 0,23 | 0,19 | nt | 0,04 |
| **SKIN 10** | normal skin | HLA-A2 | 0,07 | 0,05 | 0,04 | 0,66 | 0,00 |
| **SKIN 12** | normal skin | HLA-A2 | 0,07 | 0,30 | 0,13 | 0,23 | 0,03 |
| **SKIN 13** | normal skin | HLA-A2 | 0,09 | 0,14 | 0,20 | 0,21 | 0,05 |
| **SKIN 15** | normal skin | HLA-A2 | 0,05 | 0,18 | 0,11 | 0,43 | 0,02 |
| Significance level of "vit PL" versus "vit PBMC"§ | | | *p>0.66* | *p>0.26* | *p>0.40* | *p>0.37* | *p<0.01* |
| Significance level of "vit PL" versus "Skin"§ | | | *p<0.02* | *p<0.03* | *p<0.04* | *p<0.02* | *p>0.27* |

*: Polyclonal T cells grown out of perilesional (PL) or lesional skin biopsies from vitiligo patients, or from normal healthy donor skin were tested for their antigen-specificity using HLA-peptide tetramers composed of HLA-A2 molecules and peptides derived from melanocyte antigens (MART-1, tyrosinase, gp100) or control antigen influenza virus (flu); **nt**, not tested; #: HLA typing was performed by genotyping using allele specific probes, or by flowcytometry using an HLA-A2-specific mAb; ^: Results of the 5 HLA-A2+ donors of 12 donors yielding well-growing T cells; 16 healthy skin donors analyzed in total; §: Perilesional vitiligo T cells were compared to autologous PBMC and healthy skin-infiltrating T cells, for the difference in specific melanocyte antigen recognition using the non-parametric Mann-Whitney U-test (95% confidence interval), the difference between compared samples was significant if P<0.05.

## Perilesional T cells become activated and cytotoxic upon melanocyte antigen-specific stimulation *in vitro*.

We tested the functional activation of perilesional T cells upon recognition of melanocyte differentiation antigens. To this end, T cells were stimulated with a pool of HLA-A2-binding peptides; tyrosinase$_{369-377}$, gp100$_{280-288}$, gp100$_{209-217}$ and MART-1$_{26-35}$, or flu$_{58-66}$ control, loaded onto EBV-transformed B cells (JY) as a target. We analyzed 5

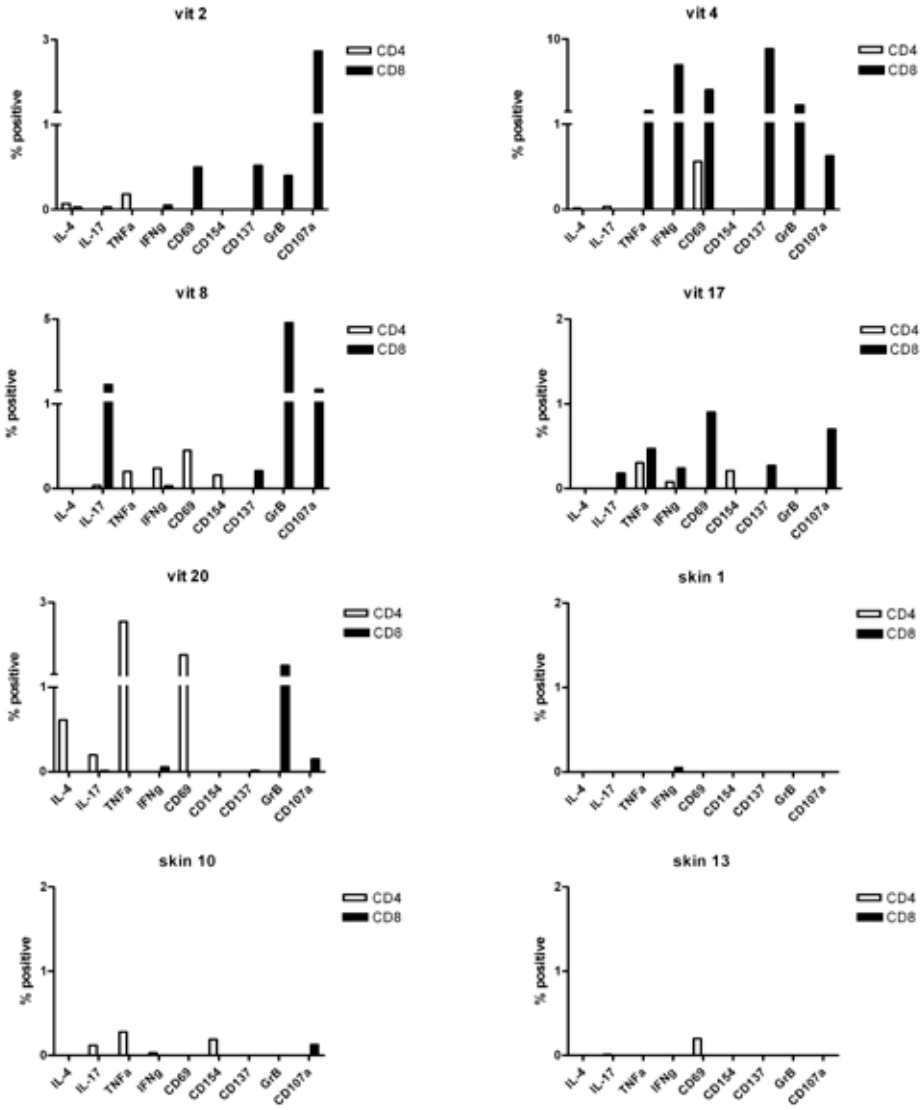


**Figure 2. Perilesional T cells become activated and express cytotoxic markers upon melanocyte antigen-specific stimulation *in vitro*.** Multi-parameter flowcytometric analysis of perilesional T cells (panels designated "vit") and healthy skin-infiltrating T cells (panels designated "skin") stimulated in vitro with pooled tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$ peptides loaded on EBV transformed B cells (JY). Comparison of the data required normalization; therefore the percentages depicted represent the percentages of CD4$^+$ or CD8$^+$ T cells reactive to the peptide pool minus the percentage of these T cells reactive to unloaded JY cells (background). Stimulations using flu$_{58\text{-}66}$ control peptide-loaded JY produced negative results for both perilesional- and healthy skin-infiltrating T cells, while positive control incubations with PMA/ionomycin resulted in maximal expression of the markers tested in all samples.

**Table 2:** Clinical data of the vitiligo patients tested for melanocyte antigen recognition and functional T cell response.

| Patient | Vitiligo since(yrs) | Poliosis | Koebner phenomen. | Inflammatory vitiligo | Halo nevi | Body surface(s) involved | Approx. body surface (%) | Responsive to UVB after (months)[$] |
|---|---|---|---|---|---|---|---|---|
| **vit 1** | 3 | yes (scalp) | yes | no | no | face, torso, arms, legs, feet, genitalia | 30 | 3 |
| **vit 2** | 2 | yes (scalp) | yes | no | no | face, chest, hands | 20 | 8 |
| **vit 4** | 18 | yes (genitalia) | yes | no | no | face, torso, legs, arms, genitalia | 20 | 12 |
| **vit 5** | 1 | no | yes | no | no | face, hip, hands | 5 | 4 |
| **vit 8** | 8 | yes (lesions) | no | no | yes | scalp, torso, genitalia | 10 | 3 |
| **vit 16** | 4 months | no | no | no | no | face, neck, torso | 10 | 12 |
| **vit 17** | 40 | no | no | no | no | fingertips, face (since 4 years) | < 5 | 3 |
| **vit 19** | 4 | no | yes | no | no | armpits, chest, hands, legs | 5 | 6 |
| **vit 20** | 4 months | no | yes | yes | no | back, neck | 10 | 3 |

[$] : Indicates the time period elapsed before the first repigmentation of vitiligo lesions occurred

vitiligo patients and 3 healthy controls, which all were HLA-A2 positive and yielded a sufficient number of T cells within 14 days of biopsy culture, to rule out bias introduced by prolonged in vitro culture. T cell activation was measured by the expression of CD69 for early T cell activation, CD137 specific for $CD8^+$ T cell activation (Myers and Vella, 2005) and CD154 for $CD4^+$ T cell activation (Chattopadhyay et al., 2005). The cytolytic function during T cell activation, was determined by the expression of the cytotoxic marker granzyme-B (Chowdhury and Lieberman, 2008) and the surface mobilization of CD107a (Betts et al., 2003), which indicates cytotoxic degranulation. Additionally, we analyzed the production of the humoral response-promoting cytokine IL-4, and the pro-inflammatory cytokines IL-17, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ).

Upon melanocyte antigen-specific stimulation, the perilesional $CD8^+$ T cells became activated, as indicated by upregulated CD69 and CD137 expression, as well as granzyme-B and CD107a expression, in 4 out of 5 patients (Fig. 2; *black bars, panels designated "vit"*). In contrast, these cells were not activated when stimulated with the $flu_{58\text{-}66}$ control peptide (data not shown), which demonstrates the melanocyte antigen-specific activation of the perilesional CD8+ T cells. In addition, the perilesional $CD4^+$ T cells showed bystander activation in concurrence with the $CD8^+$ T cell response, as shown by the upregulation of CD69 and CD154 in 4 out of 5 patients tested (Fig. 2; *white bars, panels designated "vit"*). The activated $CD4^+$ cells did, however, not upregulate granzyme-B upon stimulation, which confirms their antigen-independent activation in the HLA-class I restricted stimulation setup used (Casazza *et al.*, 2006). Depending

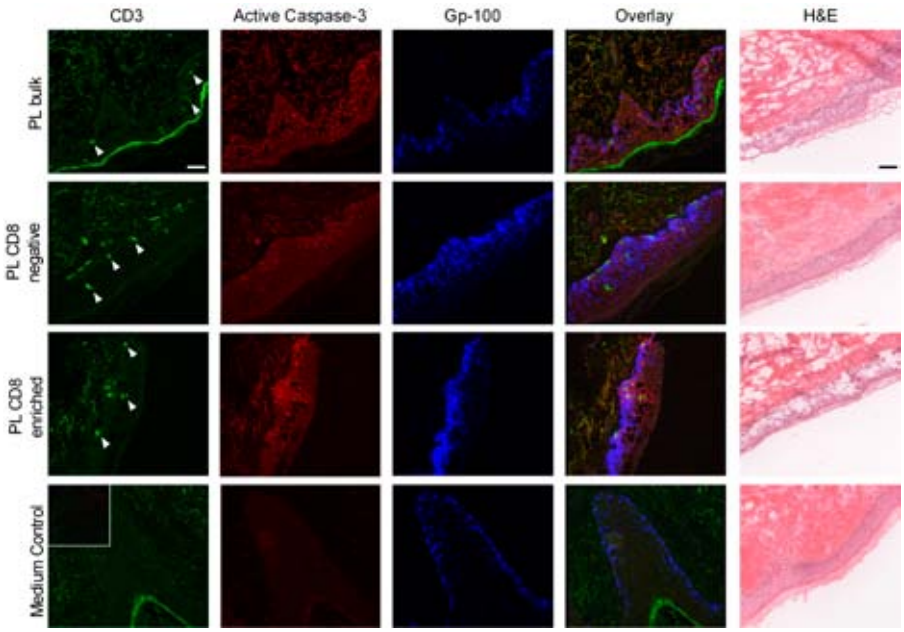


**Figure 3. Induction of melanocyte apoptosis by perilesional T cells infiltrating autologous skin explants.** CLSM and hematoxylin & eosin (H&E) analysis of skin explants following co-culture of different autologous perilesional T cell populations with autologous *non*-lesional skin. CD3 (green; membrane) was used as a T cell marker (several indicated by arrows), active caspase-3 (red; cytoplasmic) as an apoptosis indicator and gp100 (blue; cytoplasmic) to detect melanocytes. *Upper panel:* Total perilesional T cells (PL bulk) infiltrated into the explant epidermis, and induced apoptosis in melanocytes and keratinocytes. The epidermal tissue structure appeared damaged. *Second panel:* The PL $CD8^+$ T cell-depleted population infiltrated the explant dermis and epidermis. Apoptosis of melanocytes was not detected. *Third panel:* Upon explant infiltration, the PL $CD8^+$ T cell-enriched population migrated to the epidermis, where apoptosis of melanocytes was induced. Furthermore, epidermal tissue damage and apoptosis of keratinocytes was visible, clearly co-localizing with T cell presence. *Lower panel:* No residing T cells or detectable melanocyte apoptosis was found in the skin explant cultured without the addition of T cells. Antibody isotype-control analyses (insert) were all negative. These data are representative of 4 independent assays in 3 different patients. Quantitative data are summarized in Table 3. White scale bar for CLSM panels = 40 μm, black scale bar for H&E panels = 60 μm.

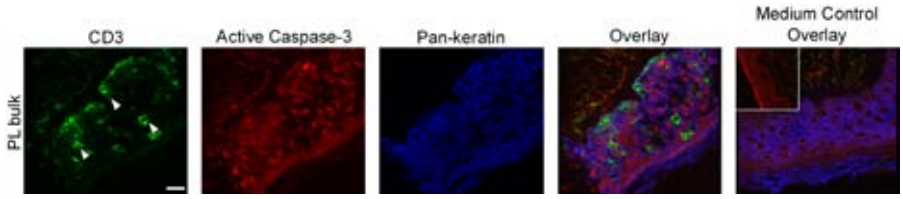


**Figure 4. Keratinocyte bystander apoptosis induced by the skin infiltration of cytotoxic T cells.** CLSM analysis detected the infiltration of perilesional T cells and bystander keratinocyte apoptosis. CD3 (green; membrane) was used for staining T cells (several indicated by arrows), active caspase-3 (red; cytoplasmic) for detecting apoptosis, pan-keratin (blue; membrane) for identifying keratinocytes. The control explant without the addition of T cells showed no detectable apoptosis of keratinocytes, and the antibody isotype-control staining (insert) was negative. Per explant, these data are representative for 15 photomicrographs showing T cell infiltration out of 15 analyzed in total. Scale bar = 20 μm.

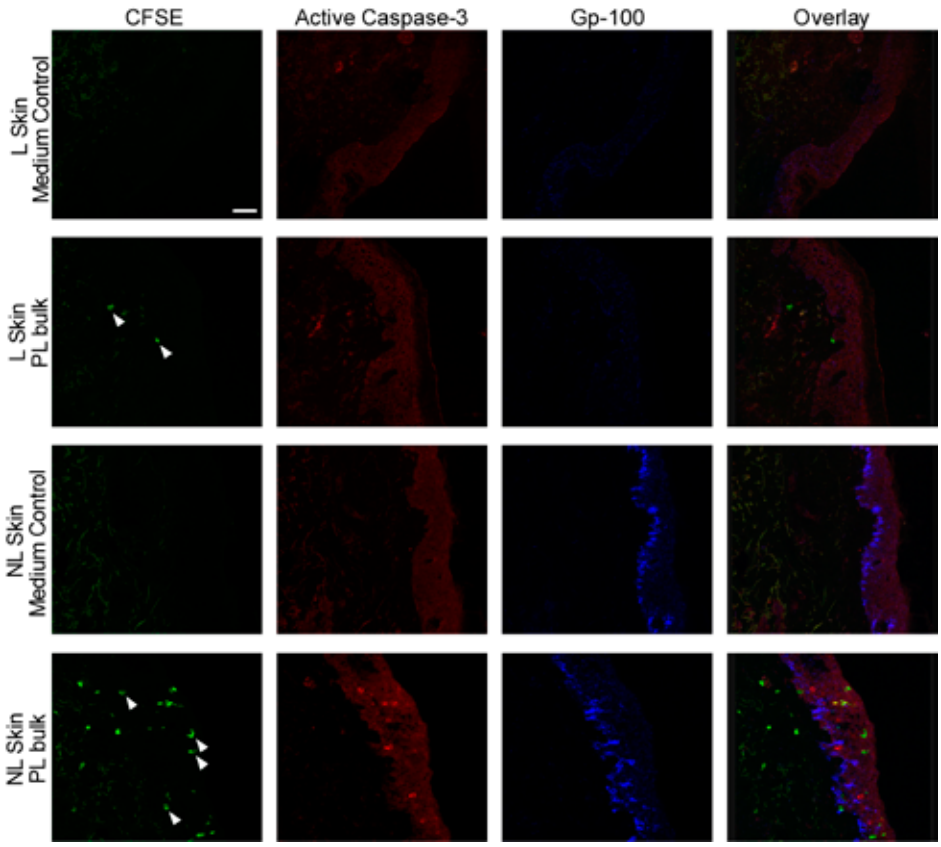


**Figure 5. Skin-infiltrating cytotoxic T cells do not induce apoptosis in lesional skin.** CLSM analysis of lesional and non-lesional skin explants cultured in medium or with autologous CFSE-labeled perilesional T cells. T cell were detected by their CFSE labeling (green, several indicated by arrows), apoptosis by active caspase-3 (red; cytoplasmic) and melanocytes were detected by gp100 expression (blue; cytoplasmic). Upper and third panel: No apoptosis was found in lesional and non-lesional explants cultured in medium, respectively. Absence of gp100 staining in the lesional skin confirms the absence of melanocytes in these explants. Second panel: perilesional T cells do not induce apoptosis in lesional skin. Lower panel: perilesional T cells induce apoptosis in non-lesional skin, especially in the basal layer containing melanocytes. These data are representative of 2 independent assays in 2 different patients. Quantitative data are summarized in Table 3. White scale bar for CLSM panels = 40 μm.

on the patient analyzed, the $CD4^+$ and $CD8^+$ perilesional T cells produced varying amounts of cytokines, especially pro-inflammatory TNF-α and IFN-γ supporting a cytotoxic T cell response. IL-17-producing $CD8^+$ T cells were found in patient vit 8, which interestingly coincided with patient vit 8 being the only one experiencing halo nevi, and poliosis in nearly all lesions. IL-17 has recently been related to T cell-mediated immunity against established melanoma (Muranski *et al.*, 2008) as well as in several autoimmune diseases such as atopic dermatitis (Koga *et al.*, 2008), psoriasis (Teunissen *et al.*, 1998), and rheumatoid arthritis (Aarvak *et al.*, 1999). Furthermore,

**Table 3:** Quantification of the skin explant assays using autologous T cells

| | | cells added: | medium | | | PL bulk | | |
|---|---|---|---|---|---|---|---|---|
| **exp** | **patient** | **tissue** | **casp** | **T cells** | **epi T cells** | **casp** | **T cells** | **epi T cells** |
| 1 | vit 4 | NL | 0.0 | 0.8 | 0.0 | 94.3 | 32.0 | 29.3 |
| 2 | vit 8 | NL | 0.0 | 0.8 | 0.8 | 21.0 | 7.0 | 4.5 |
| 3 | vit 20 | NL | 3.3 | 0.0 | 0.0 | 7.3 | 23.2 | 14.8 |
| | | L | 2.8 | 0.0 | 0.0 | 2.5 | 5.5 | 0.5 |
| 4 | vit 8 | NL | 0.7 | 0.0 | 0.0 | n.e. | | |
| | | L | 2.7 | 0.0 | 0.0 | 3.3 | 33.3 | 12.0 |
| Significance apoptosis in NL skin, versus medium control (t-test): | | | | | | *p<0.02* | | |

Numbers indicate the average number of positive cells per section: **casp**, number of active caspase-3 positive cells in the basal epidermis; **T cells**, total number of T cells infiltrating dermis and epidermis; **epi T cells**, number of T cells infiltrating the epidermis. **NL**, non-lesional skin explant; **L**, lesional skin explant, **n.e.**, tissue non-evaluable. In exp 3 and 4, the T cells were labeled with CFSE prior to co-culture, which allows selective detection of added T cells.

IL-4 was only produced by the $CD4^+$ cells in patient vit 20. In contrast, the healthy skin-residing T cells (Fig. 2, *panels designated "skin"*) showed no cytotoxic $CD8^+$ T cell response against any of the antigens tested, as evidenced by the absence of upregulated T cell activation markers CD69 and CD137, CD107a or granzyme-B upon stimulation. In addition, no cytokine production by the $CD8^+$ population was found. The minor cytokine production by $CD4^+$ T cells of donor skin 10 did however not coincide with an activated cytotoxic response. These results show that perilesional T cells are markedly enriched for functional reactivity against specific melanocyte antigens, as compared to healthy skin-residing T cells. Interestingly, the magnitude of the $CD8^+$ T cell reactions measured in these *in vitro* assays was related to vitiligo disease intensity. In particular, it seemed that the stronger the $CD8^+$ T cell response, the more elaborate the vitiligo (supported by the incidence of poliosis), and the more challenging it appeared in response to UVB therapy (Table 2). Combined with the tetramer data discussed above, these results suggest a relationship between the functional activity of the melanocyte antigen-specific T cells in the perilesional vitiligo skin and the clinical presentation of vitiligo.

## Perilesional T cells selectively induce apoptosis in autologous non-lesional skin explants

Subsequently, we examined the functional capacity of perilesional T cells to actively kill melanocytes within the skin tissue microenvironment. To test this, skin explant assays were carried out in which perilesional T cells were co-cultured for two days with *non*-lesional (normally pigmented) autologous skin biopsies. In this time period, the T cells were allowed to infiltrate the skin biopsies (now referred to as skin explants) and migrate towards the melanocytes located at the dermal-epidermal junction. This co-culture was either performed with a complete perilesional T cell population, a $CD8^+$ T cell-enriched population or a $CD8^+$ T cell-depleted population. Subsequently, skin explant cryosections were analyzed for the presence of infiltrated T cells, melanocytes

| PL CD8⁺ | | | PL CD4⁺ | | |
|---|---|---|---|---|---|
| **casp** | **T cells** | **epi T cells** | **casp** | **T cells** | **epi T cells** |
| 26.3 | 11.3 | 7.5 | 6.0 | 21.5 | 13.3 |
| 18.5 | 27.8 | 26.8 | 4.3 | 1.3 | 0.3 |
| 4.7 | 12.2 | 6.2 | 3.8 | 29.8 | 18.0 |
| 2.7 | 7.0 | 1.7 | n.e. | | |
| 10.0 | 66.0 | 52.8 | 3.7 | 30.2 | 11.7 |
| 3.3 | 23.7 | 16.5 | 1.2 | 34.0 | 16.8 |
| *p<0.02* | | | *p<0.02* | | |



and apoptosis by immunohistochemistry or immunofluorescence using confocal laser scanning microscopy (CLSM). As shown in figure 3, the complete perilesional T cell population (*green; several are indicated by arrows*) infiltrated the explant and induced apoptosis of melanocytes (*blue*), as indicated by the cytoplasmic active caspase-3 staining (*red*) in cells of the basal epidermis. Additional epidermal cells in supra-basal layers, most likely keratinocytes, also underwent apoptosis, which was accompanied by damage to the epidermal tissue structure (Fig. 3, *upper panel*). In contrast, the CD8⁺ T cell-depleted perilesional T cells (Fig. 3, *second panel*) also migrated into the epidermis, arriving in close proximity to the melanocytes, however they did not induce substantial apoptosis. Furthermore, no tissue structure damage was induced. Infiltration of the explant by the CD8⁺ T cell-enriched population (Fig. 3, *third panel*) induced the most prominent apoptosis of melanocytes and keratinocytes, clearly co-localizing with epidermal T cell infiltration, and was accompanied by profound tissue structure disruption. As a control, neither T cells nor measurable melanocyte apoptosis were found in explants cultured without the addition of T cells (Fig. 3, *lower panel*).

The previous experiments suggest that killing of melanocytes by CD8⁺ T cells is accompanied by substantial bystander keratinocyte apoptosis. CLSM analyses using pan-keratin-specific mAb as a keratinocyte marker showed that the apoptotic epidermal cells adjacent to the melanocytes were indeed keratinocytes, as these cells co-expressed active caspase-3 with pan-keratin (Fig. 4). These results show that keratinocytes undergo bystander apoptosis alongside melanocyte killing. This effect can be caused by activated cytotoxic T cells producing pro-inflammatory cytokines (i.e. TNF-α and IFN-γ) infiltrating the skin, since this effect was only visible along with melanocyte apoptosis. These cytokines can sensitize keratinocytes to undergo apoptosis (Arnold *et al.*, 1999).

Our data so far showed that perilesional T cells were found to induce not only apoptosis of melanocytes but also of keratinocytes in the *non*-lesional skin. To rule out that this is non-specific activity of perilesional T cells independent of melanocyte antigen recognition, we compared the cytotoxic effect of perilesional T cells in *non*-lesional- and lesional skin, which is devoid of melanocytes. To detect the transferred

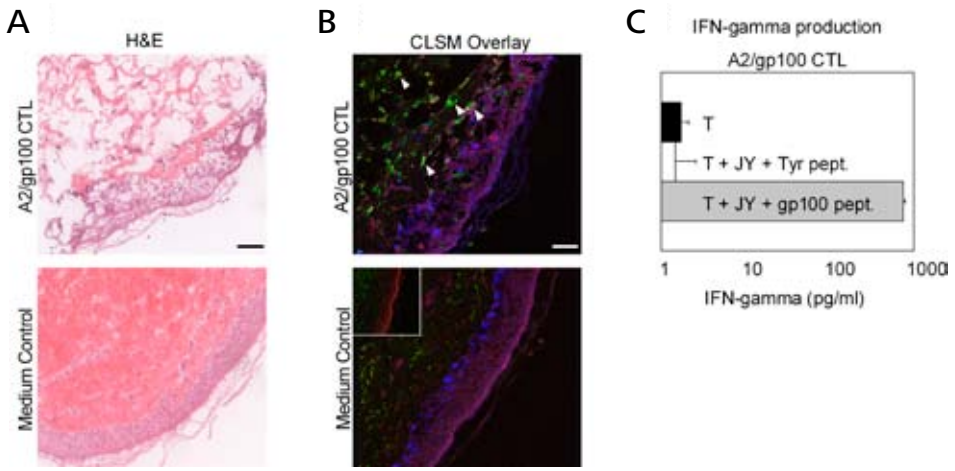


**Figure 6. Upon infiltration of the skin melanocyte antigen-specific T cells kill melanocytes.** CLSM and H&E analysis of a skin explant assay using $gp100_{280-288}$ specific CTLs co-cultured with HLA-A2 matched *non*-lesional skin from a vitiligo patient. **6a**: H&E staining of the explant shows extensive tissue damage upon CTL infiltration. The explant cultured without the addition of T cells shows normal tissue morphology. **6b**: CLSM analysis using CD3 (green; membrane), active caspase-3 (red; cytoplasmic) and gp100 (blue; cytoplasmic). A large T cell infiltration was seen in the dermis and epidermis (several T cells indicated by arrows). Very few epidermal cells had remained intact, which explains the limited caspase-3 activity at this time point. The explant without added T cells shows no detectable apoptosis. The antibody isotype-control staining (insert) was negative. Per explant, these data are representative for 15 micrographs showing T cell infiltration, out of 15 analyzed in total. **6c**: An anti-IFN-γ ELISA showed that the $gp100_{280-288}$ specific CTLs produced a large amount of IFN-γ upon stimulation with $gp100_{280-288}$ peptide loaded onto EBV-transformed B cells (JY; *lower bar*). When stimulated by JY loaded with tyrosinase peptide, the CTLs produced no IFN-γ (*middle bar*), comparable to CTLs without stimulation (*upper bar*). White scale bar for CLSM panels = 40 μm, black scale bar for H&E panels = 60 μm.

T cells in the explants, the T cells were labeled with CFSE prior to co-culture. Figure 5 shows the infiltration of CFSE-labeled perilesional T cells into lesional and *non*-lesional explants. Upon perilesional T cell infiltration, apoptosis of epidermal cells was only found in the *non*-lesional skin explants, whereas this was absent in the lesional skin. These results indicate that the keratinocyte apoptosis present in *non*-lesional skin is dependent on melanocyte antigen-recognition by infiltrating perilesional T cells, and does not result from aspecific cytotoxic activity of these cells against keratinocytes.

To quantify the level of apoptosis of melanocytes as well as the level of epidermal or dermal T cell infiltration, skin explant assays were repeated 4 times in 3 different patients. Extensive CLSM analyses were performed on at least three sections of each combination of patient and culture condition, by acquiring series of CLSM micrographs spanning the full length of the epidermis. The CLSM data comprised 13 to 15 micrographs of the 3 cryosections per condition, in which the number of positive cells was counted by two independent observers. To estimate the level of melanocyte apoptosis, only active caspase-3 positive cells in the basal epidermis were counted. The average number of positive cells, per cryosection of these skin explant

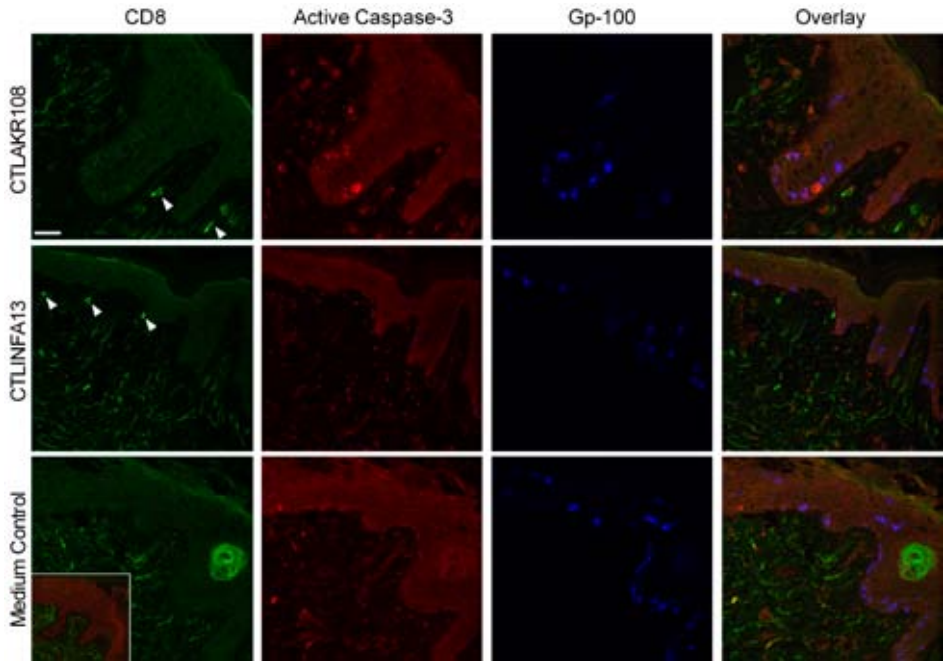


**Figure 7. Melanocyte apoptosis requires skin infiltration by melanocyte antigen-specific T cells.** CLSM analysis of skin explant assays using HLA-A2-matched *non*-lesional skin from a vitiligo patient, performed with the tyrosinase$_{369\text{-}377}$ -specific cytotoxic T cell clone CTLAKR108 and the flu$_{58\text{-}66}$-specific T cell clone CTLINFA13. CD8 (green; membrane) was used for detection of the T cell clones (several indicated by arrows), active caspase-3 (red; cytoplasmic) for apoptosis and gp100 (blue; cytoplasmic) to stain for melanocytes. *Upper panel:* CTLAKR108 infiltrated the skin tissue, migrated to the epidermis and induced apoptosis in several melanocytes. *Second panel:* CTLINFA13 also infiltrated the skin explant and migrated to the epidermis, but did not induce detectable apoptosis. *Lower panel:* The explant cultured without the addition of T cells showed neither CD8$^+$ T cell presence, nor detectable apoptosis. The antibody isotype-control (insert) was negative. These data are representative of 3 independent assays in 3 different patients. Quantitative data are summarized in Table 4. Scale bar = 40 μm.

assays using autologous perilesional T cells, is summarized in Table 3. The quantitative results show that all T cell populations were able to infiltrate the dermis and epidermis of the explants. However, only total or CD8+ enriched perilesional T cells induced elevated levels of apoptosis in all assays, whereas less apoptosis was found the explants incubated with CD4+ enriched perilesional T cells. Interestingly, in case epidermal CD8+ T cell infiltration in *non*-lesional skin was visible as patches of dense T cell infiltration, apoptosis of epidermal cells was also concentrated at these sites.

## Melanocyte antigen-specific T cells kill melanocytes within the skin microenvironment

To verify whether the effects of melanocyte and keratinocyte apoptosis and tissue damage shown in Figure 3 and 4 can be induced by purified melanocyte antigen-specific

**Table 4: Quantification of the skin explant assays using melanocyte- or flu-specific T cell clones.**

| | | | cells added: | medium | |
|---|---|---|---|---|---|
| **exp** | **patient** | **tissue** | **casp** | **T cells** | **epi T cells** |
| **5** | vit 5 | NL | 1.5 | 0.0 | 0.0 |
| **6** | vit 15 | NL | n.e. | | |
| **7** | vit 8 | NL | 0.7 | 0.0 | 0.0 |
| | | L | 2.7 | 0.0 | 0.0 |
| Significance apoptosis in NL skin, versus medium control (t-test): | | | | | |

Numbers indicate the average number of positive cells per section: **casp**, number of active caspase-3 positive cells in the basal epidermis; **T cells**, total number of T cells infiltrating dermis and epidermis; **epi T cells**, number of T cells infiltrating the epidermis. **NL**, non-lesional skin explant; **L**, lesional skin explant, **n.e.**, tissue non-evaluable. In exp 7, the T cells were labeled with CFSE prior to co-culture, which allows selective detection of added T cells.



T cell populations, we tested the effect of $gp100_{280-288}$ specific cytotoxic T cells (CTLs) in a skin explant co-culture with HLA-A2-matched *non*-lesional skin from a vitiligo patient. These CTLs were sorted from a T cell culture obtained previously from vitiligo lesional skin biopsies, and were 98% specific for the HLA-A2/$gp100_{280-288}$ epitope (data not shown). When these CTLs were co-cultured with the skin explant, they infiltrated the explant and caused extensive disruption of the skin tissue (Fig. 6a). CLSM analysis revealed CTLs infiltrating the epidermis and dermis, while very few melanocytes had remained intact (Fig. 6b). Moreover, the dermal-epidermal junction was severely damaged. When these CTLs were stimulated *in vitro* with the specific $gp100_{280-288}$ peptide, they produced large amounts of IFN-γ (Fig. 6c). In contrast, the CTLs did not produce IFN-γ when stimulated with irrelevant tyrosinase peptide nor in the absence of peptide stimulation, indicating the antigen-specific activation of these cells.

To further define whether melanocyte apoptosis required antigen-specific recognition of melanocytes by infiltrating T cells, we performed explant assays using the $tyrosinase_{369-377}$-specific $CD8^+$ T cell clone CTLAKR108, the MART-$1_{26-35}$-specific T cell clone CTLAKR4D8, and the $flu_{58-66}$-specific $CD8^+$ T cell clone CTLINFA13 as a control (Hooijberg *et al.*, 2000; Verra *et al.*, 2004). During co-culture, both CTLAKR108 and CTLINFA13 (Fig. 7; *several indicated by arrows*) had migrated towards the basal layer of the epidermis of the explants. The tyrosinase-specific T cell clone induced apoptosis in several melanocytes (Fig. 7, *upper panel*), while the influenza virus-specific T cell clone was ineffective (Fig. 7, *second panel*). Furthermore, the explant cultured without the addition of T cells did not show any detectable T cells or apoptosis (Fig. 7, *lower panel*). In addition, the CFSE-labeled MART-1-specific T cells extensively infiltrated the explants and induced widespread apoptosis in the basal and supra-basal epidermis (data not shown), similar to the apoptosis observed in figure 3 and 5. To quantify these data, epidermal and dermal T cell infiltration and melanocyte apoptosis was investigated in 3 skin explant assays in 3 different patients, as described for the assays in Table 3. This analysis showed that all the CFSE-labeled T cell clones were able to infiltrate the explants, however only the tyrosinase- and MART-1-specific T cells induced apoptosis (Table 4). No apoptosis was found when these T cells were incubated with lesional skin,

| AKR108/4D8 | | | INFA13 | | |
|---|---|---|---|---|---|
| **casp** | **T cells** | **epi T cells** | **casp** | **T cells** | **epi T cells** |
| 17.0 | 27.0 | 4.5 | 0.8 | 9.3 | 1.3 |
| 4.5 | 1.0 | 0.0 | 1.5 | 6.5 | 3.0 |
| 42.0 | >200 | 50.0 | 1.7 | 44.0 | 34.0 |
| 1.3 | 12.0 | 1.7 | 1.0 | 105.0 | 6.7 |
| *p<0.02* | | | *p>0.09* | | |



demonstrating that the activation of the tyrosinase- or MART-1-specific T cell clones in *non*-lesional skin was antigen-specific and depended on the presence of melanocytes (Table 4). Taken together, these results show that melanocyte antigen-specific cytotoxic T cell populations actively induce depigmentation of the skin by killing of melanocytes in the skin microenvironment. To rule out possible effects of antigen-independent T cell activation in the skin explants, we carried out 3 assays with HLA-mismatched donor skin and the $flu_{58-66}$- and $tyrosinase_{369-377}$ antigen-specific clones. In all tests, the clones infiltrated the skin explant up into the epidermis, but did not induce detectable apoptosis of melanocytes (data not shown).

## DISCUSSION

In this study, we co-cultured perilesional vitiligo skin-infiltrating T cells under different conditions with autologous pigmented skin, in order to perform a functional analysis of the effector phase of depigmentation in vitiligo vulgaris. We show that: (i) T cells infiltrating the perilesional vitiligo skin can be efficiently obtained by *in vitro* culture of perilesional skin biopsies; (ii) the perilesional T cell population displays elevated levels of T cells that recognize melanocyte antigens, in contrast to T cells residing in healthy skin; (iii) perilesional T cells are markedly enriched for cytotoxicity against specific melanocyte antigens in comparison to healthy skin-residing T cells; (iv) upon infiltration of autologous pigmented skin, perilesional T cells efficiently kill melanocytes; and (v) this active killing of melanocytes depends on melanocyte-specific cytotoxic T cells infiltrating the epidermis.

Previous studies have shown the presence of cytotoxic melanocyte antigen-specific T cells in the blood of vitiligo patients (Ogg *et al.*, 1998), the presence of cytotoxic effector T cells clustering near melanocytes in vitiligo skin (van den Wijngaard *et al.*, 2000; Wankowicz-Kalinska *et al.*, 2003), and the ability of adoptively transferred melanocyte antigen-specific T cells to home to incipient vitiligo lesions (Yee *et al.*, 2000). In our skin explant system we were able to combine these observations and investigate the T cell effector phase of depigmentation in vitiligo. In these assays, we observed that perilesional T cells can kill melanocytes within their physiological tissue microenvironment directly *ex vivo*. These results not only provide new insight into the pathogenesis of vitiligo, but are also relevant for melanoma immunotherapy.

In melanoma patients, circulating melanocyte antigen-specific T cells are of a much lower affinity compared to those present in vitiligo patients, illustrated by a decreased cytokine production and an elevated activation threshold (Palermo *et al.*, 2005). This difference probably results from tuning of the immune response in melanoma patients (van den Boorn *et al.*, 2006). The observed keratinocyte apoptosis in our assays is not an artifact. Firstly, in several control assays using vitiligo lesional skin explants we have shown that this apoptosis depended on melanocyte antigen-recognition by infiltrating T cells. Secondly, cytokines such as IFN-γ and TNF-α, both produced by perilesional T cells following this recognition (Fig. 2), sensitize keratinocytes to undergo apoptosis (Arnold *et al.*, 1999). Furthermore, keratinocyte damage in vitiligo skin has been described earlier in light- and electron-microscopic studies of vitiligo skin biopsies (Montes *et al.*, 2002; Bhawan and Bhutani, 1983; Hann *et al.*, 1992; Moellmann *et al.*, 1982). In these studies damaged keratinocytes were found to a varying extent in normal appearing skin adjacent to amelanotic skin. These damaged keratinocytes showed vacuoles and focal deposits of extracellular granular material were present. Likewise, in atopic dermatitis and contact dermatitis infiltration of the skin by activated T cells is found to induce keratinocyte apoptosis (Trautmann *et al.*, 2000; Raj *et al.*, 2006). Consistent with these findings is the case of a melanoma patient who was treated recently with anti-MART-1 T cell receptor gene transfer therapy at the National Cancer Institute (NIH, Bethesda, MD). This patient experienced a severe skin reaction following the T cell-based treatment, which resolved by spontaneous healing of the skin after two weeks. Immunohistochemistry of skin biopsies revealed a large T cell infiltrate in the damaged epidermis accompanied by widespread keratinocyte apoptosis, directly resembling our observations in the skin explants (Dr. N.P. Restifo – personal communication). Not only do these observations confirm that keratinocyte bystander-apoptosis can accompany a strong melanocyte antigen-specific T cell response, but also indicate that unexpected adverse effects can occur during clinical application of new immunotherapy protocols. In this context, the use of *ex vivo* models of autoimmunity, like the current skin explant model, could prove useful in predicting the risks of these immunological approaches.

The clinical data of the patients tested (Table 2) show a correlation with the functional T cell data in figure 2. These results suggest that the stronger the $CD8^+$ T cell response, the more elaborate the vitiligo (supported by the incidence of poliosis). Furthermore, the patients showing a more intense $CD8^+$ T cell reaction also appeared to need a longer time period of UVB irradiation before the first repigmentation occurred. This suggests a relationship between the functional reactivity of melanocyte antigen-specific T cells in the skin, and the clinical appearance of vitiligo. When combined with the skin explant assay results which demonstrated that perilesional T cells actively induced depigmentation through the induction of melanocyte apoptosis, this indicates a causal relationship between the perilesional T cells and the depigmentation in vitiligo. Nonetheless, the T cell effector function mediating depigmentation shown here, does not exclude a potential role for melanocyte antigen-specific auto-antibodies that are frequently found in progressive vitiligo patients. These auto-antibodies may

well aid and amplify an ongoing T cell response by specifically enhancing the uptake and presentation of melanocyte-specific antigens by professional antigen presenting cells, for example through Fc receptor-mediated antigen ingestion.

Furthermore, patient vit 8 displayed substantial numbers of IL-17 producing $CD8^+$ T cells in response to melanocyte antigen-specific stimulation. These cells have been directly related to inflammation and autoimmunity (Korn *et al.*, 2007) as well as anti-tumor immunity (Muranski *et al.*, 2008; Kryczek *et al.*, 2007). The fact that this patient is also the only one with halo nevi and poliosis in nearly all lesions is merely an observation; nevertheless it could hint at a role for IL-17 in more florid cases of vitiligo. Indeed, further exploration of IL-17 producing T cells in vitiligo could provide new pathogenic insights.

In summary, this study establishes that the vitiligo perilesional skin-infiltrating T cell population is enriched for melanocyte-reactive cytotoxic T cells, and that these T cells kill melanocytes within the skin, thereby causing the loss of pigmentation characteristic for vitiligo vulgaris. Additionally, the skin explant model can be used as a useful predictive tool for assessing the effects and risks of future melanoma immunotherapy protocols.



## MATERIALS & METHODS

### Patients

All patient and donor material was collected after written informed patient consent using protocols approved by the medical ethical committee of the Academic Medical Center in Amsterdam, according to the Helsinki guidelines. All vitiligo patients (n=14) were included at the Netherlands Institute for Pigment Disorders and presented with active vitiligo vulgaris indicated by progression of depigmentation within 6 months prior to inclusion. The macular depigmentation showed a random, more or less symmetrical distribution pattern typical for vitiligo vulgaris. Samples of healthy donor skin (n=16) were obtained from anonymous residual specimens discarded after plastic surgery of the breast or abdomen.

### HLA typing

HLA typing of vitiligo patients was performed at the Department of Immunohematology and Bloodtransfusion of the Leiden University Medical Centre, via genotyping on peripheral blood collected in EDTA-tubes. HLA typing of donor skin-derived T cells and lesional vitiligo skin-derived T cells was performed via flowcytometry using a fluorescein isothiocyanate (FITC)-conjugated mouse anti-human HLA-A2-specific mAb (HLA-A2 FITC, 1 µl/sample, BD Pharmingen, USA).

### Perilesional T cells

Two millimeter punch biopsies were obtained flanking the depigmented macule. These biopsies were cultured in a humidified atmosphere at 37 °C and 5% $CO_2$ in 24-well plates in 1 ml IMDM (Cambrex Bio Science, USA) supplemented with 10% heat-inactivated

human serum type AB (Cambrex, USA), 20 U/ml interleukin-2 (Eurocetus, The Netherlands), 5 ng/ml interleukin-15 (Strathmann Biotec AG, Germany), 15 µg/ml gentamycin (Duchefa, The Netherlands), 2 mM L-glutamine (Gibco Invitrogen, USA), 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco Invitrogen, USA) and 50 mM 2-mercaptoethanol (Sigma-Aldrich, The Netherlands). Additionally 1.25 µl/ml anti-CD3/CD28 mAb-coated T cell expander beads (Dynal Biotech – Invitrogen, USA) were added to promote T cell outgrowth.

### PBMC and T cell clones

PBMCs were isolated from peripheral blood by Ficoll gradient centrifugation (Lymphoprep, Fresenius Kabi Norge AS, Norway), and resuspended in IMDM with supplements as stated above. PBMC were cultured using equal conditions and time periods compared to isolated perilesional T cells prior to the skin explant assays. The T cell clones CTLAKR108, CTLAKR4D8 and CTLINFA13 were obtained, cultured and restimulated as previously described (Hooijberg *et al.*, 2000; Verra *et al.*, 2004), in the same medium as the perilesional T cells.

### Tetramer synthesis and T cell specificity analysis

R-phycoerythrin (PE)- or allophycocyanin (APC)-conjugated HLA-A2/peptide complex tetramers were synthesized as described before (Altman *et al.*, 1996), for the antigens tyrosinase$_{369\text{-}377}$ (YMDGTMSQV), gp100$_{280\text{-}288}$ (YLEPGPVTA), gp100$_{209\text{-}217}$ (ITDQVPFSV), MART-1$_{26\text{-}35}$ (modified position 27 (A>L): ELAGIGILTV) and the control antigen influenza virus$_{58\text{-}66}$ (GILGFVFTL). T cells were incubated with HLA-A2/peptide tetramers at 37 °C and 5% $CO_2$ in T cell culture medium for 15 minutes. Subsequently cells were counterstained with FITC-conjugated mouse anti-human CD8 mAb (CD8-FITC, 1 µl/sample, BD biosciences, USA). Antibody and tetramer binding to T cells was subsequently analyzed by flowcytometry (FACS Canto II, Beckton Dickinson, USA).

### Melanocyte antigen-specific T cell stimulation and flowcytometric analysis

T cells were stimulated in a 1:1 ratio with $10^5$ JY cells loaded with a pool of melanocyte antigen-specific peptides (500 ng/ml each): tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$. Flu$_{58\text{-}66}$ peptide-loaded JY were used as a separate negative control incubation, PMA/Ionomycin incubations (1/500; Leukocyte activation cocktail, Beckton Dickinson, USA) as separate positive controls. Cells were incubated for 5 hours at 37 °C and 5% $CO_2$, in 200 µl per 96-well IMDM with supplements and culture conditions as stated above, in the presence of protein transport inhibitor brefeldin A (1/1000; Golgiplug, Beckton Dickinson, USA). If required CD107a mAb was present during the co-culture (1/200, lot. 32100, BD Pharmingen, USA). Subsequently cells were stained on ice for 20 minutes for the surface markers (0,5 µl/sample): CD4 (PerCP-Cy5.5, RPA-T4, 1 µl/sample, Biolegend, USA), CD8 (APC-Cy7, ref. 348813, BD Bioscience, USA), CD69 (PE-Cy7, FN50, Biolegend, USA), CD137 (FITC, MCA1612F,

AbD serotec, UK) and CD154 (APC, 24-31, Biolegend, USA). Upon permeabilisation using the cytofix/cytoperm kit according to the manufacturer's instructions (Beckton Dickinson, USA) cells were stained for intracellular markers for 20 minutes on ice (0,5 µl/sample): IL-4 (FITC, MP4-25D2, Biolegend, USA), IL-17 (PE, eBio64CAP17, eBioscience, USA), granzyme-B (Alexa 700, lot. 01702, BD Pharmingen, USA), TNF-α (PerCP-Cy5.5, Mab11, Biolegend, USA) and IFN-γ (Alexa 700, 4SB3, Biolegend, USA). Antibody binding to T cells was subsequently analyzed by flowcytometry, measuring 7 fluorochromes simultaneously (FACS Canto II, Beckton Dickinson, USA). Comparison of the data required normalization; therefore the percentages depicted in figure 2 represent the percentages of $CD4^+$ or $CD8^+$ T cells reactive to the peptide pool *minus* the percentage of these T cells reactive to unloaded JY cells (background).



### Cell sorting

The $CD8^+$ T cell-enriched and $CD8^+$ T cell-depleted perilesional T cell populations were prepared using anti-human CD8 mAb microbeads and magnetic cell separation columns (Miltenyi Biotec, Germany), according to the manufacturer's instructions. The vitiligo lesional skin-derived $gp100_{280\text{-}288}$ specific T cell population was purified using HLA-A2/$gp100_{280\text{-}288}$ tetramer staining and subsequent flowcytometric cell sorting (FACS Aria, Beckton Dickinson). Sorted T cells were analyzed for HLA-A2/gp-$100_{280\text{-}288}$ tetramer binding, and antigen specific activation.

### CFSE labeling of T cells

T cells were centrifuged in a 15 mL tube to remove supernatant. Cell pellet was resuspended in 1 ml of PBS containing 5 µM of carboxyfluorescein succinimidyl ester (CFSE, Invitrogen). Cells were incubated 10 minutes at 37 °C in the dark, whereafter 14 ml of T cell culture medium was added. Cells were washed in T cell culture medium three times prior to use in skin explant assays.

### Skin explant assay

Two millimeter punch biopsies were obtained from the *non*-lesional (normally pigmented) or lesional skin of vitiligo patients. Each biopsy was co-cultured in a 96-well round bottom plate with $3\text{-}5x10^5$ autologous perilesional T cells, CTLs or T cell clones of interest, for two days in 200 µl per well IMDM with supplements and culture conditions as stated above. Subsequently, explants were washed three times in Dulbecco's phosphate buffered saline (PBS-D, Gibco Invitrogen, USA), and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek Europe, The Netherlands) for further immunohistochemical and/or CLSM analysis on cryosections of the explants.

### Immunofluorescence staining

Acetone-fixed 5 µm skin cryosections of skin explants were incubated with 10% normal goat serum (DAKO, Denmark) in Tris-buffered saline (TBS) for 15 minutes. Subsequently series of sections were stained with the following antibodies: NKI-beteb

(mouse anti-gp100 mAb, 1/40, Monosan, The Netherlands), rabbit anti-active caspase-3 mAb (1/100, affinity purified, BD Pharmingen, Switzerland), mouse anti-pan cytokeratin mAb (1/400, Abcam, United Kingdom), diluted in TBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich), and incubated for 1 hour at room temperature (RT) in the dark. Subsequently the murine antibodies were detected by biotinylated polyclonal goat-anti-mouse immunoglobulins (GAM-bio, DAKO) in TBS with 10% normal human serum (NHS, Sanquin) for 30 minutes RT and Cy5-conjugated streptavidin (1/90, Jackson Immunoresearch Laboratories) in TBS with 1% BSA for 30 minutes in the dark. To detect the rabbit primary antibodies, sections were incubated in the dark with Cy3-conjugated goat anti-rabbit IgG (1/400, Jackson Immunoresearch Laboratories, United Kingdom) at RT. Sections were subsequently blocked with 10% normal mouse serum (DAKO) in TBS for 15 minutes RT in the dark, and were incubated with mouse-anti-human-CD3-FITC (1/125, clone SK7, BD Biosciences Europe, Belgium) in TBS with 1% BSA for 1 hour RT in the dark. For all antibodies the corresponding isotype controls were used (mouse IgG2b κ, 1/250 (BD Pharmigen), rabbit IgG1 1/1000 (Vector laboratories, USA), mouse IgG1-FITC, 1/100, BD Biosciences). Sections were mounted using Vectashield mounting medium for CLSM analyses (Vector laboratories).



### Immunohistochemistry

Endogenous peroxidase was blocked on acetone-fixed 5 μm explant cryosections by incubation with 0.25% hydrogen peroxide (Sigma) and 0.001% sodium-azide in TBS for 20 minutes at RT. Subsequently sections were incubated with 10% normal goat serum (DAKO) in TBS for 15 minutes at RT. The following primary antibodies were incubated for 60 minutes at RT in TBS with 1% BSA: mouse anti-human CD3 FITC mAb (1/125, clone SK7, BD Biosciences), mouse anti-human CD8 mAb (1/100, BD Biosciences, USA) or rabbit anti-active caspase-3 mAb (1/100, affinity purified, BD Pharmingen). For all antibodies the corresponding isotype controls were used (mouse IgG1, 1/250 (BD Biosciences, USA), rabbit IgG1 1/1000 (Vector laboratories, USA) or mouse IgG1-FITC, 1/100, BD Biosciences, USA). Subsequently, bound antibody on the tissue sections was detected by either biotinylated goat anti-mouse or goat anti-rabbit immunoglobulins (1/200 and 1/50 respectively, DAKO) in TBS with 10% NHS. All sections were incubated with streptavidin-HRP (1/400, DAKO) in TBS with 1% BSA for 30 minutes RT, and antibody reactivity was detected by incubation with AEC substrate (Vector laboratories) according to manufacturer's instructions. Sections were counterstained with hematoxylin (Sigma-Fluka) for 4 minutes RT and coverslips were mounted using Kaiser's glycerine gelatine (Sigma).

### CLSM data analysis

Immunofluorescence staining was analyzed using a Leica TCS-SP2 confocal laser scanning microscope system, equipped with argon/krypton and helium/neon lasers and using a 40.0x1.25 (OIL UV - HCX PL APO CS) numerical aperture 1.25 objective

(Leica Microsystems Heidelberg GmbH, Germany). Possible crosstalk between different fluorochromes, which could lead to false-positive colocalisation, was avoided by sequential measurement of individual channels. Approximately 5 photomicrographs spanning the full length of the biopsy epidermis were acquired for each cryosection, and 3 serial cryosections were analyzed for each mAb per individual skin explant analysis. Color images were taken from each channel, and electronic 3-color overlays were made using Leica LCS-Lite confocal software (v2.00, Leica Microsystems).

**IFN-γ measurement.**

T cells were stimulated overnight in a 1:1 ratio with JY cells loaded with the gp100-derived peptide YLEPGPVTA or the tyrosinase-derived peptide YMDGTMSQV or left unstimulated. The amount of IFN-γ present in the culture supernatants was measured using the enzyme-linked immunosorbent assay (ELISA) with a PeliKine Compact Human IFN-γ ELISA kit (Sanquin), according to the manufacturer's instructions.



## CONFLICT OF INTEREST

**The authors state no conflict of interest.**

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## AUTHOR CONTRIBUTIONS

D.K. and J.G.B. conducted all experiments, CLSM and immunohistochemical analysis. J.G.B. prepared figures and drafted and prepared the manuscript. T.A.D. and F.A.V. provided technical assistance for CLSM and immunohistochemistry. J.P.W.V. provided patients for this study. R.M.L., F.A.V. and J.D.B. supervised the project. R.M.L., F.A.V. and C.J.M.M. initiated and supervised the project.

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# CHAPTER 4

## THERAPEUTIC IMPLICATIONS OF AUTOIMMUNE VITILIGO T CELLS

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## ABSTRACT

Vitiligo is an autoimmune disease presenting with progressive loss of skin pigmentation. The disease strikes 1% of the world population, generally during teenage years. The progressive loss of melanocytes from depigmenting vitiligo skin is accompanied by cellular infiltrates containing both $CD4^+$ and $CD8^+$ T lymphocytes. Infiltrating cytotoxic T cells with high affinity T cell receptors have likely escaped clonal deletion in skin draining lymph nodes, allowing such T cells to enter the circulation. Through the expression of CLA, these T cells home to the skin where they express type 1-cytokine profiles and mediate melanocyte apoptosis via the granzyme/perforin pathway. T cells found juxtapositionally apposed to remaining melanocytes can be isolated from the skin. Vitiligo T cells have demonstrated reactivity to antigens previously recognized as target antigens for T cells infiltrating melanoma tumors. In a comparison to existing melanoma-derived T cells, vitiligo T cells displayed superior reactivity towards melanoma cells. It is thought that genes encoding the TCRs expressed by vitiligo skin infiltrating T cells can be cloned and expressed in melanoma T cells, thereby generating a pool of circulating T cells with high affinity for their targets that can re-direct the immune response towards the tumor.




**Bullet points:**

- Vitiligo is characterized by progressive loss of melanocytes from the epidermis accompanied by elevated titers of anti-melanocyte antibodies and by inflammatory skin infiltrates.
- Melanosomes are lysosome-like organelles where melanogenic enzymes reside that form prime target antigens for T cells infiltrating melanoma tumors or depigmenting vitiligo skin.
- Melanocytes secrete cytokines, and can phagocytoze, process and present antigens to T cells in the context of MHC class II, accounting in part for their immunogenicity.
- Depigmenting vitiligo skin is characterized by MHC class II expressing melanocytes by granzyme-expressing, skin infiltrating T cells with type 1 cytokine profiles.
- Polyclonal cytotoxic T cells derived from vitiligo skin are highly reactive towards melanoma cells and may serve as a superior source of high affinity TCR genes to treat melanoma.

## THE HISTORY OF AUTOIMMUNE VITILIGO

Vitiligo is defined as a skin disorder characterized by progressive loss of pigmentation (1). The age of onset is most frequently found in the 2nd decade of life, coinciding with major hormonal changes, yet a subset of patients acquires vitiligo much later in life (2). The distribution of lesions appears to vary accordingly (3). It is thought that as much as 1% of the world population is afflicted by vitiligo (1). Convincing epidemiologic studies to assess the distribution of vitiligo among ethnic groups are lacking and although there is currently no evidence for a bias towards a particular skin type, the appearance of milky-white skin lesions is particularly devastating against a background of darkly pigmented skin (4). Coincidentally, vitiligo lesions can resemble depigmented skin patches in patients with tuberculoid leprosy, a disease not yet fully eradicated (5). Here vitiligo patients can be ostracized within their communities, with severe social and economical consequences.

Existing therapies for vitiligo are considered inadequate (6). Patients with a recent diagnosis of vitiligo who are seeking treatment may consider their treatment effective, yet (unpublished observation) the majority of long-standing patients are not currently seeking treatment and prior treatment attempts are generally considered ineffective. We speculate that this apparent difference of opinion reflects a true difference in efficacy, as the majority of newly diagnosed patients is young and therefore likely more responsive to therapy in general, and newly diagnosed patients are more likely to receive novel and more effective therapeutic measures based on recent findings regarding vitiligo etiology.



In order to investigate whether vitiligo etiology involves an autoimmune response to melanocytes, it was necessary to first establish that milky-white skin patches were devoid of melanocytes rather than lesions being caused by melanocytes having lost the ability to generate melanin. Immunostainings with a panel of antibodies reactive with different melanocyte antigens supported the loss of differentiated melanocytes from vitiligo lesional skin. (7) The question remains whether the epidermis harbors undifferentiated melanocyte stem cells, and whether these stem cells will be left unharmed in vitiligo skin. As shown in Figure 1, c-KIT expression is not observed in lesional vitiligo epidermis. Since c-KIT signaling is considered crucial for the proper distribution and maintenance of melanoblasts in the epidermis, these findings suggest that vitiligo skin is devoid of melanocyte precursors (7). Thus, it may not be possible to repigment vitiligo skin through the differentiation of stem cells already present within the epidermis. However, a source of stem cell factor receptor , c-KIT expressing, melanocyte precursors is often maintained in the bulge region of the hair follicle, within vitiligo lesional skin. The stimulation of melanocyte differentiation and migration from hair follicles can be a successful treatment for vitiligo. This is supported by reports of 'freckly' repigmentation in patients undergoing PUVA therapy (8).

The concept of vitiligo as an autoimmune disease was introduced following reports of autoantibodies found in serum from patients with active disease (9). This phenomenon has been studied in great detail, and antibodies directed against

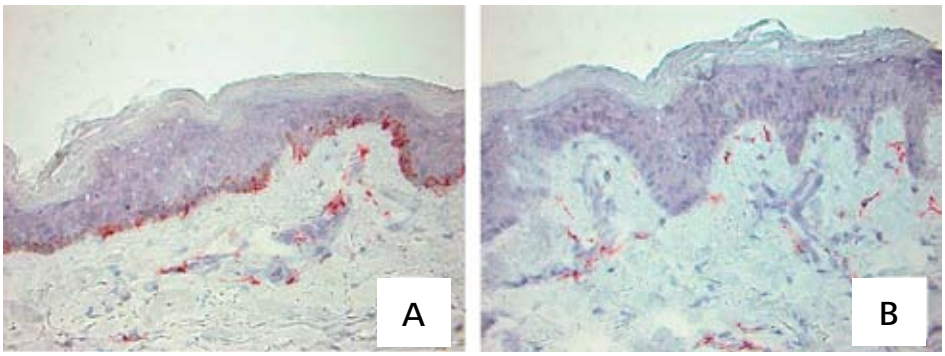


**Figure 1. Lack of expression of c-kit in lesional vitiligo skin.** Sections of (A) non-lesional and (B) lesional vitiligo skin were subjected to indirect immunoperoxidase staining. Expression of the steel factor receptor was observed in non-lesional, but not in lesional skin. Original magnification 400x.



melanocytes are clearly more prevalent in patients with active vitiligo. These antibodies are generally reactive with intracellular antigens, and humoral immunity to pigment cells may constitute an epiphenomenon that occurs in response to cell damage. In this respect the discovery of the melanin-concentrating receptor-1 (MCR1) as a target for vitilgo autoantibodies is of interest (10). This can explain at least in part why adoptive transfer of vitiligo serum can induce depigmentation of xenografted human skin in SCID mice (11). Further evidence in support of a role for antibodies is provided by demonstrating antibody-dependent cellular cytotoxicity (ADCC) *in vitro* (12). At the very least, humoral responses indicate active anti-melanocyte reactivity.

We have demonstrated the consistent presence of an inflammatory infiltrate surrounding expanding vitiligo lesions (13). This has long been overlooked, likely because infiltrates are found only in active disease and because the infiltrates are generally small (13). Frequently, patients report progression of their lesions to be associated with itchy skin (unpublished observation). Infiltrates consist of macrophages, as well as dendritic cells and T cells. In contrast to psoriasis, no B cells are found in vitiligo skin (14). T cells found in peri-lesional skin of active lesions express the skin homing receptor CLA (cutaneous lymphocyte antigen), indicating that these T cells were 'en route' to the skin (13). These finding greatly evoked our interest in cell mediated autoimmunity and its role in vitiligo pathogenesis.

It should be mentioned, that depigmentation will develop only in individuals prone to develop vitiligo. In this respect there are many reports of melanocytes with aberrant morphology in pigmented skin from vitiligo patients, suggesting that these melanocytes are increasingly sensitive to stress (15,16). Indeed, 'occupational vitiligo' in response to phenolic agents will occur only in a subset of individuals (17). It appears that melanocytes from vitiligo patients will respond differently to stress: we have demonstrated that vitiligo melanocytes will release more HSP70 in response to model stressor compound 4-TBP (18). Stress proteins can function as peptide chaperones and

are capable of activating dendritic cells (19, 20). In fact, stress protein/ tumor antigen fusion proteins are currently in clinical trials for the treatment of human tumors, including melanoma. Interestingly, it has been shown that vitiligo development in melanoma patients is an indicator of effective immunity against the tumor, and vitiligo in melanoma patients is considered a positive prognostic factor (21). It is therefore of great importance to analyze the similarities and discrepancies among effective immunity to melanocytic cells in autoimmune vitiligo, and the lack thereof in patients with malignant melanoma (22).

## A GLANCE AT MELANOCYTE IMMUNOBIOLOGY

Melanocytes are unique in their ability to confer pigmentation to the skin. The process is based on melanin synthesis within specialized organelles, the melanosomes. Melanosomes contain enzymes and structural components required for effective melanization. MART-1 appears to lay the groundwork for the deposition of the melanosomal matrix, assisted by gp100 (23). These 2 molecules are also considered the most immunogenic antigens of the melanocytic cells and T cells infiltrating melanoma tumors are most frequently reactive with either of these antigens (24). The subsequent arrival of tyrosinase and tyrosinase-related enzymes TRP-1 and TRP-2 completes the arsenal of melanosomal enzymes. Eumelanin (brown/black melanin) offers better protection against UV whereas in pheomelanogenesis, more radicals are generated than can be scavenged by the yellow/reddish brown end product, explaining in part why pheomelanized individuals (including orientals) will develop skin tumors more frequently than darkly pigmented individuals (25).



The melanosome is unique in particular because it is transferred to neighboring cells in a process that remains poorly understood to date. This process is of particular interest to immunologists. Organelle transfer has otherwise been described only for exosomes, small lipid vesicles serving to discard or transfer membrane proteins. Exosomes are considered important for DC activation and the induction of immune responsiveness to antigens contained within these organelles (26). Interestingly, organelles transferred from melanocytes to keratinocytes are in fact equivalent to lysosomes in other cells. It is well possible that lysosome transfer can likewise occur between cells as well but cannot be easily observed for lack of a marker as readily detectable as melanin. The transfer of lysosome-like organelles may allow for sharing of antigens destined for presentation in the context of MHC class II. It should be noted that ripe melanosomes do not contain intact melanogenic enzymes as shown by melanocyte fractionation (27). But peptide fragments may be generated within the acidic environment of the melanosome. Resulting peptides may retain their immunogenicity when transferred to dendritic cells. Thus peptides derived from gp100, MART-1 and other melanosomal proteins, may be transferred to infiltrating dendritic cells in the absence of melanocyte death within the skin. This may explain why melanocytes are highly immunogenic whereas this cell type is otherwise very resistant to apoptosis (28).

Melanocyte immunogenicity has also been ascribed to their Langerhans cell-like morphology and distribution. Melanocytes synthesize primary cytokines, and are thus capable of eliciting a local immune response (29). Moreover, we have previously demonstrated that melanocytes are capable of phagocytosis and can process and present antigens in the context of MHC class II to CD4+ proliferative and cytotoxic T cells (30,31). As melanocytes lack the mobility of Langerhans cells and other professional antigen presenting cells, it is conceivable that antigen presentation within the skin renders the melanocyte a 'sitting duck' for an immune attack. Of course, this situation will arise only when the melanocyte expresses MHC class II molecules, which happens to be the case only in vitiligo and in melanoma (32). These are also situations where T cell responses to melanocytes prevail. In conclusion, a role for melanocytes within the Skin Immune System cannot be overlooked as we assess the etiology of vitiligo.

## INVOLVEMENT OF T CELLS IN DEPIGMENTATION



T cells are more prevalent in vitiligo perilesional skin than in surrounding non-lesional or lesional skin. An occasional T cell remains in lesional skin but most of the infiltrate appears to migrate with the depigmenting epidermal border. Among the T cells, the infiltrate consists mainly of CD8 but also of CD4 T cells (33). Since cytotoxic T cells are especially prevalent and colocalize with remaining melanocytes, it was postulated that these T cells are actively cytolytic towards remaining melanocytes (34). T cells express granzyme and the prevailing theory is that cytotoxicity is mediated by the granzyme/perforin pathway rather than through Fas/FasL in vitiligo (unpublished observations). It is well possible that cytotoxicity is executed in part by CD4+ T cells as HLA-DR expression by melanocytic cells appears to be limited to melanocytes in vitiligo marginal skin and melanoma cells within tumors. In this respect it is of interest that the T cell clones originally isolated from a patient with leprosy that were used to demonstrate reactivity towards MHC class II expressing melanocytes were reactive with mycobacterial stress protein hsp65 (31). This is noteworthy, particularly since Schwann cells harbor the causative *M. leprae* organism in leprosy, whereas Schwann cells are developmentally very closely related to melanocytes (35). This similarity may ultimately contribute to cross reactivity or epitope spreading from Schwann cell antigens to melanocytes, explaining the similarity among lesions observed in tuberculoid leprosy and autoimmune vitiligo.

The involvement of T cells in vitiligo is further supported by the observation that depigmentation is accompanied by the expression of type 1 cytokines. We found that IFN-γ inducible CDw60, representative of the ganglioside GD3, was upregulated in perilesional skin (36). The type 1 cytokine profile observed for T cells isolated directly from depigmenting lesions likewise support a T cell mediated autoimmune response.

## PROMISING ASPECTS OF HIGH AFFINITY AUTOIMMUNE TCR

Skin infiltrating T cells can be isolated from tissue biopsies of interest and grown in bulk. These T cells can be collected and propagated without antigen selection in presence of IL-2 and anti-CD3/anti-CD28 antibody coated beads. T cells have been isolated from perilesional skin of an HLA-A*0201+ patient with active vitiligo. These unselected T cells were found to have similar reactivity towards HLA-A*0201+ melanocytes when compared with established T cell lines from melanoma patients that are >90% melanoma reactive (unpublished observation). Importantly, our vitiligo T cells demonstrated superior reactivity towards HLA-A*0201+ melanoma cells. We have since determined that >15% of our T cells were reactive with the gp100 209-217 epitope as shown in Fig. 2. Interestingly, among six T cell clones reactive with the gp100 209-217 epitope we have thus far identified two different TCRs (unpublished). Our data support the current hypothesis, that T cells isolated from vitiligo skin are highly reactive with melanoma-associated antigens (37). High affinity TCRs, once expressed in PBMC from melanoma patients, can more effectively target the tumor (38). It is well possible that high affinity T cells previously escaped clonal deletion in skin-draining lymph nodes and were inadvertently allowed to enter the circulation, emigrating to the skin to inflict damage to the melanocyte population. (39).

An ongoing autoimmune response may be allowed to further develop and mature in the absence of functional T regulatory cells (T regs) (40). These cells actively mediate suppression of the immune system generally by secreting IL-10 and TGF-β to prevent autoimmunity (40). Very recent data showing a decrease in the number of T regs in vitiligo skin indicate that this may apply to vitiligo as well (unpublished observation). In the absence of regulatory T cells, cytotoxic T cells with increasing affinity for their

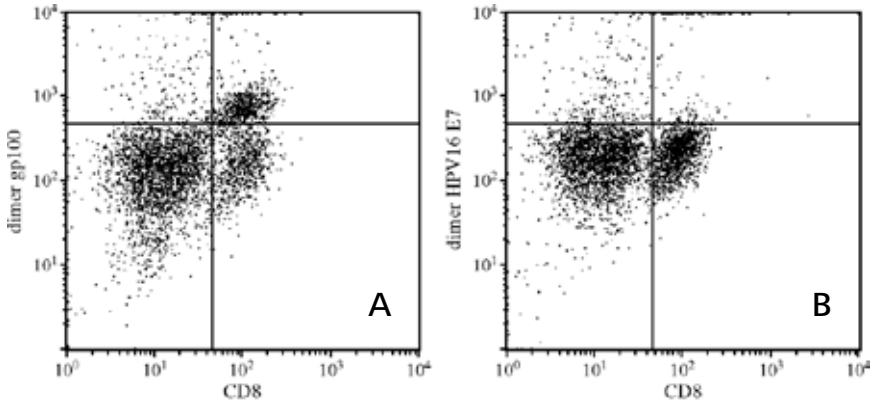


**Figure 2. Abundance of gp100 reactive T cells among lymphocytes from perilesional vitiligo skin.** Lymphocytes from a progressive generalized vitiligo patient were expanded in presence of CD3/CD28 coated beads and IL-2, then reacted with fluorescently detected HLA-A2 dimers loaded with either (A) gp100 209-217 or (B) irrelevant human papillomavirus-derived immunogenic peptide, HPV16 E7 86-93, plus a fluorescent antibody to CD8, and analyzed by flow cytometry.

targets enter the skin and continuously proliferate and migrate towards novel target cells, causing depigmentation. This unfortunate event of disfiguring depigmented skin patches left in vitiligo patients may turn out to be a blessing in disguise if the T cell receptors expressed by high affinity T cells provide an effective treatment for melanoma patients.

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# CHAPTER 5

## MONOBENZONE INDUCES AUTOIMMUNE SKIN DEPIGMENTATION BY SPECIFIC IMMUNOGENIC MELANOCYTE MODULATION

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*Submitted*

## ABSTRACT

Monobenzone is a phenolic compound with the capacity to induce progressive skin depigmentation that is clinically and histologically indistinguishable from vitiligo vulgaris. The exact mode of action by which monobenzone induces vitiligo has remained unknown. In the present study we show that monobenzone specifically endows exposed pigmented cells with immunogenicity. Using autologous healthy human donor DC-T cell stimulations, we established that monobenzone-exposed melanoma cells induced a robust melanoma-reactive CD8+ T cell response *in vitro* within 7 days, as compared to unexposed melanoma cells. Importantly, 78% of the melanoma cell-reactive CD8+ T cell clones generated with monobenzone were found to recognize monobenzone-treated and –untreated melanoma cells equally well. Furthermore we have found that upon enzymatic conversion of monobenzone by the enzyme tyrosinase, quinone metabolites form haptens specifically to cysteine residues in proteins. Moreover, its conversion was found to generate reactive oxygen species, mediate the release of tyrosinase- and MART-1-containing CD63+ exosomes from monobenzone-exposed pigmented cells, and activate dendritic cells. These data show that monobenzone, by its specific effects on the enzyme tyrosinase, induces potent CD8+ T cell immunity against auto-antigens expressed by melanocytes and melanoma cells. Monobenzone thereby presents a powerful new drug in the field of melanoma immunotherapy.

## INTRODUCTION

In 1939 Oliver *et al.* discovered the monobenzylether of hydroquinone, (MBEH or monobenzone) to be a powerful inducer of occupational leukoderma[1]. Early clinical studies on monobenzone report skin sensitization[2-4] and the progressive spread of depigmentation beyond the skin application site[5, 6], suggesting a systemic reaction. In healthy individuals who applied the cream to acquire a lightened skin tone it can induce depigmentation that is clinically and histologically indistinguishable from vitiligo vulgaris[3, 5]. Importantly, vitiligo patients applying monobenzone to achieve complete depigmentation, often experience an application-related dermatitis exclusively in pigmented areas of skin[7], illustrating the dependence of monobenzone on melanocyte presence. Nevertheless, the precise mode of action of monobenzone has remained unknown. Studies on the structurally related, less potent[8] skin-depigmenting agent monomethylether of hydroquinone, have revealed that this compound is converted into a reactive benzoquinone product by the enzyme tyrosinase[9], present in the melanosome granule of the skin melanocyte. Benzoquinone subsequently forms haptens to cysteine residues in proteins[10, 11], and was thereby identified as a skin sensitizer[12]. Furthermore, studies in animal models have demonstrated that the skin depigmenting action of phenols or catechols depend on their conversion by the enzyme tyrosinase, and the subsequent formation of benzoquinone-haptens to proteins[13]. Thereby skin-depigmentation by this type of compound largely depends on the enzymatic conversion by tyrosinase, and indeed it was found that the resulting quinone-metabolites induced more profound depigmentation than the parental compounds[14].

Recently we have shown that vitiligo vulgaris is mediated by the autoimmune destruction of skin melanocytes by CD8+ T cells[15] and we here investigated whether monobenzone induces a destructive CD8+ T cell-mediated reaction specifically against pigmented cells, by a selective interaction with the enzyme tyrosinase in the pigmented cell. Our present study shows that monobenzone confers potent immunogenicity selectively to exposed pigmented cells via the generation of quinone-haptens, the secretion of melanocyte antigen-containing exosomes and the activation of dendritic cells. Furthermore, we establish here that monobenzone-exposed pigmented cells initiate a robust pigment-cell reactive CD8+ T cell response within 7 days *in vitro*, using autologous DC-T cell stimulations from 9 healthy human donors. Interestingly, the majority of these CD8+ T cells were found to be equally responsive to monobenzone-treated and –untreated cells on a clonal T cell level, providing an immunological basis for the spread of skin depigmentation beyond the monobenzone application site. Based on the shared origin of melanocyte antigens between melanocytes and melanoma cells, the potent immune response induced by monobenzone can also be employed against melanoma cells. Thereby monobenzone-evoked anti-melanocyte immunity presents a promising advance for melanoma immunotherapy.

## RESULTS

### Tyrosinase is inactivated by monobenzone through quinone-hapten formation.

Monobenzone is an alternative substrate for the natural tyrosinase substrate L-3,4-dihydroxyphenylalanine (L-DOPA)[16] and by reacting with tyrosinase it can inactivate the enzyme[8, 11]. To investigate the molecular effects of monobenzone on the tyrosinase enzyme, we performed spectrophotometry studies with purified mushroom tyrosinase. Figure 1A shows the natural reaction of tyrosinase to form dopachrome[17] from L-DOPA and the subsequent autoconversion of dopachrome into 5,6-dihydroxyindole (DHI)[18], as measured by absorption peaks for the formation of dopachrome at 473 nm and DHI at 290- and 310 nm. Reaction kinetics are depicted in the corresponding lower panels. In contrast, the interaction between tyrosinase and monobenzone revealed a distinct absorption pattern after 45 minutes of incubation, composed of a 260 nm absorption peak, characteristic of a covalent quinone-cysteine bond (quinone-hapten)[19] and a 420 nm absorption peak indicative of the reduced state of a copper-enzyme[20]. Tyrosinase being the copper-containing enzyme in this reaction, this result suggests that quinone-haptens formed by the monobenzone-metabolite 4-benzoxy-1,2-benzoquinon (figure 1B) inactivate the enzyme. The reaction of monobenzone with tyrosinase reached a plateau-state after 30 minutes. Furthermore, it seems that a certain level of quinone-hapten formation is required for the enzyme to become inactivated, since the 420 nm product starts to emerge following 15 minutes of incubation. When tyrosinase was pre-incubated with a higher concentration of monobenzone, addition of L-DOPA at the plateau state of the reaction did not result in subsequent dopachrome formation. This demonstrates tyrosinase was completely inactivated by the preceding incubation. Importantly, since the previous reaction had reached a plateau, mere substrate competition between monobenzone and L-DOPA is excluded.

These data reveal at the molecular level that the enzyme tyrosinase is inactivated by monobenzone, likely by the formation of quinone-haptens that reduce the enzyme's catalytic core.

### Quinone-haptens occur specifically at cysteine residues in peptide chains.

To test whether the quinone metabolite 4-benzoxy-1,2-benzoquinon binds cysteine residues within peptides upon monobenzone conversion by tyrosinase, we incubated various 9-mer peptides containing 0-, 1- or 2 cysteine residues with synthetically prepared 4-benzoxy-1,2-benzoquinone (table 1). Peptides containing cysteine were incubated with a 2-fold excess of the metabolite to allow rapid quinone-hapten formation. As a control, a peptide containing no cysteine residues was incubated with an 8-fold excess of the quinone. The liquid chromatography/mass spectrometry (LC/MS) results are summarized in table 1. The peptides containing cysteines both formed quinone-haptens (peptide 1 & 2). In contrast, the control peptide 3 not containing any cysteine, did not display any quinone haptenation. These results demonstrate that 4-benzoxy-1,2-benzoquinon is able to form quinone haptens by selectively binding to cysteine residues in peptides.

**Table 1.** Binding of the quinone hapten to peptide chains is cysteine-specific.

| | | | | quinone hapten observed? | |
|---|---|---|---|---|---|
| **peptide number** | **peptide sequence** | **number of cysteines** | **Excess of 4-benzoxy-1,2-benzoquinon** | **unmodified peptide** | **modified peptide** |
| **1** | CTELKLSDY | 1 | 2x | – | + |
| **2** | KCDICTDEY | 2 | 2x | – | + |
| **3** | KVPRNQDWL | 0 | 8x | – | – |

–: specific mass of the quinone-hapten not detected
+: specific mass-increase of quinone hapten detected

### Cellular pigment synthesis is suppressed by monobenzone.

To determine the effects of monobenzone on melanogenesis, we incubated human cutaneous melanoma cells and normal human melanocytes with various concentrations of monobenzone for 72 hours *in vitro*. Melanin synthesis was induced in the melanoma cells by isobutylmethylxanthine (IBMX) and melanocyte-stimulating hormone (αMSH). The normal human melanocytes did not require additional pigment synthesis stimulation. The melanoma cells showed a significant increase in pigmentation upon IBMX/αMSH exposure (figure 1C). When monobenzone was present simultaneously with the IBMX/αMSH stimulus, a significant dose-dependent decrease in cellular melanin content was observed. Likewise, melanin content in normal human melanocytes was significantly reduced upon monobenzone exposure. These cells appeared more sensitive to monobenzone, since the lowest concentration of monobenzone already gave near-maximal reduction of cellular melanin content. Importantly, equal numbers of viable cells were used for each experiment, to exclude effects of divergent cell numbers on melanin determinations.

These data demonstrate that the molecular effects of monobenzone on the enzyme tyrosinase translate to effective inhibition of melanogenesis at the cellular level.

### Monobenzone is not selectively toxic to pigmented, tyrosinase-positive cells.

It has been reported previously that high concentrations of monobenzone can be selectively toxic to tyrosinase-expressing pigment cells[21]. We carried out experiments to identify if monobenzone is selectively toxic to pigmented cells at the concentrations used in our experiments. As shown in figure 1D, sensitivity to monobenzone varied between cell lines, however this did not correlate to presence of pigmentation. Interestingly, the amelanotic tyrosinase-negative melanoma cell line BLM displayed no decreased survival in monobenzone when tyrosinase was re-expressed in these cells by tyrosinase-cDNA transfection (BLM-tyr). These data show that the sensitivity of cells to monobenzone is independent of their pigmentation level, or the presence of the tyrosinase enzyme. As a control, we have incubated the same cell panel with different concentrations of $H_2O_2$. It has been shown that melanin protects cells against oxidative stress, increasing cell survival by acting as an antioxidant[22]. As shown in figure 1D (*right panel*), pigmented cells clearly show a survival benefit as compared to amelanotic

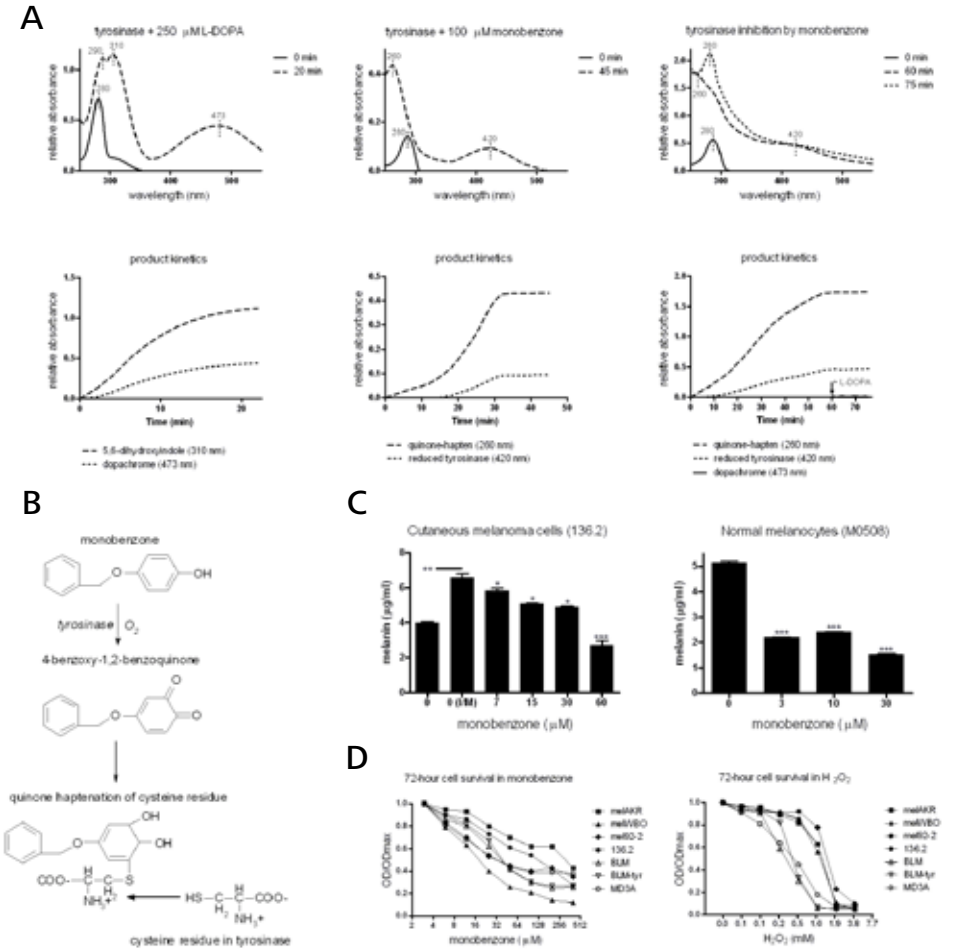


**Figure 1. Monobenzone is converted into a reactive benzoquinone metabolite by the enzyme tyrosinase, which reduces cellular pigmentation but is not selectively toxic to pigmented cells. A,** Spectrophotometry showing the reaction of L-3,4-dihydroxyphenylalanine (L-DOPA) or monobenzone with purified mushroom tyrosinase. The reactants described at t=0 min are represented by the 280 nm absorption peak. *Left panels:* Tyrosinase converted L-DOPA (250 µM) into the melanin-intermediates dopachrome and 5,6-dihydroxyindole (DHI), represented by the 473- and 310/290 nm absorption peaks respectively. Product formation kinetics showed gradual increase of the metabolites in time. *Middle panels:* Upon interaction with 100 µM monobenzone, covalent quinone-cysteine bonds were formed (260 nm peak). Furthermore, the tyrosinase enzyme was reduced (420 nm). Product kinetics displayed a reaction plateau-phase after 30 minutes of incubation. *Right panels:* When tyrosinase was incubated with 300 µM monobenzone, the enzyme was completely inactivated. After 60 minutes of tyrosinase incubation with monobenzone, absorption peaks of the reduced enzyme (420 nm) and the quinone-cysteine bonds (260 nm) were visible. At this time point, 250 µM L-DOPA was added to the reaction, evidenced by the appearance of an additional 280 nm absorption. Following an additional 15 minutes of incubation, no dopachrome- or DHI formation was observed. Product kinetics showed gradual formation of the 260- and 420 nm products over time, until a plateau-phase after 60 minutes. L-DOPA addition to the mixture (arrow) did not result in production of dopachrome in an additional 15 minutes of incubation. Data represent the results of 5 independent experiments. **B,** Schematic overview of the conversion of monobenzone into 4-benzoxy-1,2-benzoquinon by the enzyme tyrosinase. ▷

▷ 4-benzoxy-1,2-benzoquinon can bind covalently to cysteine residues by nucleophilic attack of sulfhydryl groups. **C,** Monobenzone suppresses melanogenesis in melanoma cells and melanocytes. Melanoma cells (136.2; left), cultured under pigment synthesis-stimulating conditions using isobutylmethylxanthine and melanocyte-stimulating hormone (I/M), or melanocytes (M0508; right) were incubated with increasing concentrations of monobenzone. Graphs show a dose-dependent decrease of cellular melanin content. All culture conditions contained equal numbers of viable cells for comparison. Both panels are representative of two independent experiments using either two melanoma cell lines, or two lines of normal melanocytes respectively. *: $p<0.05$, **: $p<0.01$, ***: $p<0.0001$ using paired t-test (95% CI). Graphs depict mean with SEM. **D,** Monobenzone is not selectively toxic to pigmented cells. *Left panel:* Pigmented (*black symbols)* or amelanotic (*open symbols*) human melanoma cell lines were incubated for 72 hours in increasing concentrations of monobenzone. Pigmented and amelanotic melanoma cells showed variable survival in the presence of monobenzone, not correlating to cellular pigmentation. Expression of tyrosinase enzyme in the amelanotic melanoma cell line BLM (BLM-tyr) did not affect the survival of this cell line. *Right panel:* The same cell panel was incubated for 72 hours in the presence of $H_2O_2$, showing the protective effect of pigmentation on cell survival under oxidative stress.

cells in the presence of $H_2O_2$-induced oxidative stress. Pigmented melanoma cells were markedly less sensitive to $H_2O_2$, likely by the radical-scavenging properties of melanin.

### Monobenzone-exposed pigmented cells induce melanoma-reactive CD8⁺ T cells within 7 days in vitro.

Since monobenzone is known to induce leukoderma, which can spread to non-exposed sites, we investigated the nature of this systemic reaction. In particular, we studied whether monobenzone can induce CD8+ T cell-reactivity to pigmented cells. To characterize the immunological impact of monobenzone exposure to pigmented cells, we performed a series of cross-presentation experiments using monocyte-derived dendritic cells (DC) and autologous (resting) T cells from 9 healthy human donors. The DCs were loaded with either a freeze-thaw melanoma cell lysate, or with melanoma cells exposed overnight to 20 or 40 μM monobenzone. Melanoma cell lysate is well suited to prime tumor antigen-specific T cells by DCs[23], and thereby functions as a reference stimulation. Furthermore, it provides an unmodulated and representative source of melanoma-specific antigen to the DCs, since standard procedures such as UV- or γ-irradiation of melanoma cells are known to significantly alter autoantigens and their expression, -processing- and -presentation[24]. Importantly, no additional DC-activating stimuli such as TLR-ligands were included in the cultures. The DCs were confirmed to be immature prior to co-culture with T cells by flowcytometry (figure 4 *upper row*).

Figure 2 depicts a representative healthy-donor DC-T cell stimulation. The columns designated "*ex vivo*" show that CD8+ T cells from healthy donors did not *ex vivo* recognize autologous DCs presenting melanoma cells or melanoma cells previously exposed to 40 μM monobenzone (both either lysed or intact). Upon 7 days of co-culture under these conditions, and subsequent re-stimulation, a population of CD8+ T cells reactive to DCs cross-presenting monobenzone-exposed melanoma cell lysate had emerged, while DCs loaded with unexposed melanoma cell lysate (or intact cells) did not induce T cell activation. Control stimulations neither revealed T cell

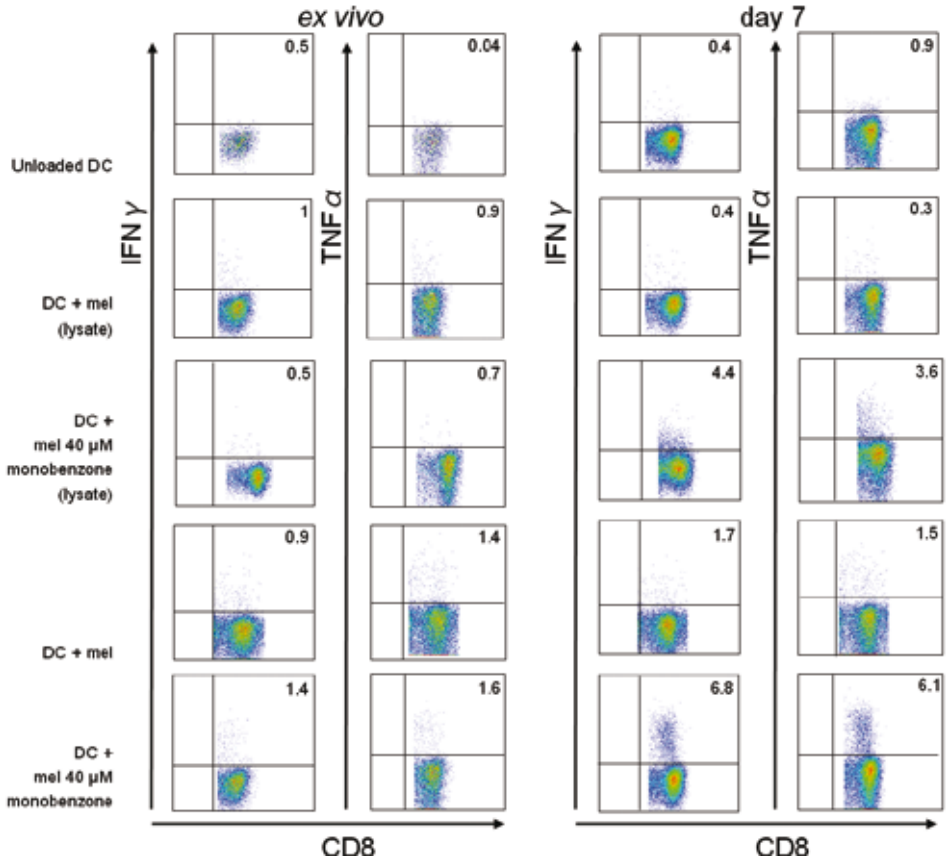


**Figure 2. Monobenzone-exposed melanoma cells induce activation of human melanoma-reactive CD8+ T cells within 7 days.** *Left panels: In vitro* overnight stimulation directly *ex vivo* of T cells with autologous DCs loaded with melanoma cells previously exposed overnight to 40 µM monobenzone, or unexposed melanoma cells (both either lysed or intact). CD8+ T cells were not activated by these culture conditions, as evidenced by the absence of TNF-α or IFN-γ production. *Right panels:* After 7 days of incubation, cultures were re-stimulated under equal conditions. Now, CD8+ T cells reactive to DCs loaded with monobenzone-exposed melanoma cells (intact or as a lysate) were detected, while T cell reactivity against DCs loaded with monobenzone-untreated melanoma cells was absent. Interestingly, intact monobenzone-exposed melanoma cells induced the most profound T cell activation.

reactivity against unloaded DCs, nor direct T cell recognition of intact melanoma cells (data not shown). Importantly, profound T cell activation was induced by DCs cross-presenting intact monobenzone-exposed melanoma cells, illustrating monobenzone confers functional immunogenicity to intact pigmented cells. Moreover, the use of intact monobenzone-treated melanoma cells in the DC-T cell co-cultures more closely represents the *in vivo* situation of topical monobenzone exposure than a pigmented cell lysate. Therefore, we used intact melanoma cells in our further experiments to verify the immunogenicity of monobenzone-exposed pigmented cells.

Monobenzone-induced T cell activation (figure 2) was found in 8 additional healthy human donors as summarized in figure 3. In these donors, significant activation of CD8+ T cells reactive to 20 μM monobenzone-exposed intact melanoma cells was induced by day 7, as evidenced by IFN-γ and TNF-α production. Moreover, melanoma cells exposed to 40 μM monobenzone induced even more CD8+ T cell activation. Increasing exposure of melanoma cells to 80 μM monobenzone did not further increase T cell reactivity (data not shown). In contrast, no T cells reactive to untreated melanoma cells were found, indicating that monobenzone increased the immunogenicity of the melanoma cells.

To exclude the possibility that the CD8+ T cell reactivity to monobenzone-exposed melanoma cells was mediated by allo-reactivity against the melanoma cells, we performed cross-presentation experiments using the melanoma cell line mel-WBO and the syngeneic EBV-transformed B cell line EBV-WBO. As shown in figure 3B,

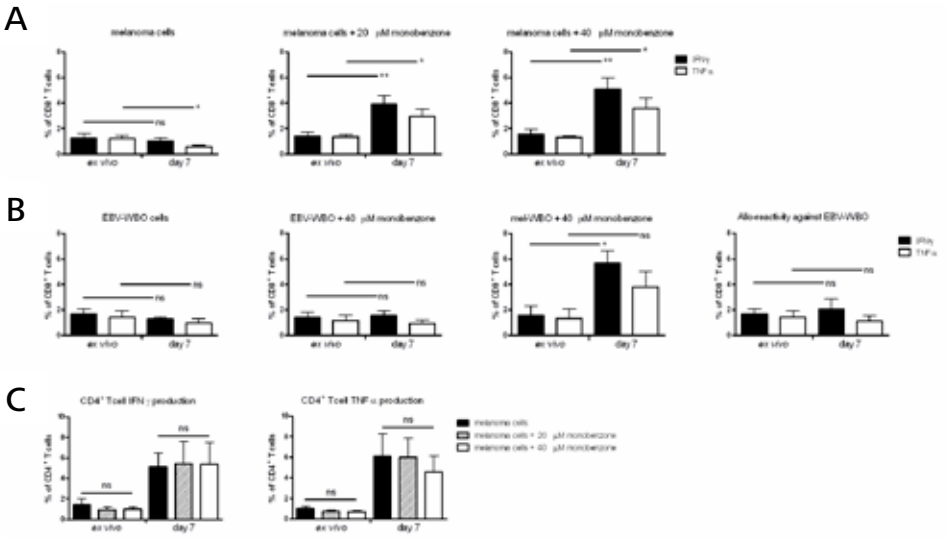


**Figure 3. Monobenzone-exposed melanoma cells induce melanoma-reactive CD8+ T cells in healthy donors. A,** CD8+ T cell reactivity against 20- or 40 μM monobenzone-exposed intact melanoma cells was found in 9 healthy human donors. Graphs show the T cell activation, as measured by IFN-γ and TNF-α production of CD8+ T cells that were stimulated during 7 days of co-culture. Monobenzone-exposed melanoma cells increased cross-presentation of melanoma antigens by DCs and CD8+ T cell activation after 7 days of stimulation, whereas monobenzone-unexposed melanoma cells did not trigger CD8+ T cell activation. Data show the mean result of 9 healthy donors. **B,** Monobenzone treatment of control EBV-transformed B cell line EBV-WBO did not induce a reactive CD8+ T cell population within 7 days, similar to untreated EBV-WBO, indicating that the effect of monobenzone is specific for pigmented cells and depends on the presence of tyrosinase. CD8+ T cell reactivity induced by monobenzone-exposed melanoma cell line melWBO did not react with syngeneic EBV-WBO cells, indicating the absence of allo-reactivity. Data show the mean result of 4 healthy donors. **C,** CD4+ T cell activation was induced by DCs incubated with either monobenzone-exposed or –unexposed melanoma cells. Data show the mean of 5 out of 9 healthy donors, since the CD4+ T cell population had disappeared in 4 out of 9 donors after 7 days of DC-T cell co-culture. Ns: not significant ($p>0.05$), *: $p<0.05$, **: $p<0.01$, using unpaired t-test (95% CI) in B and C, paired t-test in C. Graphs depict mean with SEM.

**Table 2. Reactivity pattern of CTL clones isolated from healthy donor stimulations following day-7 re-stimulation.**

| Autologous DC + target: | irradiated MCF7 | | melanoma cells | | mel + 40 μM monobenzone | | Equally reactive* to both stimuli? |
|---|---|---|---|---|---|---|---|
| Cytokine produced: | IFN-γ | TNF-α | IFN-γ | TNF-α | IFN-γ | TNF-α | |
| CTL clone | | | | | | | |
| 1 | – | – | + | + | + | + | yes |
| 2 | – | – | +/- | + | +/- | + | yes |
| 3 | – | – | – | +/- | – | + | no |
| 4 | – | – | + | + | + | + | yes |
| 5 | – | – | + | + | + | + | yes |
| 6 | – | – | + | + | + | + | yes |
| 7 | – | – | + | + | + | + | yes |
| 8 | – | – | – | +/- | – | +/- | yes |
| 9 | – | – | – | – | +/- | + | no |

+: clone is activated by co-culture to produce relevant cytokine (25-100% produces relevant cytokine in response to stimulation); –: clone is not activated by co-culture (<5% produces relevant cytokine in response to stimulation); +/–: clone displays intermediate activation in co-culture (5-25% produces relevant cytokine in response to stimulation); *: For all clones classified as "equally reactive", no significant difference was found in the production levels of the individual cytokines, when comparing between the two stimulation conditions (Two-tailed Wilcoxon signed rank test, considered significant in case $p<0.05$); **mel**: melanoma cells



EBV-WBO lysate did not induce a significant CD8+ T cell response within 7 days, also not when this cell line was exposed to 40 μM monobenzone prior to DC-T cell co-culture. Importantly, this shows that monobenzone did not increase the immunogenicity of these unpigmented cells. Furthermore, the CD8+ T cells that were activated by monobenzone-exposed mel-WBO during 7 days of co-culture, were unresponsive to syngeneic EBV-WBO cells. These data demonstrate that the monobenzone-exposed melanoma cells did not induce T cell allo-reactivity but selectively activated melanoma-reactive T cells.

As shown in figure 3C, CD4+ T cell activation was found in both stimulation conditions of monobenzone-exposed and -unexposed melanoma cells. Melanoma cells were able to induce a CD4+ helper T cell response which was not increased by monobenzone. These results demonstrate that the monobenzone exposure of the melanoma cells selectively affected the generation of melanoma-reactive CD8+ T cells.

Taken together, these results demonstrate that the interaction of monobenzone with tyrosinase elevated the immunogenicity of melanoma cells, resulting in the effective induction of a melanoma cell reactive CD8+ T cell response.

### Monobenzone-induced melanoma-reactive CD8+ T cells also recognize unexposed melanoma cells.

Next, we tested whether the CD8+ T cells induced by the monobenzone-exposed melanoma cells had the capability to recognize both monobenzone-exposed and –unexposed melanoma cells. Therefore, we cloned the activated T cells by single cell

flowcytometry sorting of the IFN-γ-producing CD8+ T cells following the re-stimulation at day 7. CD8+ T cell clones were established from 5 healthy donor cultures. As shown in table 2, all T cell clones recognized the stimulus they were raised against, namely autologous DCs presenting intact 40 μM monobenzone-exposed melanoma cells. None of the T cell clones recognized unloaded DCs, or DCs loaded with MCF7 non-melanoma control tumor cells, indicating their melanoma-specific activation. Importantly, 7 out of 9 clones (78%) equally recognized autologous DCs loaded with either melanoma cells or 40 μM monobenzone-exposed melanoma cells. Interestingly, 2 out of 9 T cell clones were less reactive with monobenzone-untreated melanoma cells (clones 3 and 9), one of which was unresponsive to unexposed melanoma cells (clone 9). Possibly, these two clones were reactive with an epitope containing a quinone-hapten. These modified epitopes are absent from monobenzone-unexposed melanoma cells, which explains the lack of reactivity by these clones. Importantly, since 78% of the clones recognized both monobenzone-treated and –untreated melanoma cells, these data provide immunological evidence for the clinical observation that monobenzone-induced vitiligo eventually spreads beyond the monobenzone application site to unexposed skin areas. 5 out of 9 T cell clones were HLA-A2-positive, and were tested for their recognition of the melanocyte differentiation-antigens tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$ by HLA-A2/peptide tetramers, however specific recognition of these epitopes was not found.

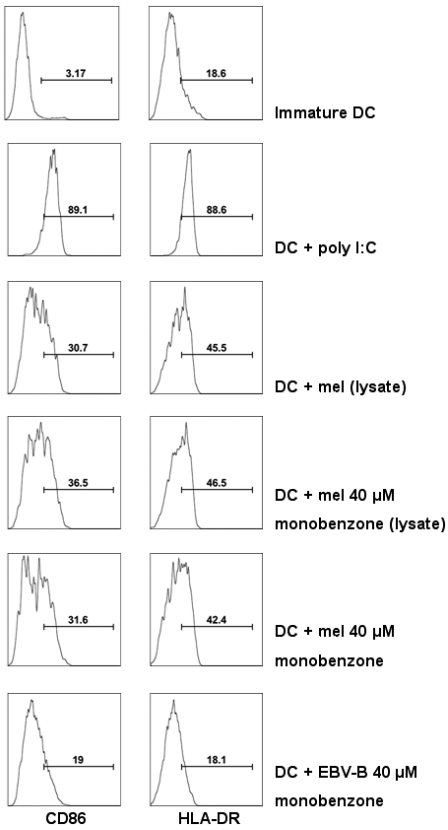


**Figure 4. Monobenzone-exposed intact melanoma cells activate DCs.** DCs were incubated for 24 hours with melanoma cells, with or without monobenzone pre-treatment. Graphs show the level of DC activation following incubation. DC activation was analysed by expression of activation markers CD86 and HLA-DR. Polyinosinic:polycytidylic acid (poly I:C) was used to induce optimal DC activation. Results show that intact monobenzone-treated melanoma cells induce DC activation to the same extent as melanoma cell lysate with or without monobenzone. Monobenzone-treated, intact EBV-B tyrosinase-negative control cells did not induce DC activation. Data is represents the results of 4 independent experiments.

Taken together, these data show that the monobenzone exposure of melanoma cells increases their immunogenicity, leading to the activation of a T cell response against antigens present on both monobenzone-treated and –untreated melanoma cells.

### Monobenzone-exposed, intact melanoma cells activate DCs

Monobenzone-treated melanoma cells, either intact or as a lysate, induced melanoma-antigen cross-presentation by DCs and CD8+ T cell activation. To explore the mechanism of this effect, we investigated whether incubation of DCs with monobenzone-exposed melanoma cells induced DC activation. Shown in figure 4A, immature DCs express low levels of the co-stimulatory molecule CD86, and the MHC class-II molecule HLA-DR. Expression of the monocyte lineage-associated CD14 marker was negative in all conditions (data not shown). DCs were incubated with polyinosinic:polycytidylic acid (poly I:C) to induce optimal DC maturation, as evidenced by increased surface expression of CD86 and HLA-DR. Lysate of monobenzone-exposed melanoma cells induced DC activation comparable to untreated melanoma cell lysate. Interestingly, monobenzone treatment of intact melanoma cells induced DC activation, while monobenzone treatment of intact tyrosinase-negative EBV-B cells did not. This demonstrates the dependence of the observed DC activation on the interaction of monobenzone with tyrosinase. This finding corresponds to the absence of immune reactivity against monobenzone-treated EBV-B cells, as shown in figure 3B. Importantly, monobenzone did not disrupt melanoma cells during overnight incubation (data not shown). Control incubations of DCs with monobenzone, melanoma- or EBV-B cells alone did not trigger DC activation (data not shown).



These results demonstrate that monobenzone treatment of pigmented cells confers an activation stimulus to DCs. This concomitantly augments melanoma-specific antigen cross-presentation by these DCs, and subsequently induces melanoma-reactive CD8+ T cells as shown in figures 2 and 3. These effects are not simply resulting from the low level of cytotoxicity associated with monobenzone incubation (figure 1D) since melanoma cell lysate did not induce a melanoma-reactive CD8+ T cell response (figure 3A).

To analyse the DC activation stimulus mediated by monobenzone, we compared the uptake of cell-derived matter from monobenzone-treated and –untreated melanoma cells by DCs, using carboxyfluorescein succinimidyl ester (CFSE)-labeled melanoma cells. CFSE fluorescently labels intracellular proteins covalently[25], and does not diffuse out of labeled cells. CFSE-labeled melanoma cells were co-cultured with immature DCs for 3 hours. Subsequently, uptake of CFSE-labeled matter by DCs was analysed by confocal laser scanning microscopy. As figure 5 shows, control incubations with DCs alone showed DCs to be CFSE-negative. DCs co-cultured with intact CFSE-labeled melanoma cells did not ingest any CFSE-labeled matter from these cells. In contrast, monobenzone treatment of intact melanoma cells led to the uptake of CFSE+ cell fragments. DCs had also taken up CFSE-labeled debris from lysed melanoma cells, either monobenzone-treated or unexposed. These results show that

monobenzone-exposure of melanoma cells triggers the release of intracellular-derived antigenic matter which is readily taken up by local DCs. These DCs subsequently induce CD8+ T cell activation, as shown in figures 2 and 3. This effect was exclusively found when melanoma cells were exposed to monobenzone. A well-known mechanism, by which cells can release antigenic particles for rapid uptake by DCs, is the release of antigen-containing exosomes.

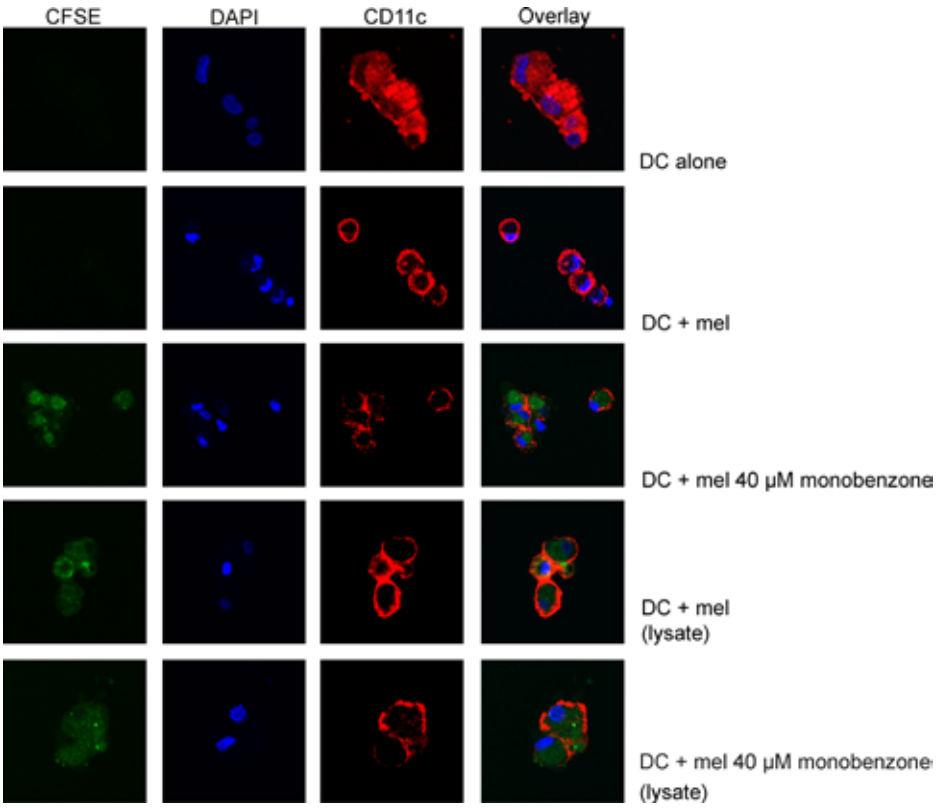


**Figure 5. Monobenzone-exposed melanoma cells release matter which is efficiently taken up by DCs.** DCs were incubated for 3 hours with carboxyfluorescein succinimidyl ester (CFSE)-labeled 136.2 melanoma cells either as intact cells or lysate with or without monobenzone pre-treatment. Uptake of cellular matter from the melanoma cells was then analysed by confocal laser scanning microscopy. DCs were specifically stained with CD11c (red), DAPI identifies the cell nucleus (blue) and uptake of CFSE-labeled debris from melanoma cells is detected in green. Data shows the uptake of CFSE-labelled cell fragments from melanoma cell lysate, but not from intact melanoma cells. Monobenzone pre-treatment induced the uptake of cell matter from intact cells to the same extent as from lysed melanoma cells. Data represents the results of 6 independent experiments.

## Monobenzone augments the release of tyrosinase- and MART-1-containing exosomes by human melanocytes and -melanoma cells.

Monobenzone has been suggested to induce cellular oxidative stress[8] upon its conversion by tyrosinase. Since oxidative stress promotes the release of exosomes[26], we here tested for the generation of reactive oxygen species (ROS) in monobenzone-exposed pigmented cells. Therefore, a panel of pigmented melanoma cells, melanocytes and the tyrosinase-negative control MCF-7 was incubated with 20 μM monobenzone for 90 minutes. As shown in figure 6A, monobenzone induced ROS in exposed melanoma cells and melanocytes, while MCF7 control cells displayed background levels of ROS during monobenzone exposure. Importantly, monobenzone-induced ROS were not cytotoxic, as monobenzone was not selectively toxic to pigmented cells (figure 1D). This may relate to the gradual decline in ROS generation following the initial burst after 20 minutes, and is probably mediated by the progressive inactivation of the tyrosinase enzyme by monobenzone.

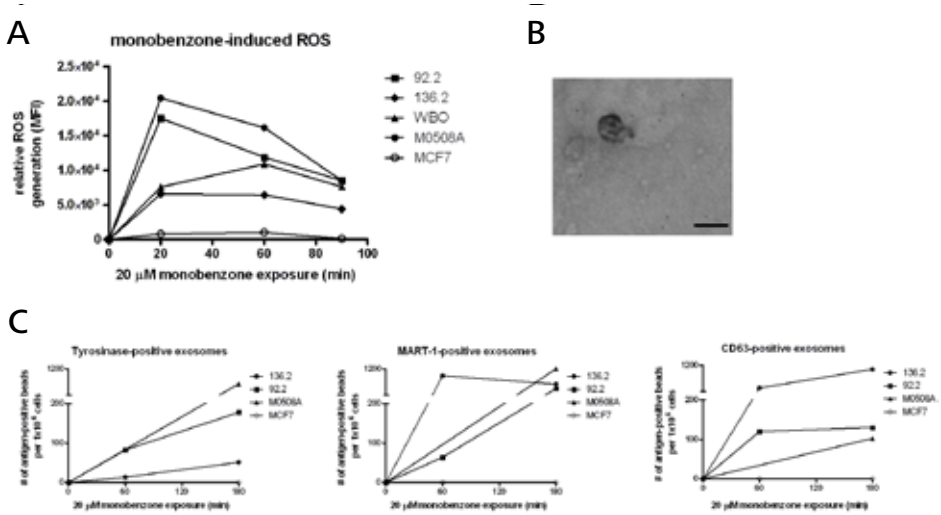


**Figure 6. Monobenzone-exposure of pigmented cells induces reactive oxygen species and increases the release of tyrosinase- and MART-1-containing CD63+ exosomes. A,** Human melanocytes (M0508A), human melanoma cells (92.2, 136.2, WBO) and tyrosinase-negative MCF7 control cells were incubated with 20 μM monobenzone for 90 minutes. During monobenzone exposure reactive oxygen species (ROS) are formed in the melanocytes and melanoma cells. In contrast, MCF7 control cells display background levels of ROS upon monobenzone exposure. Data are normalized to the ROS levels present in each cell line without monobenzone addition. **B,** Transmission electron microscopy image of an exosome, as found in the prepared culture supernatants of melanoma cells treated with 20 μM monobenzone for 3 hours (136.2 and 92.2, data represents the results of 2 independent experiments). Bar indicates 100 nm. **C,** Human melanocytes (M0508A), human melanoma cells (136.2 and 92.2) or tyrosinase-negative control cells (MCF7) were incubated with 20 μM monobenzone for 180 minutes. During monobenzone exposure, increasing numbers of tyrosinase- and MART-1 antigen-containing exosomes were released by M0508A, 136.2 and 92.2 cells, as analysed by flowcytometry. Data are normalized to the exosome levels of untreated cells for each cell line. Released exosomes were found to express the exosome-specific marker CD63. In contrast, MCF7 control cells did not increase exosome release under influence of monobenzone.

Subsequently, we analysed exosome release from monobenzone-exposed cells. Supernatants from different pigmented- and control cells exposed to 20 μM monobenzone for 0, 60 and 180 minutes were analysed for the presence of exosomes. Exosomes were isolated by ultracentrifugation of the cell culture supernatant[27] and identified by transmission electron microscopy, in analogy to the previously reported structure of tumor cell-derived exosomes[28] (figure 6B). To quantify the presence of exosomes, we adsorbed these exosomes on aldehyde/sulphate latex beads and determined the presence of tyrosinase and MART-1 protein, together with the expression of the CD63 molecule by flowcytometry. The concomitant presence of immunogenic proteins and the CD63 marker is a typical exosomal determinant[29]. Tumor- as well as untransformed cells are known to release exosomes regularly for different functions[30]. The active release of cell specific antigen-containing exosomes is a potent inducer of adaptive immune responses[31-33]. Figure 6C shows that melanocytes or melanoma cells release increasing numbers of exosomes when exposed to monobenzone over time. These exosomes were found to carry both tyrosinase- and MART-1 antigens, and to be positive for CD63. In contrast, the tyrosinase-negative control cell line MCF7 did not enhance its exosome release under influence of monobenzone-exposure, nor did MCF7-exosomes contain tyrosinase or MART-1 (data not shown).

These data demonstrate that monobenzone, by inducing cellular oxidative stress, elevates the release of melanocyte differentiation antigen-containing exosomes from exposed pigmented cells. These findings provide the functional link between the effect of monobenzone on pigment cell physiology and the induction of a pigment cell-specific immune response (figure 2 and 3).



## DISCUSSION

The present study demonstrates how monobenzone is able to induce selective immunity against pigmented cells. We have established that the interaction of monobenzone with tyrosinase results in covalent quinone binding to tyrosinase, and complete inactivation of its enzymatic activity. The 4-benzoxy-1,2-benzoquinon metabolite of monobenzone was found to specifically bind to cysteine residues in peptide chains, forming quinone-haptens. Moreover, monobenzone-exposed melanoma cells induced a significant melanoma-reactive CD8+ T cell response within 7 days *in vitro* in 9 healthy human donors. The potency of monobenzone to induce such immune responses was found to be pigment cell-dependent and did not represent allo-reactivity. The increased immunogenicity of monobenzone-exposed melanoma cells was further mediated by the generation of ROS in monobenzone-exposed melanocytes and melanoma cells, the release of tyrosinase- and MART-1-containing CD63+ exosomes from these cells, and the subsequent activation of DCs. Accordingly, we have found that intracellular-derived matter from intact CFSE-labeled and monobenzone-treated melanoma cells is effectively taken up by local DCs, a transfer likely mediated by these exosomes.

In the present study we have identified several different immunological mechanisms that cooperate to elevate the immunogenicity of the monobenzone-exposed pigmented

cell. First of all, the generation of quinone-haptens was identified to occur upon monobenzone conversion by tyrosinase. A recent study by Palm and Medzhitov[34] has found that haptenated proteins can be highly immunogenic, while their native counterparts are not. Here, protein haptenation was found to overcome the need for TLR signalling in inducing immunity against an otherwise non-immunogenic protein in TLR-signalling deficient mice. This study reports that haptenation predominantly induces immunity against the haptens, rather than the protein they are attached to. Interestingly, in our present study we found that the majority of the CD8+ T cells generated in the DC-T cell co-cultures were equally reactive to monobenzone-exposed and –unexposed melanoma cells. This indicates that monobenzone is able to induce a T cell response not only reactive with quinone-haptenated antigens, but also displaying auto-reactivity against pigmented cells. This combination of hapten- and auto-reactivity induced by monobenzone may be caused by the concurrence of this hapten-generation with two other immunological processes: the formation of ROS and the release of antigen-containing exosomes.

Specific antigen-containing exosomes released by tumor cells have been shown to considerably enhance the CD8+ T cell response against these antigens[31-33]. Moreover, secretion of exosomes from melanoma cells has been ascribed to cellular oxidative stress[26], similar to our findings in monobenzone-exposed melanoma cells. Monobenzone-induced exosomes were found to contain tyrosinase and MART-1 antigen. The tyrosinase protein in these exosomes will carry quinone-haptens, while MART-1 is likely present in its native form. The excretion of haptenated and native antigen in exosomes explains why monobenzone-exposed melanoma cells can induce T cells either selectively reactive to monobenzone-exposed melanoma cells, or reactive to both monobenzone-exposed and –unexposed melanoma cells.

Increased exosome secretion alone can not account for the observed DC activation by monobenzone-exposed melanoma cells. Recent research has shown that contact-sensitizing compounds generating haptens, such as trinitro-chlorobenzene, can activate DCs via triggering of the NALP3 inflammasome[35, 36]. Furthermore, ROS are known to induce adenosine tri-phosphate (ATP) release from tumor cells[37], and this ATP-release is a potent metabolic danger stimulus able to activate the NALP3 inflammasome[38]. Thereby, it is possible that monobenzone-exposed pigmented cells activate DCs in two different ways: Firstly, the generation of ROS may mediate ATP release from monobenzone-exposed cells, stimulating activation of the NALP3 inflammasome in local DCs. Secondly by augmenting the excretion of quinone-haptenated tyrosinase-containing exosomes. This hapten might additionally activate the NALP3 inflammasome.

The HLA-A2+ T cell clones we have obtained during our experiments were analysed for their recognition of the melanocyte specific antigens tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$ and MART-1$_{26\text{-}35}$ by HLA-A2/peptide tetramers. In the 5 clones analyzed we did not identify specificity for these antigens. While this only represents a test on a small number of T cell clones with a limited antigen-panel, it could be that the immunological mechanisms employed by monobenzone-exposed pigmented

cells alter typical antigen-processing pathways and shift immunodominance towards other melanocyte differentiation antigens. Alternatively, the quinone-haptenation of tyrosinase may alter proteasome handling of the protein, changing its antigen processing pattern. Both mechanisms may shift the focus of immunity to less dominant melanocyte antigen-specific epitopes, or mediate the generation of cross-reactive epitopes. Since central- and peripheral T cell tolerance to these kind of epitopes is generally low or absent, this may explain the relative swiftness by which the CD8+ T cell populations observed in figure 2 and 3 emerged.

In conclusion, we here show that conversion of monobenzone by the tyrosinase enzyme has specific effects on pigmented cells. Various immunological mechanisms were found to be at play, which confer profoundly elevated immunogenicity on monobenzone-exposed pigmented cells. The majority of the CD8+ T cell clones that were efficiently induced by monobenzone-exposed melanoma cells were equally reactive to unexposed melanoma cells. The immunological mechanism we have established in the present study provides the immunological basis for our successful and easily-applicable monobenzone-based MIC-therapy for melanoma, which we previously developed in the B16-B6 model of murine melanoma (chapter 7). Taken together, monobenzone represents a potent and completely new way of inducing immunity, with great value to the field of melanoma immunotherapy. By combining different melanocyte antigen-specific immunogenic mechanisms in exposed pigmented cells and evoking DC activation, it can effectively break tolerance and induce effective immunity against melanocyte differentiation antigens.



## MATERIALS & METHODS

### Cell culture

DCs were isolated and generated as described previously[39]. Briefly, monocytes were isolated from peripheral blood mononuclear cells (PBMC) by density centrifugation, and subsequently cultured at 37 degrees and 5% $CO_2$ for 6 days in IMDM (for DC- and DC-T cultures: Life Technologies, Paisley, U.K.) supplemented with 10% FBS (Hyclone, Erembodegem-Aalst, Belgium), 2mM L-glutamine (Gibco Invitrogen, Breda, The Netherlands), 50 U/ml penicillin and 50 mg/ml streptomycin (Gibco Invitrogen), GM-CSF (500 U/ml, Schering-Plough, Uden, The Netherlands) and IL-4 (250 U/ml; PBH, Hannover, Germany). MelWBO and 136.2 melanoma cells were maintained in IMDM (PAA), 92.2 in RPMI-1640 (PAA) with 10% FBS, penicillin, streptomycin and L-glutamine supplements as stated above. Human melanocytes (M0508 and M0109) were cultured in Ham's F-10 medium with specific growth supplements (FeTi medium) as described previously[40].

### Spectrophotometric studies

Purified mushroom tyrosinase (Sigma Aldrich, Steinheim, Germany) was dissolved in 50 mM phosphate buffer (pH 7.1) at 1000 U/ml aliquots and stored at -20 degrees. Reactions consisted of 10 U/ml tyrosinase supplemented with either 250

μM L-DOPA (Fluka Sigma Aldrich, Steinheim, Germany) or 100 μM monobenzone (1,4-benzyloxyphenol, 99+% pure, Sigma Aldrich). For complete inactivation of tyrosinase 300 μM monobenzone was used. Reactions were carried out in quartz cuvettes (Suprasil quartz; Hellma, Aartselaar, Belgium) at room temperature and mixtures were throroughly oxygenized at start of incubation by vortexing. At 2 minute intervals the 250-550 nm absorption spectrum was determined by a spectrophotometer (V-560; Jasco, Great Dunmow, U.K.).

### Peptide and protein analysis

Synthesis of 4-benzoxy-1,2-benzoquinon: To an ice-cooled solution of $KH_2PO_4$ (50 mg) in water (25 ml) was added Fremy's salt (potassium peroxylamine disulfonate, 500 mg). After the addition of monobenzone (1,4-benzyloxyphenol, 150 mg) the mixture was stirred for 80 min on ice and NMR indicated about 90% conversion. The reaction mixture was extracted with chloroform (3 x 25 ml) and the collected organic layers were dried on $MgSO_4$. The solvent was evaporated *in vacuo* after which the residue was used directly (the product is not stable during overnight storage at -20 degrees). Reaction of 9-mer peptides with 4-benzoxy-1,2-benzoquinon: A 5 mM solution of the peptide in a 9:1 mixture of 50 mM PBS, pH 7.2 / acetonitrile, total volume 250 μl, was treated with various amounts (0-40 μl of a 0.25 M stock solution in acetonitrile, 0-8 fold excess) of 4-benzoxy-1,2-benzoquinon. After one hour the reaction was quenched with 30 μl trifluoroacetic acid and the reaction mixture was analyzed by LC-MS (rpHPLC, 10-90% gradient of acetonitrile). Quinone adduct was detected when $MH^+$ and $MH_2^{2+}$ of the 4-benzoxy-1,2-benzoquinon-incubated peptides showed an increase of 214.2 or 107.1 respectively, as compared to the starting material.

### Cellular melanin determination

Normal human melanocytes (M0508 and M0109) or human melanoma cells (mel-136.2 and mel-88.23) were seeded out in 4 separate T25 flasks (Corning, NY) $1{,}5x10^5$ per cell-line and exposed to 0-, 3-, 10- or 30 μM of monobenzone in 3 ml of melanocyte medium, or IMDM (Cambrex) for melanoma cells, for 72 hours. Melanoma cell melanogenesis was stimulated by adding isobutylmethylxanthine (100 μM) and melanocyte-stimulating hormone (1 μM). Normal human melanocytes did not require additional melanogenesis stimuli. Monobenzone was added daily to maintain stable concentration. Subsequently, cells were washed using PBS-D (Dulbecco's PBS, PAA, Pasching, Austria). Cells were harvested and pelleted in eppendorf tubes and dissolved in 1M NaOH with 10% dimethylsulfoxide (DMSO; Sigma-Aldrich) for 60 minutes at 80 degrees under mild shaking. Melanin content was measured using a spectrophotometer at 405 nm (Jasco V-560). As a reference synthetic melanin (Sigma Aldrich) was dissolved similarly and included in a concentration range between 0-20 μg/ml.

### Cell survival assay

The pigmented human melanoma cell lines, melAKR, melWBO, 136.2 and 92.2, the amelanotic melanoma cell lines MD3A, BLM and BLM-tyr (transfected with the tyrosinase gene), were seeded at a density of $8x10^3$ cells/well in a 96-well flatbottom plate. Monobenzone was added at final concentrations from 3 to 400 μM, and $H_2O_2$ at concentrations from 4 to 0.03 mM. Cells were cultured 72 hours. Cell viability was determined using by staining with 50 μl/well 3,(4,5-dimethylthiazol-2-yl)2,5diphenyl-tetrazolium bromide (MTT) at 2.5 mg/ml in PBS for 2 hours at 37 degrees, subsequently dissolving the blue formazan crystals with 100 μl/well SDS/MDF lysis solution (2:1 v/v mixture of 30% SDS in water and N,N-dimethyl formamide) overnight at 37 degress and measuring the optical density (OD) at 590 nm. The percentage cytotoxicity was calculated relative to the OD of an MTT staining of untreated cells (ODmax) for each cell line.

### DC-T cell autologous co-cultures and re-stimulation

DCs were cultured in 48-well plates at $10^5$ DC/well in 1 ml IMDM (Life Technologies) supplemented with GM-CSF (500 U/ml, Schering-Plough), and were co-cultured with $2x10^5$ melanoma cells (1:1 mixture of melanoma cell lines melWBO and mel-136.2, or melWBO alone) or EBV-WBO previously incubated overnight with 0-, 20- or 40 μM of monobenzone in 100μl of IMDM (Life Technologies) per $2x10^5$ cells at 37 degrees 5% $CO_2$. When required, melanoma cells were lysed by 3x freeze-thawing using liquid nitrogen prior to DC loading. After 6 hours, autologous PBMC were added to the DCs at $2x10^5$ cells/well. PBMC were previously cultured for 7 days in IMDM (PAA GmbH, Pasching, Austria) with 10% FBS (Cambrex Bio Science, Verviers, Belgium), supplemented with 15 μg/ml gentamycin (Duchefa, Haarlem, The Netherlands), 20 U/ml IL-2 (Novartis, Arnhem, The Netherlands) and 5 ng/ml IL-15 (CLB/Sanquin, Amsterdam, The Netherlands). IL-2, IL-15 and gentamycin were added to the DC-T co-culture. To test T cell activation in this co-culture, Brefeldin-A was added (Golgiplug, 1:1000; BD Biosciences, San Diego, CA) during overnight incubation to allow determination of cytokine production by flowcytometry the following day. Alternatively, the co-culture was cultured for 7 days followed by an overnight re-stimulation and T cell activation analysis. Unloaded DCs and PMA (phorbol 12-myristate 13-acetate)/ionomycine-stimulated T cells were included as controls in the stimulations (Leukocyte activation cocktail, 1:500, BD Biosciences).

### Flow Cytometry

DCs were tested for activation and cytokine production as reported previously[39]. For T cell activation assays, T cells were harvested and stained on ice for 20 minutes for surface markers (0,5 μl antibody/sample) CD4 (FITC, Becton Dickinson, San Diego, CA) and CD8 (APC, Becton Dickinson). Subsequently, cells were permeabilized using the cytofix/cytoperm kit according to the manufacturer's instructions (BD Biosciences), and stained for intracellular cytokines TNFα (PerCpCy5.5, BioLegend,

Uithoorn, The Netherlands) and IFNγ (PE, BD-Pharmingen, San Diego, CA) for 20 minutes on ice (0,5 µl antibody/sample). Cells were analysed on a FACS Canto-II flowcytometer (Becton Dickinson). For testing DC-uptake of CFSE labelled cell debris, melanoma cells were labelled with CFSE as described previously[15]. Hereafter these cells were incubated overnight with 0- or 40 µM monobenzone. DCs were incubated for 3 hours with untreated lysed melanoma cells or monobenzone-exposed intact or lysed melanoma cells. Subsequently, DCs were stained for CD11c (PE, Becton Dickinson) for 20 minutes on ice. Intracellular presence of CFSE in CD11c+ DCs was analysed on a confocal laser scanning microscope (TCS-SP2 microscope, Leica microsystems, Heidelberg, Germany). ROS detection was performed on 70% confluent cell cultures using the total ROS-detection kit for flowcytometry (Enzo life sciences, Zandhoven, Belgium) according to the manufacturer's protocol.

### Exosome isolation and analysis

Exosomes were isolated as described before[41], with the modification of filtering supernatants at 0.2 µm and subsequent centrifugation of filtered supernatants at 100.000 x g. Flowcytometric analysis of exosomes was performed using aldehyde/sulphate latex, 4% w/v 4 µm beads (Invitrogen), as described before[42] using monoclonal antibodies specific for tyrosinase (clone T311, 1:200, Invitrogen), melanA (1:50, DAKO cytomation, Glostrup, Denmark) and CD63 (1:100, BD Pharmingen). Antibody binding was detected by goat-anti-mouse Alexa-488 for IgG2a-isotype primary antibodies (1:400, Invitrogen), and goat-anti-mouse Cy3 for IgG1-isotype primary antibodies (1:100, Jackson immunoresearch, West Grove, PA). Exosome electron microscopy was performed as described previously[28].

### T cell cloning by single cell sorting

Following overnight re-stimulation on day 7 (without Brefeldin-A), IFN-γ production by activated CD8+ T cells was determined using an IFN-γ secretion assay according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Subsequently single-cell CD8+ T cell clones were established from the CD8+/IFN-γ+ population by single cell sorting of T cells using a FACS-Aria cell sorter (Becton Dickinson).

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# CHAPTER 6

## TARGETING OF MELANOSOMES TO MHC CLASS-II COMPARTMENTS BY MONOBENZONE-INDUCED AUTOPHAGY

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*Submitted*

## ABSTRACT

The tyrosinase enzyme is essential for the process of melanogenesis, which takes place in the melanosome organelle of pigmented cells. Tyrosinase can be inactivated by the skin-depigmenting compound monobenzone and monobenzone-exposed pigmented cells release tyrosinase- and MART-1 antigen-containing exosomes. These results suggest that monobenzone, by its interference with cellular pigment synthesis, induces the processing- and shedding of melanosome-derived antigens in exposed pigmented cells. How monobenzone induces the processing of these antigens is unknown. In the present study we show that monobenzone induces melanosome autophagy. Using pigmented human melanoma cells we reveal that these cells generate melanin-containing autophagosomes and autolysosomes upon monobenzone exposure. We also demonstrate in human melanocytes and melanoma cells that the autophagic degradation of the melanosome induced by monobenzone is a lysosomal degradation process leading to the targeting of tyrosinase to MHC class-II compartments. Moreover, we found that monobenzone induces the ubiquitination of tyrosinase in exposed melanoma cells. These processes together reveal the catabolic response to monobenzone exposure in pigmented cells, and provide the mechanistic basis for the melanocyte-reactive immunity evoked by monobenzone.

## INTRODUCTION

Monobenzone is a phenolic skin-depigmenting compound able to induce progressive vitiligo upon prolonged skin exposure[1, 2]. This depigmenting effect depends on its interaction with the enzyme tyrosinase, located in the melanosome organelle of pigmented cells, and is illustrated by the fact that monobenzone selectively provokes an inflammatory reaction in pigmented areas of skin[3]. We have shown previously that monobenzone-exposed pigmented cells are highly immunogenic (chapter 5). Two mechanisms appeared to confer this immunogenicity to the exposed cells, namely the generation of non-cytotoxic levels of reactive oxygen species (ROS) and the release of antigen-containing exosomes. The combination of oxidative stress and the active shedding of antigen by exposed cells suggest active melanosome degradation is engaged in monobenzone-exposed cells. An established cellular catabolic response against ROS is the initiation of autophagy which actively degrades organelles damaged by the oxygen radicals[4]. This degradation by autophagy may involve either engulfment and breakdown of small portions of cytoplasm (microautophagy), or processing of entire damaged organelles (macroautophagy)[5]. Autophagy can effectively deliver antigen for presentation via the MHC class-I[6] and -II[7, 8] pathways. Moreover, microautophagy can deliver antigen to the internal vesicles of multivesicular bodies which can be released as exosomes[9].

The phenolic skin depigmenting compound hydroquinone, structurally related to monobenzone, can disrupt the melanosome organelle probably via ROS-mediated oxidation[10]. Combined with our previous findings on monobenzone-induced ROS generation and our observation that monobenzone reduces cellular pigment content (chapter 5), we here investigated how monobenzone interferes with melanogenesis. Moreover, we explored whether monobenzone induces melanosome autophagy, since this could explain the augmented immunogenicity of exposed pigmented cells we have observed before both *in vitro* (chapter 5) and *in vivo* (chapter 7).



## RESULTS

### Monobenzone induces melanosome degradation and –macroautophagy.

To identify the effects of monobenzone on melanogenesis we investigated the ultrastructural effects of monobenzone exposure on pigmented cells. In particular we studied whether monobenzone disturbs melanosome formation, an effect previously shown for the less potent phenolic depigmenting compound hydroquinone[10]. To this end, we incubated pigmented human melanoma cells with 20 μM monobenzone for 3 hours, and analyzed these cells by electron microscopy (EM). Figure 1A depicts monobenzone-untreated melanoma cells, which contained numerous electron-dense fully melanized stage IV melanosomes in the cytoplasm. Moreover, all 4 melanosome developmental stages[11, 12] could be identified. Melanosomes displayed normal cigar-like morphology and the striations typical for stage II and III melanosomes were clearly visible. Exposure of the melanoma cells to monobenzone for 60 minutes

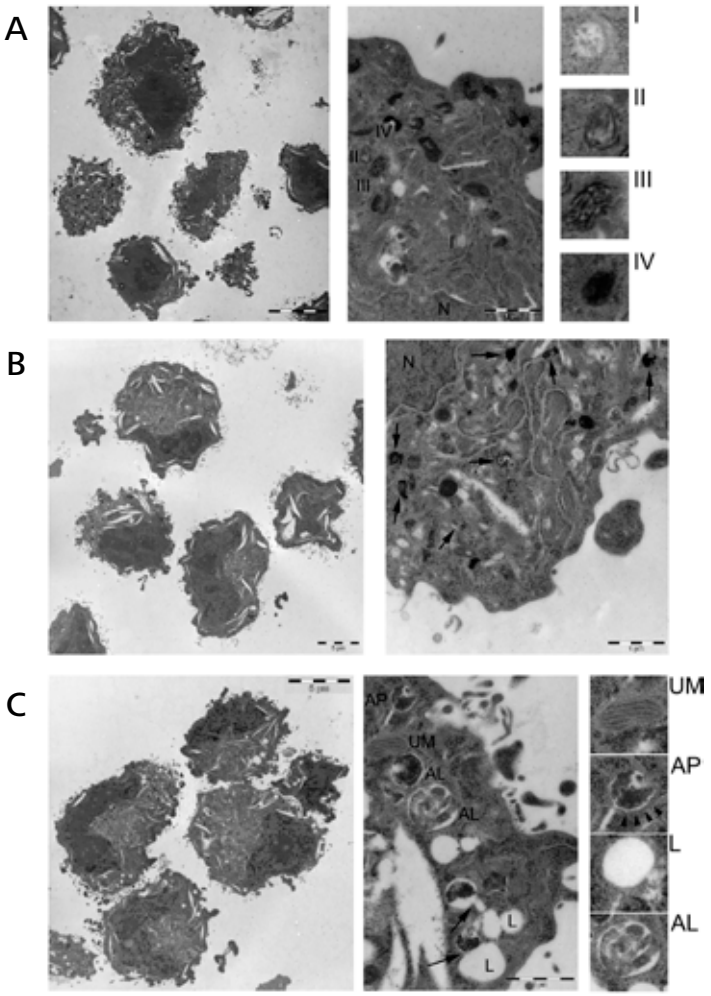


**Figure 1. Monobenzone induces melanosome autophagy.** Electron microscopy analysis of pigmented human melanoma cells (92.2) exposed to 20 μM monobenzone for 3 hours. **A,** Melanoma cells not exposed to monobenzone contain many fully-melanized stage IV melanosomes, visible as electron-dense organelles throughout their cytoplasm. All melanosome developmental stages were identified in the cytoplasm of these cells. Melanosomes displayed the typical cigar-like shape, and the regular striations of stage II and III melanosomes were clearly visible. **B,** Upon 60 minutes of monobenzone exposure, the melanoma cells contained notably less stage IV melanosomes in their cytoplasm, while appearing morphologically normal. Closer investigation showed that stage II and II melanosomes appeared disorganized and smaller in size, even ruptured (arrows). **C,** While morphologically normal, melanoma cells no longer contained stage IV melanosomes upon 3 hours of monobenzone exposure. Large vacuoles could occasionally be seen in the cytoplasm. The cytoplasm of these cells showed unmelanized stage III melanosomes (UM). Double membrane-walled structures containing electron-dense material were found, characteristics typical for a melanin-containing autophagosome (AP; arrowheads for double membrane). These autophagosomes were found to regularly fuse with electron-lucent lysosome-like structures (L; arrows for fusion), forming single membrane-walled autolysosomes (AL) containing electron-dense debris in various stages of degradation. Data represents the results of 2 independent experiments on 2 human pigmented melanoma cell lines (92.2 and 136.2). N = nucleus.

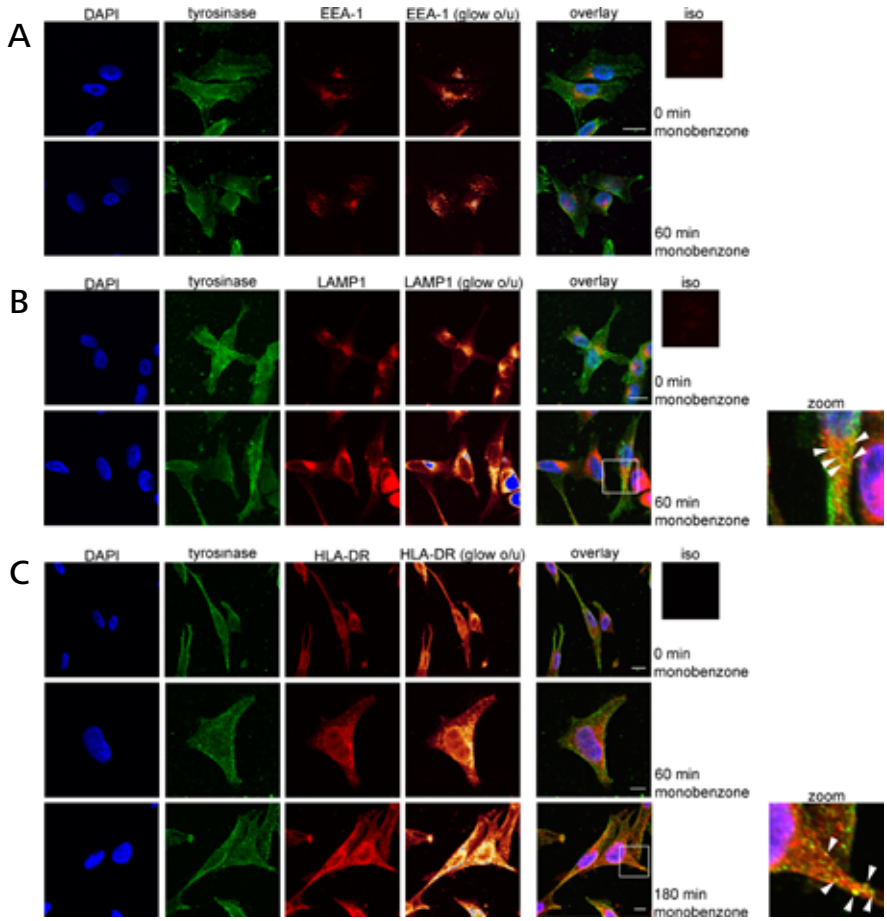


**Figure 2. Monobenzone induces lysosome-dependent melanosome degradation in melanoma cells.** Confocal laser scanning microscopy (CLSM) analysis of pigmented human melanoma cells (92.2) exposed to 20 μM monobenzone, showing the melanosome degradation induced by monobenzone. Tyrosinase (green) was used to identify melanosomes, 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain (blue). Glow over/under panels (glow o/u) show the fluorescence intensity of the designated markers, indicating the expression level from low (red/orange) to high (white/blue). **A,** Monobenzone exposure does not initiate early-endosomal degradation routes in melanoma cells. The cells did not upregulate their early endosome antigen-1 ($EEA_1$; red) expression upon 60 minutes of monobenzone exposure, neither was co-localization of tyrosinase (green) with $EEA_1$ observed. Scale indicates 20 μm for all panels. Isotype monoclonal antibody (mAb) control incubations were negative (iso panel). **B,** In contrast, melanoma cells degraded melanosomes via the lysosomal route. Cells exposed to monobenzone for 60 minutes showed increased lysosome formation, indicated by upregulated lysosome-associated membrane protein-1 expression (LAMP1; red). Tyrosinase was found to co-localize with these lysosomes (arrowheads). Scale indicates 20 μm for all panels. Isotype mAb incubations were negative (iso panel). **C,** Melanosomal antigen is eventually routed to MHC class-II compartments. Melanoma cells increased their MHC class-II expression (HLA-DR; red) after 60 minutes of monobenzone exposure, reaching maximum HLA-DR expression after 180 minutes of exposure. At 180 minutes of incubation, tyrosinase co-localized to HLA-DR containing vesicles (arrowheads). Scale indicates 20 μm for 0 minutes, and 8 μm for 60 and 180 minutes. Isotype mAb incubations were negative (iso panel). Data represents the results of 2 independent experiments on 2 pigmented human melanoma cell lines (92.2 and 136.2).

reduced the number of stage IV melanosomes in the cytoplasm without altering overall cell morphology (figure 1B). Closer examination of these cells revealed that the melanosomes, especially stages II and III, were smaller in size and appeared disorganized (arrows). Some melanosomes seemed swollen or ruptured. Prolonging monobenzone exposure to 3 hours induced distinct cytoplasmic changes in the melanoma cells. Shown in figure 1C, the cells no longer contained electron-dense stage IV melanosomes and the stage III melanosomes present had an unmelanized appearance (UM), indicating that monobenzone interfered with their melanization. Also, while the cells overall appeared normal in morphology, their cytoplasm showed areas of vacuolization and structural changes characteristic of melanosome macroautophagy: The lower right panel shows electron-dense melanin-like matter enclosed in a double membrane structure of approximately 500 nm diameter, the hallmarks of the autophagosome[13-15] (AP; figure 1C, arrowheads for double membrane). Moreover, these structures were found to fuse with electron-lucent lysosome-like vesicles (L; arrows for fusion sites), thereby forming characteristic single-membrane walled autolysosomes (AL)[5] able to progressively degrade their protein contents.

These results show that monobenzone disturbs melanogenesis, and mediates the disruption- and subsequent macroautophagy of melanosomes. This process allows melanosome antigens to be efficiently degraded for antigen processing[6-8].

### Melanosome autophagy directs tyrosinase into MHC class-II compartments via lysosomal degradation

Autophagy typically involves fusion of the autophagosome with lysosomes[5], excluding endosome participation[5], and can thereby lead to antigen delivery to MHC class-II compartments[7, 8, 16]. We carried out experiments to identify the organelles with which the autophagosome fuses. Therefore, we incubated pigmented human melanocytes and melanoma cells with 20 μM monobenzone for 3 hours and analyzed the cells by confocal laser scanning microscopy (CLSM). In these studies we used the T311-clone monoclonal antibody[17] against tyrosinase to detect melanosomes. Importantly, this antibody specifically recognizes an immunodominant, linear determinant in tyrosinase (tyrosinase$_{233-247}$)[18] which allows the identification of specific tyrosinase antigen beyond the protein degradation stage.

After 1 hour of monobenzone exposure, melanoma cells had selectively initiated a lysosomal degradation response (figure 2). The cells upregulated their expression of the lysosome-specific marker LAMP1 (lysosome-associated membrane protein-1) while their expression of the endosome-associated antigen EEA$_1$ (early endosome antigen-1) was unaffected (figure 2A and -B). Moreover, tyrosinase was found to co-localize with LAMP1-positive vesicles (arrowheads in magnification). During this lysosomal response, the number of vesicles expressing HLA-DR increased after 60 minutes of monobenzone exposure (figure 2C). HLA-DR expression reached a maximum at 3 hours of exposure. At this point, a high level of co-localization between tyrosinase and HLA-DR-positive vesicles was observed (arrowheads in magnification). MCF7

non-melanoma control cells were found not to express tyrosinase, and these cells did not upregulate $EEA_1$, LAMP1 nor HLA-DR in response to 3 hours of monobenzone exposure (data not shown). These data indicate that the autophagy response depends on the presence of tyrosinase. The interaction of monobenzone with tyrosinase in pigmented cells leads to the generation of ROS (chapter 5), which likely play an active role in the initiation of autophagy. Subsequently, this interaction also leads to the upregulation of LAMP1 and HLA-DR. In contrast, MCF7 non-pigmented control cells did not generate ROS when exposed to monobenzone (chapter 5), and subsequently did not upregulate LAMP1 and HLA-DR during exposure.

Since the vitiligo-inducing effect of monobenzone is mediated by topical exposure of the skin, we incubated human melanocytes with 20 μM monobenzone to verify whether the effects of monobenzone in melanoma cells are also found in melanocytes. As figure 3A and -B show, melanocytes also exclusively initiate a lysosomal response upon 1 hour of monobenzone exposure. While $EEA_1$ expression was unaffected by monobenzone, LAMP1 is visibly upregulated and co-localizes to cytoplasmic areas with tyrosinase. HLA-DR expression was upregulated by the exposed melanocytes after 60 minutes, and reached a maximum at 2 hours of exposure. In these cells, tyrosinase clearly co-localized with HLA-DR positive vesicles (arrowheads in magnification).

These results demonstrate that monobenzone in pigmented cells induces the degradation of melanosomes via lysosomal uptake and subsequent fusion with HLA-DR containing vesicles. These HLA-DR/tyrosinase positive vesicles were slightly larger organelles, suggesting that these are the multi-vesicular bodies characteristic for late-stage autophagosomes processing antigen for MHC class-II presentation[8].

### Autophagy by monobenzone involves tyrosinase ubiquitination.

Recent evidence has shown that ubiquitination of protein aggregates provides a receptor for macroautophagy[19]. We investigated if ubiquitination of tyrosinase occurs following monobenzone-exposure of pigmented cells. In particular, we sought to identify ubiquitinated aggregates of tyrosinase in later phases of monobenzone exposure. Consequently, we incubated human melanoma cells with 20 μM monobenzone and investigated the cells with CLSM (figure 4). Unexposed melanoma cells displayed low ubiquitin expression, with small foci of non tyrosinase-associated ubiquitination (arrows). Cellular ubiquitin upregulation and foci of tyrosinase-associated ubiquitination were occasionally observed after 60 minutes of monobenzone exposure (arrows). In contrast, following 3 hours of monobenzone-incubation many small foci and larger complexes were identified showing co-localization of tyrosinase and ubiquitin (arrowheads). Tyrosinase-negative MCF7 control cells did not show ubiquitin upregulation in response to 3 hours of monobenzone exposure (data not shown).

These results demonstrate that the protein degradation response induced in pigmented cells by monobenzone involves ubiquitination of the tyrosinase protein. Besides aiding the autophagy process, ubiquitination of tyrosinase protein can provide antigen for the MHC class-I presentation route.

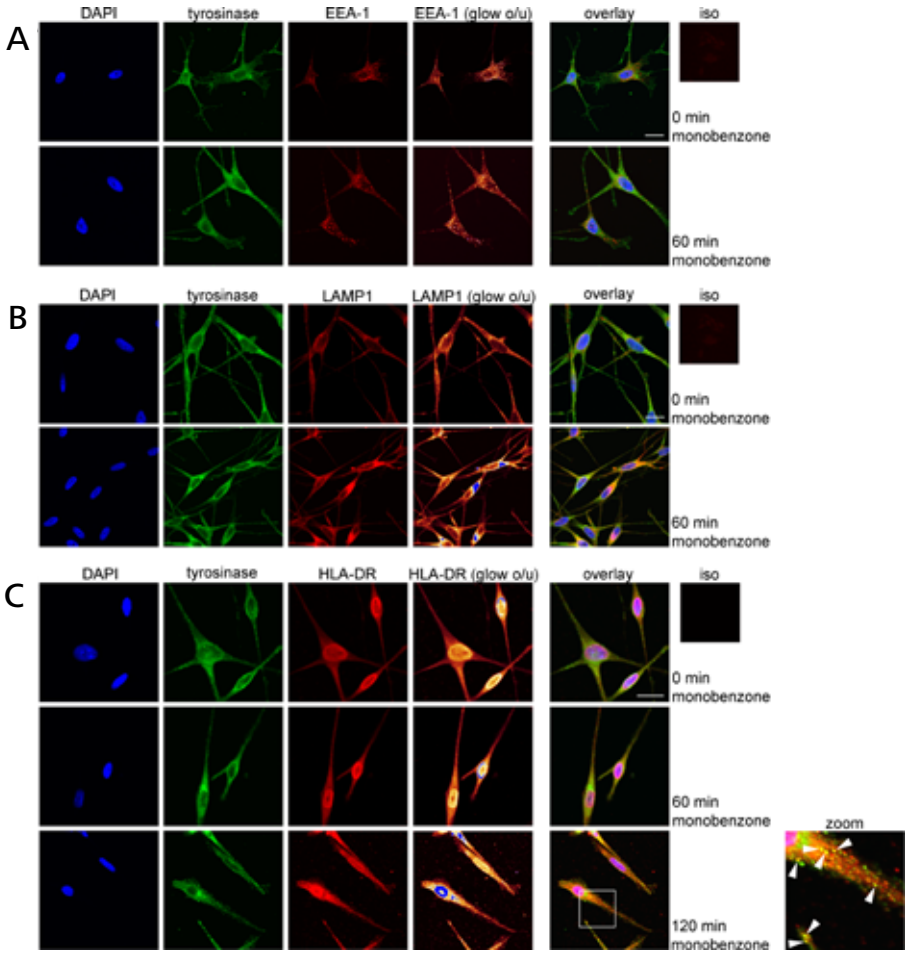


**Figure 3. Melanocytes also display lysosomal degradation of melanosomes upon monobenzone exposure.** Confocal laser scanning microscopy (CLSM) analysis of pigmented human melanocytes (M0508A) exposed to 20 μM monobenzone, demonstrating the melanosome degradation induced by monobenzone. Tyrosinase (green) was used to identify melanosomes, 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain (blue). Glow over/under panels (glow o/u) show the fluorescence intensity of the designated markers, indicating the expression level from low (red/orange) to high (white/blue). **A,** Melanocytes exposed to monobenzone for 60 minutes did not initiate early-endosomal degradation routes since tyrosinase did not co-localize with the early-endosome antigen-1 ($EEA_1$; red) and $EEA_1$-expression was unaffected. Scale indicates 20 μm for all panels. Isotype monoclonal antibody (mAb) control incubations were negative (iso panel). **B,** On the contrary, melanocytes did initiate a lysosomal melanosome-degradation response. Following 60 minutes of incubation, melanocytes upregulated expression of lysosome-associated membrane protein-1 (LAMP1; red), signifying increased lysosome formation. Scale indicates 20 μm for all panels. Isotype monoclonal antibody (mAb) control incubations were negative (iso panel). **C,** Tyrosinase routing into MHC class-II compartments in melanocytes occurred after 120 minutes of monobenzone exposure. MHC class-II (HLA-DR; red) was upregulated in melanocytes after 60 minutes of exposure, and was maximal after 120 minutes. At this point, tyrosinase was found to co-localize with HLA-DR containing vesicles (arrowheads). Scale indicates 20 μm for all panels. Isotype monoclonal antibody (mAb) control incubations were negative (iso panel). Data represents the results of 4 independent experiments using 2 human melanocyte lines (M0508A and M0109).

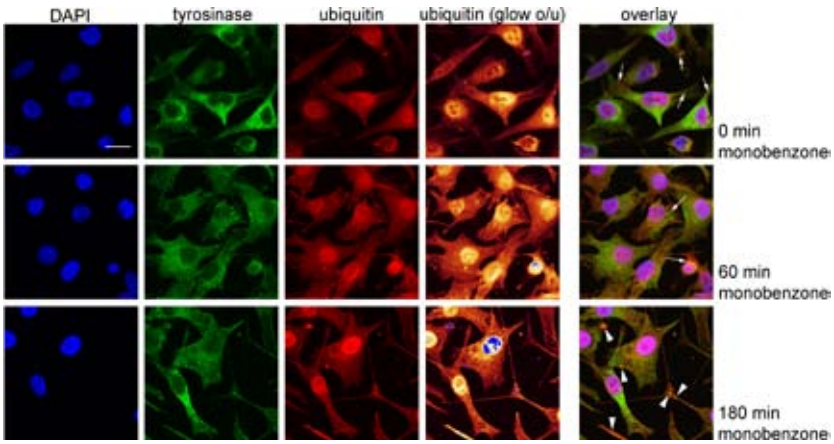


**Figure 4. Tyrosinase is ubiquitinated following monobenzone exposure.** Confocal laser scanning microscopy (CLSM) analysis of pigmented human melanoma cells (92.2) exposed to 20 μM monobenzone, showing tyrosinase ubiquitination following monobenzone exposure. 4',6-diamidino-2-phenylindole (DAPI) was used as a nuclear stain (blue). Glow over/under panels (glow o/u) show the fluorescence intensity of ubiquitin, indicating its expression level from low (red/orange) to high (white/blue). **Upper row,** Expression of ubiquitin (red) in untreated melanoma cells is low, and only small foci of tyrosinase (green)-unrelated ubiquitination complexes could be found (arrows). **Middle row,** Upregulation of cellular ubiquitin expression was found upon 60 minutes of monobenzone incubation, as well as foci of tyrosinase-associated ubiquitination (arrows). **Lower row,** Melanoma cells displayed large complexes of tyrosinase and ubiquitin co-localization (arrowheads) after 180 minutes of monobenzone exposure, as well as many areas and foci of tyrosinase-ubiquitin co-localization. Scale indicates 16 μm for all panels. Isotype monoclonal antibody (mAb) control incubations were negative (iso panel). Data represents the results of 2 independent experiments in 2 different melanoma cell lines (92.2 and 136.2).



## DISCUSSION

In the present study we show that monobenzone induces melanosome autophagy. Using electron-microscopy we have shown that monobenzone interferes with melanogenesis by disrupting melanosomes and impeding the melanization of these organelles. Moreover, monobenzone induced the formation of autophagosomes containing electron-dense melanin-like material, and these structures were found to fuse with lysosome-like electron-lucent vesicles forming autolysosomes. Using tyrosinase as a melanosome-specific marker in CLSM experiments, we identified that under influence of monobenzone pigmented cells degrade their melanosomes via selective initiation of a lysosomal response within 60 minutes. After 2 or 3 hours of monobenzone exposure (for melanocytes and melanoma cells, respectively) tyrosinase antigen was found to be localized in MHC class-II compartments. Also, we found that tyrosinase protein is being ubiquitinated after 1 hour of monobenzone exposure. Our results establish that monobenzone induces a melanosome degradation response via ubiquitination and autophagy.

Autophagy is a catabolic response against cellular stress, and is a typical cellular reaction to nutrient starvation or oxidative damage to organelles[5]. Although autophagy

can be a sign of imminent cell apoptosis under conditions of enduring stress[20], this is likely not the case for monobenzone-exposed cells since we previously found monobenzone not to be selectively toxic to pigmented cells (chapter 5). Interestingly, this may relate to our finding that after an initial burst of ROS generation after 20 minutes of monobenzone exposure, the ROS levels gradually declined. This decline is likely caused by the progressive inactivation of the tyrosinase enzyme by monobenzone (chapter 5). Following this initial ROS insult, a low level of ROS will probably be maintained since new tyrosinase protein is constantly synthesized. As a response, the exposed cell will degrade its oxidatively damaged melanosomes in an initial wave of autophagy. Thereafter, autophagic degradation of newly formed melanosomes will continue, since the cell is constantly exposed to monobenzone. The monobenzone exposure of pigmented cells leads to cellular depigmentation (chapter 5 and this study) and the constant engagement of exposed cells in the degradation-, processing- and presentation of melanosome proteins. This enhanced melanosomal antigen presentation can result in the activation of autoreactive T cells, and provides the most likely explanation for the elevated immunogenicity of monobenzone-exposed pigmented cells.

Autophagy has a clear relationship to the generation of antigen for presentation in MHC class-I and –II molecules. MHC class-II antigen-loading compartments regularly receive antigen-input from autophagosomes[7, 8], while it was also found that autophagy enhances endogenous antigen presentation in MHC class-I[6]. Interestingly, melanocytes are quite unique cells in the sense that they express MHC class-II, like dendritic cells (DC), and even display effective antigen-presenting capabilities[21]. This implies that the exposed melanocyte by itself may initiate melanocyte antigen-specific CD4+ or CD8+ T cell immunity. Besides this direct immunogenicity of the exposed melanocyte, we have now established that monobenzone-exposed melanoma cells activate DC (chapter 5). Moreover, we observed that these DC take up melanoma cell-derived matter and activate melanoma-reactive T cells in cross-presentation experiments (chapter 5). In these studies we found that monobenzone-exposed pigmented cells release melanosome antigen-containing exosomes, which can trigger the induction of melanoma-reactive T cell immunity. The generation of these exosomes may result from the autophagy process. While macroautophagy degrades whole organelles, microautophagy engulfs small portions of the cytoplasm[5]. This typically occurs via membrane invaginations of the multivesicular endosome[9], a late endosomal compartment, which in this way forms many small cytosol-containing vesicles. This structure can release these vesicles as exosomes upon fusing with the plasma membrane[22]. In taking up cytosol, microautophagy can form exosomes containing proteins released by damaged melanosomes, such as tyrosinase and MART-1.

During the present study we also observed ubiquitination of the tyrosinase enzyme, which suggests protein ubiquitination in damaged melanosomes. Besides marking proteins for degradation by the proteasome and subsequent presentation of antigenic peptide fragments in MHC class-I, ubiquitination seems to have a distinct

role in autophagy. Recent evidence shows that ubiquitination of peptide aggregates, for example in destroyed melanosomes, functions as a receptor for the initiation of macroautophagy[19, 23]. Furthermore, ubiquitination of transmembrane proteins, like tyrosinase and MART-1 found in the melanosome, serves as a sorting signal directing these proteins into the vesicles of the multivesicular endosome[24-28]. Thereby, active shuttling of these proteins into the exosomal pathway may be induced by monobenzone. The ubiquitinated tyrosinase complexes we observed, by their sub-cell membrane localization, might represent multivesicular bodies ready to release their exosomal content. Moreover, these complexes may consist of aggregates of ubiquitinated tyrosinase tagged for the initiation of autophagy.

In conclusion, this study shows that monobenzone exposure induces a melanosome degradation response in exposed pigmented cells. Involving autophagy and ubiquitination, these results establish the mechanism by which monobenzone induces melanocyte antigen-specific immunity leading to skin depigmentation and vitiligo.

## MATERIALS AND METHODS

### Cell culture

136.2 melanoma cells were maintained in IMDM (PAA, Pasching, Austria), 92.2 melanoma cells in RPMI-1640 (PAA) both supplemented with 10% FBS (Hyclone, Erembodegem-Aalst, Belgium), 2mM L-glutamine (Gibco Invitrogen, Breda, The Netherlands), 50 U/ml penicillin and 50 mg/ml streptomycin (Gibco Invitrogen). Human melanocytes (M0508 and M0109) were cultured in Ham's F-10 medium with specific growth supplements (FeTi medium) as described previously[29].

### Confocal microscopy analysis

$1.5\text{-}2x10^4$ cells were grown overnight on poly-D-Lysine (Sigma-Aldrich, Zwijndrecht, The Netherlands) coated coverslips. After treatment with 20 μM monobenzone the coverslips were fixed with 3.7 % paraformaldehyde buffered with methanol (Sigma), hereafter the coverslips were permeabilized with 0.01% Triton-X (Sigma) in PBS (PAA). For the tyrosinase/EEA1, tyrosinase/LAMP1, and tyrosinase/ubiquitin double stainings the cells were incubated for 1h with anti-tyrosinase monoclonal antibody (mAb; clone T311, Invitrogen, Merelbeke, Belgium) mixed with anti-EEA1 mAb (BD Biosciences, Breda, The Netherlands), anti-LAMP-1 mAb (BD) or anti-ubiquitin mAb (Sigma). This was followed by alexa fluor 488-conjugated goat-anti-mouse IgG2A for tyrosinase detection, alexa fluor 568-conjugated goat-anti-mouse IgG1 (both Invitrogen) for EEA1 and LAMP-1 detection and Cy3-conjugated goat-anti-rabbit for ubiquitin (Jackson Immunoresearch, Suffolk, United Kingdom). For the tyrosinase/HLA-DR double staining the cells were incubated for 1h with anti-tyrosinase mAb (clone T311, Invitrogen), followed by an IgG-specific alexa fluor 488-conjugated goat-anti-mouse IgG2A. Aspecific binding was blocked by incubation with 10% normal mouse serum (Dako, Glostrup, Denmark). The HLA-DR antibody (IgG2B, BD) was incubated for 1h and followed by an incubation with alexa fluor 568-conjugated

goat-anti-mouse IgG2B (Invitrogen). Coverslips were mounted with Prolong Gold with DAPI for nuclear staining (Invitrogen). Samples were analysed using a Leica TCS-SP2 confocal microscope and the Leica LCS software (Leica Microsystems, Rijswijk, The Netherlands).

### Electron microscopy

Melanoma cells were incubated with 20 μM monobenzone for the time stated or left untreated and subsequently prefixed in 1% glutaraldehyde with 4% paraformaldehyde in 0.1 M phosphate buffer. Samples were fixed using a solution of 1% $OsO_4$ in phosphate buffer. Subsequently, specimens were dehydrated by alcohol series and Epon embedded. Ultrathin sections were collected on formvar-coated grids and counterstained with uranil acetate and lead citrate. Samples were analysed using a Technai T-12 G2 Biotwin microscope (FEI company, Eindhoven, The Netherlands). Images were acquired using a SIS Veleta 2K x 2K side-mounted TEM CCD camera (Olympus SIS, Münster, Germany).

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# CHAPTER 7

## EFFECTIVE MELANOMA IMMUNOTHERAPY IN MICE BY THE SKIN-DEPIGMENTING AGENT MONOBENZONE AND THE ADJUVANTS IMIQUIMOD AND CPG

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## ABSTRACT

**Background:** Presently melanoma still lacks adequate treatment options for metastatic disease. While melanoma is exceptionally challenging to standard regimens, it is suited for treatment with immunotherapy based on its immunogenicity. Since treatment-related skin depigmentation is considered a favourable prognostic sign during melanoma intervention, we here aimed at the reverse approach of directly inducing vitiligo as a shortcut to effective anti-melanoma immunity.

**Methodology and Principal Findings:** We developed an effective and simple to use form of immunotherapy by combining the topical skin-bleaching agent monobenzone with immune-stimulatory imiquimod cream and cytosine-guanine oligodeoxynucleotides (CpG) injections (MIC therapy). This powerful new approach promptly induced a melanoma antigen-specific immune response, which abolished subcutaneous B16.F10 melanoma growth in up to 85% of C57BL/6 mice. Importantly, this regimen induced over 100 days of tumor-free survival in up to 60% of the mice, and forcefully suppressed tumor growth upon re-challenge either 65- or 165 days after MIC treatment cessation.

**Conclusions:** MIC therapy is effective in eradicating melanoma, by vigilantly incorporating NK-, B- and T cells in its therapeutic effect. Based on these results, the MIC regimen presents a high-yield, low-cost and simple therapy, readily applicable in the clinic.

## INTRODUCTION

Melanoma patients could benefit greatly from immunotherapy, since melanoma is one of the most immunogenic tumors [1] and metastatic disease responds poorly to conventional therapy, such as irradiation and chemotherapy [2]. Cancer immunotherapy underwent considerable progress in recent years, since the first promising results of adjuvant immune stimulation using interferon-α (IFN-α) and interleukin-2 (IL-2) [3–6]. Recent immunotherapeutic vaccination strategies have appeared moderately effective in achieving superior clinical results than standard interventions [7–9]. Nonetheless, studies using the toll-like receptor (TLR) ligand cytosine-guanine oligodeoxynucleotides (CpG) as a TLR9 agonist or imiquimod as a TLR7 agonist in the melanoma setting [10–17], have shown encouraging results. Successful melanoma immunotherapy can lead to treatment-related vitiligo-like leukoderma as an autoimmune side-effect [18], which is considered an encouraging prognostic sign [19,20]. Therefore, as a reverse approach, we here investigated the active induction of vitiligo as an immunotherapy approach for melanoma treatment.

Skin contact with phenols or catechols, such as the monobenzylether of hydroquinone (MBEH or monobenzone), induces depigmentation in susceptible individuals upon occupational exposure, which is clinically and histologically indistinguishable from vitiligo vulgaris [21–24]. Monobenzone is the most potent skin depigmenting agent [21], discovered by Oliver *et al.* in 1939 [23]. In healthy individuals who have applied it to initially lighten their skin tone it is known to induce vitiligo vulgaris [25–27]. Moreover, it has been used in a 20% cream for patients with vitiligo universalis to induce complete depigmentation [27]. The skin depigmentation spreads to distant sites unexposed to monobenzone, indicating that monobenzone induces a progressive systemic reaction against melanocytes, by acting as a skin sensitizer [26,28,29]. Monobenzone specifically interacts with tyrosinase [21,30], the key enzyme in melanocyte pigment synthesis, and forms quinone-haptens to the tyrosinase protein [31]. Quinone metabolites of phenols or catechols have been shown to induce extensive depigmentation *in vivo* [32,33] depending on the enzymatic conversion by tyrosinase, and covalent binding as a hapten to proteins [30,31].

Since we have previously shown that vitiligo vulgaris is mediated by melanocyte antigen-specific CD8+ T cells [34], we postulate that monobenzone by its selective interaction with melanocytes, induces melanocyte-specific autoimmunity. In this report we combined the topical skin-bleaching agent monobenzone with immune-stimulating TLR7-agonist imiquimod and the TLR9-agonist CpG [35,36], designated as MIC-treatment. This combination proved to provoke a robust melanocyte antigen-specific autoimmune response in C57BL/6 *wildtype* mice. This activated response effectively abolished the growth of subcutaneous B16.F10 melanoma. Importantly, the therapeutic effect was found in up to 85% of the mice, while it also mediated over 100 day tumor-free survival in 60% of the mice on average. Innate and adaptive immunity cooperated in the observed therapeutic effect. Our MIC therapy namely induced a melanocyte antigen-specific CD8+ T cell response, a B16-specific serum

IgG response and a sustained NK cell expansion. Furthermore, the MIC treatment conferred melanocyte antigen-specific CD8+ T cell-mediated immunological memory that forcibly suppressed secondary tumor growth. Our data establish the MIC therapy as an effective new regimen in the field of melanoma immunotherapy.

## RESULTS

### Expansion and activation of melanoma-reactive CD8+ T cells and NK cells in response to monobenzone, imiquimod and CpG treatment of subcutaneous B16.F10 melanoma

To characterize the *in vivo* immune response induced by monobenzone and the immunostimulatory adjuvants CpG and imiquimod against the highly aggressive and poorly immunogenic B16.F10 melanoma, we inoculated C57BL/6 *wildtype* mice with $2.5x10^3$ B16.F10 cells subcutaneously in the right flank at day 0 (n=5 mice/group), and from day 2 treated these mice with monobenzone alone, the immunostimulatory adjuvants CpG and imiquimod combined (CI) or monobenzone with imiquimod and CpG (MIC). Importantly, tumors were injected in the flank, while topical applications of monobenzone and imiquimod were selectively applied on the shaved abdomen of the mice; CpG was injected peritumorally. On treatment day 18, mice were sacrificed and splenocytes were *ex vivo* tested for their specific recognition of B16.F10 melanoma. Syngeneic EL4 mouse thymoma cells were used as control.

As shown in figure 1A (*left panel*), MIC-treated mice showed significantly elevated percentages of CD8+ T cells producing TNF-α upon recognition of B16.F10 cells *in vitro*, as compared to untreated mice. CD8+ T cells from MIC-treated mice did not react to with the EL-4 control tumor cells, indicating the melanoma specificity of the T cell response. This T cell activation, albeit at a lower level, was also found in mice that were treated with monobenzone alone, while CI-treated mice did not show specific T cell reactivity against B16.F10 cells. Additionally, when B16.F10 target cells were interferon-γ (IFN-γ)-primed to raise their surface class-I expression (figure 1C), making them an optimal CD8+ T cell target, more CD8+ T cell activation was seen in the MIC-treated mice (figure 1A, *right panel*). The IFN-γ primed melanoma cells also evoked T cell activation in the CI-treated mice, while T cell activation was not detected in the untreated- and monobenzone-treated mice. The CD4+ T cell population did not display any significant responses (data not shown).

NK cell activity against B16.F10 melanoma cells was found in all treated mice as well as in untreated mice, as illustrated by TNF-α and IFN-γ production upon co-culture (figure 1B). These NK cells did not react with EL4 control cells, illustrating their melanoma-specific reactivity. However, NK cell activation was significantly increased in MIC-treated mice as compared to untreated mice, as indicated by the production of both TNF-α and IFN-γ. The other treatments did not enhance NK cell activity against B16.F10 cells, except for the increased IFN-γ production by NK cells from CI-treated mice.

Taken together, these *ex vivo* analyses demonstrate that MIC- and to a lesser extent CI-treatment induced the activation of TNF-α-producing melanoma reactive CD8+ T

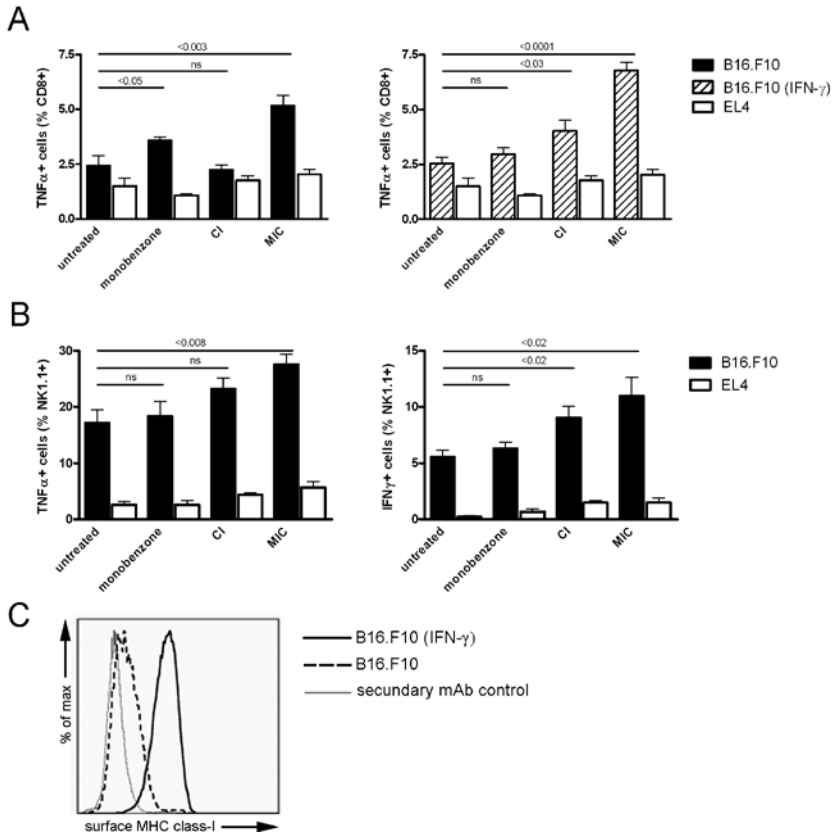


**Figure 1. MIC treatment of subcutaneous B16.F10 melanoma induced melanoma-reactive CD8+ T cells and -NK cells *in vivo*.** Splenocytes were tested for their *ex vivo* activation upon co-culture with B16.F10 melanoma or EL4 thymoma control cells (n=5 mice per group). **A,** *Left panel:* CD8+ T cells from monobenzone- and MIC-treated mice showed significant TNF-α production upon co-culture with melanoma cells (*black bars*; $p<0.05$ and $p<0.003$ respectively). In contrast, CD8+ T cells from CI-treated mice did not display significant TNF-α production upon melanoma cell co-culture (non significant difference: ns). *Right panel:* To identify CD8+ T cell activation upon co-culture with immunogenic melanoma cells with high MHC class-I expression, co-cultures with IFN-γ primed B16.F10 cells were included (*dashed bars*). Under these conditions, CI-treated mice showed significant TNF-α production as compared to untreated mice ($p<0.03$). The MIC-treated mice showed even more TNF-α production as in the non-IFN-γ-primed stimulation shown in the left panel. Monobenzone-treated and untreated mice did not display this increased T cell activation upon splenocyte co-culture with IFN-γ primed melanoma cells. T cell activation upon splenocyte co-culture with syngeneic EL4 thymoma control cells showed comparable background levels in all groups (white bars). **B,** *Left panel:* Only NK cells from MIC-treated mice showed significantly increased TNF-α production upon co-culture with melanoma cells ($p<0.008$). *Right panel:* Elevated production of IFN-γ was found in NK cells from CI- and MIC-treated mice in response to co-culture with melanoma cells ($p<0.02$ and $p<0.02$ respectively). TNF-α and IFN-γ production by NK cells was comparable in all groups upon co-culture with EL4 control cells. For the statistical analysis of the *in vivo* tumor growth kinetics of the treatments depicted in this figure, see table 1 ("Exp. 2"). **C,** B16.F10 melanoma cells upregulate their surface MHC class-I expression upon IFN-γ exposure. While IFN-γ-unexposed melanoma cells express very low levels of surface MHC class-I (*dashed line*), priming of these cells with 1000 U/ml IFN-γ restores their surface expression of MHC class-I (*black line*). Control incubations of IFN-γ-primed melanoma cells with only the IgG2a-detecting secondary antibody were negative (*grey line*).

cells. Furthermore, T cells induced by the MIC treatment were also able to recognize non-IFN-γ-primed, poorly immunogenic melanoma cells. This indicates that the MIC treatment generates a significant melanoma-reactive CD8+ T cell population with elevated functional avidity. Moreover, the MIC regimen significantly increased NK-cell melanoma-reactivity *in vivo*.

### Melanoma-reactive IgG response in MIC-treated mice

To further characterize the extent of the melanoma-specific immune activation induced by the MIC treatment, we investigated the formation of a melanoma-reactive antibody response. Of the mice described in figure 1, peripheral blood serum was obtained on day 18 of sacrifice. B16.F10 melanoma cells were permeabilized and binding of serum IgG-, IgM-and IgA was determined. Permeabilized EL4 thymoma cells were included as controls. As shown in figure 2, only the MIC-treated mice showed significant levels of melanoma-reactive serum IgG antibodies, whereas no IgG binding to melanoma cells was found in monobenzone- and CI-treated mice. Mice from all tested groups displayed comparable background reactivity to EL4 thymoma cells, illustrating the melanoma-specificity of the IgG-reactivity. IgG binding to intact, unpermeabilized melanoma cells was negligible (data not shown). Melanoma-reactive IgA was not detected and IgM did not show any significant differences between groups (data not shown). The melanoma-reactive IgG response induced by the MIC treatment *in vivo* indicates the involvement of a concurrent B cell response alongside the melanoma antigen-specific T cell activation found in the MIC-treated mice.

### MIC treatment inhibits growth of subcutaneous B16.F10 melanoma

To determine whether the melanoma antigen-specific immune responses described above were able to eradicate B16.F10 melanoma *in vivo*, we treated mice bearing subcutaneous melanoma and performed a long-term follow-up (figure 3A). On day



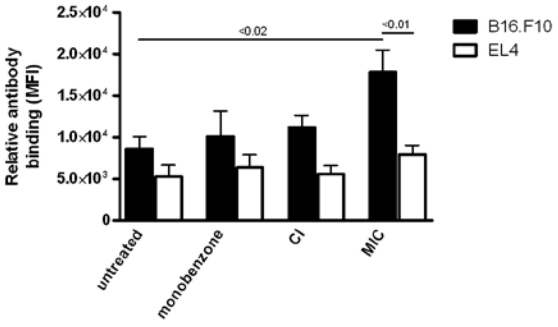


**Figure 2. A melanoma-reactive serum IgG response was found in MIC-treated mice.** Upon sacrifice peripheral blood serum was obtained from treated mice, and serum antibody binding to B16.F10 melanoma cells was analyzed using flow cytometry (n=5 mice per group). EL4 syngeneic thymoma cells were used as a control to verify the melanoma-specificity of the antibody binding, and showed comparable serum IgG binding levels in all groups ($p>0.05$, one-way ANOVA). A significant level of IgG antibody binding to the melanoma cells above the EL4 background level was found in the MIC-treated mice ($p<0.01$, paired t-test). Furthermore, only the MIC-treated mice showed melanoma-reactive IgG levels significantly above those found in untreated mice ($p<0.02$, unpaired t-test). IgA controls were negative, and IgM antibodies showed no significant binding levels (data not shown). Data is representative of three independent *in vivo* experiments.

0, C57BL/6 *wildtype* mice were inoculated subcutaneously with $2.5x10^3$ B16.F10 melanoma cells. Treatments were started on day 2, and continued for 33 days. Mice were either left untreated, treated with the individual treatment components monobenzone, CpG or imiquimod, or with CI- or MIC-treatment regimens and tumor growth was monitored (figure 3A, n=7 per group). All untreated mice showed tumor development (TD) around day 10, and none of these mice experienced a 200-day tumor-free survival (TFS). In contrast, in 85% of mice treated with MIC therapy the tumor did not grow during the treatment period (figure 3A *lower right panel*). Only one mouse did not respond to the MIC treatment and experienced a (delayed) tumor outgrowth during the treatment period, while all other mice remained tumor-free. 10 days after treatment cessation at day 35, two additional MIC-treated mice developed a melanoma. These animals were directly sacrificed for analysis without monitoring their tumor growth kinetics. This illustrates that MIC treatment effectively suppressed melanoma growth in these two mice during treatment, and that MIC therapy actively induces anti-tumor reactivity without merely preventing tumor implantation. Ultimately, the remaining 57% of the MIC-treated mice experienced tumor-free survival for more than 200 days. The individual treatment components monobenzone, CpG or imiquimod all mediated a certain degree of tumor growth delay, resulting in suppression of melanoma outgrowth in these groups. Furthermore, the combination of CpG and imiquimod acted synergistically in delaying melanoma outgrowth and inducing tumor-free survival. The survival data are summarized in figure 3B, showing the long-term tumor-free survival data of the different treatments. The therapeutic effect of the MIC treatment was found in four independent *in vivo* experiments, which all showed significant inhibition of tumor growth (table 1).

To investigate whether long-term surviving MIC-treated mice developed protective immunological memory, MIC-treated 100- and 200-day surviving mice from two independent experiments were challenged with B16.F10 melanoma cells subcutaneously in the flank. Without further treatment, tumor outgrowth was monitored and compared to untreated, naïve mice. Untreated mice showed comparable tumor outgrowth as seen in figure 3A (*upper left panel*). The MIC long-term survivors all showed a significant growth retardation following the tumor challenge (figure 3C, *upper and lower panel*). These data show that the MIC treatment had induced melanoma antigen-specific immunological memory which remained effective at 65- and 165 days after treatment cessation. Occasionally the development of vitiligo-like patches of depigmented fur at sites distant from initial monobenzone application was found in about 50% of long-term surviving mice (figure 3D). Importantly, vitiligo development occurred exclusively in mice treated with MIC therapy, and was absent in control-treated animals.

As summarized in table 1, the MIC regimen can mediate significant tumor-growth delay and increase tumor-free survival. Although monobenzone, CpG, imiquimod or the CI combination induced significant melanoma growth inhibition, they did not confer long-term tumor-free survival as found in the MIC therapy-treated mice. Combined with the immunological data described in figures 1 and 2, these results

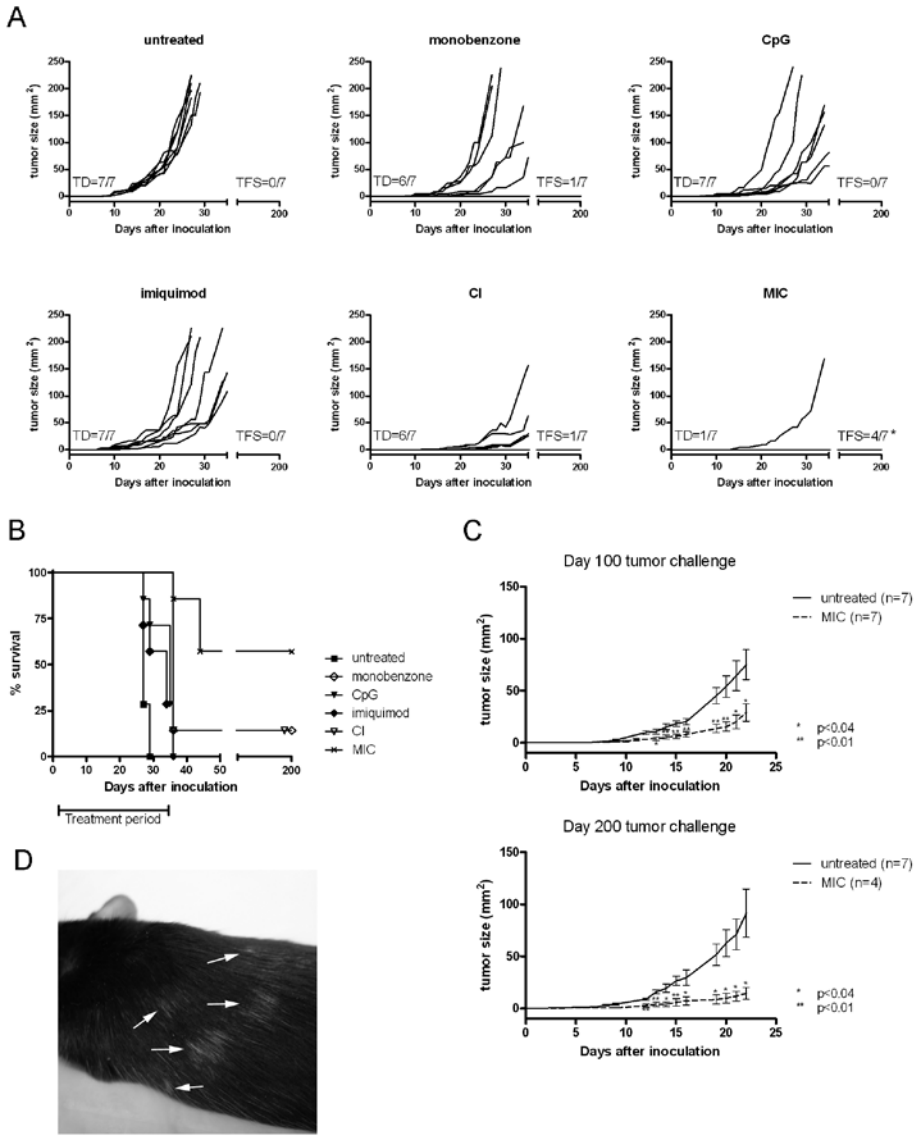


**Figure 3. Growth of subcutaneous melanoma is inhibited by MIC therapy.** Mice (n=7 per group) were treated with monobenzone, CpG, imiquimod, CI- or the MIC-regimen. Tumor growth and animal survival were monitored for 200 days. Each graph line depicts an individual tumor growth curve. **A,** *Upper left panel:* Untreated mice all show tumor development (TD) around day 10, and none of the mice experienced a 200-day tumor-free survival (TFS). In contrast, 6 out of 7 MIC-treated mice remained tumor-free during treatment, and only one mouse showed delayed TD during the treatment (*lower right panel*). *:10 days following treatment cessation at day 35, two additional mice developed a melanoma and these animals were directly sacrificed for analysis without monitoring tumor growth kinetics. Eventually, 4 out of 7 MIC-treated mice showed 200-day TFS, since 2 of the mice developed a melanoma 10 days following treatment cessation at day 35. The individual treatment components monobenzone, CpG or imiquimod all mediated a certain degree of tumor-growth delay. Interestingly, CpG and imiquimod clearly work synergistically in the CI ▷

regimen. Depicted tumor growth kinetics are representative of 4 independent *in vivo* experiments. For the statistical analysis of the *in vivo* tumor growth kinetics of the treatments depicted in this figure, see table 1 ("Exp. 4"). **B,** Kaplan-Meier survival curve for the different treatment groups depicted under A, showing 57% 200-day survival for MIC-treated mice against 14% long-term survival for monobenzone- or CI treated mice. Mice left untreated or receiving one of the individual treatment components, no TFS was found. These animals were sacrificed around day 25-35 due to maximally allowed tumor burden. **C,** The MIC therapy induced an effective immunological memory response. *Upper panel:* At day 100 (day 65 after treatment cessation) surviving mice were challenged with a melanoma tumor-inoculation, and tumor growth was monitored without further treatment. Untreated naive control mice showed rapid tumor development (*black line*, n=7). Mice treated previously with MIC therapy showed significant tumor growth delay (*dashed line*, n=7). *Lower panel:* Mice challenged at day 200 (day 165 after treatment cessation) show protective immunity to a comparable degree to that found in the day 100 tumor challenge shown in the upper panel. Untreated control mice showed normal tumor development (*black line*, n=7) while mice treated previously with the MIC therapy display significant tumor growth retardation (*dashed line*, n=4). Depicted graphs represent two independent tumor challenge experiments (follow-up on Exp. 3 & 4 in table 1). Statistical analysis using unpaired t-test comparing MIC-treated mice with untreated animals on designated time points (*: $p<0.04$, **:$p<0.01$). **D,** MIC-treated, long-term surviving mice occasionally develop progressively depigmenting patches of fur in a vitiligo-like pattern, distant from the initial monobenzone application site (arrows). This effect occurs in approximately 50% of the long-term surviving animals.

indicate that the immunological impact of the MIC regimen translates to significant tumor eradication *in vivo*.

### Activation of an NK cell response in MIC treated mice *in vivo*

We subsequently carried out immune monitoring of treated mice to investigate the *in vivo* immune activation. We determined the ratios of different peripheral blood leukocyte (PBL) populations in blood obtained from the tailvein on day 8 and 23 following melanoma inoculation (figure 4A). Interestingly, the MIC-treated mice (*right panels*) showed increased levels of NK cells (NK1.1+ CD3-) among their PBL on day 8, as compared to the untreated group. NK cell counts were also significantly increased in mice treated with CpG, imiquimod or the combination of these compounds (CI). Importantly, the increased NK cell numbers only persisted up to day 23 in MIC-treated mice (figure 4A). This MIC treatment-induced NK cell expansion has been found in three independent experiments, the statistical analysis of which is shown in table 2. Remarkably, monobenzone alone did not influence PBL ratios at either time point. Taken together, although the CI- and MIC treatment both mediated a significant increase in blood NK cell counts, this effect persisted only in MIC-treated mice.

Importantly, MIC-treated, tumor-free surviving mice included in tumor challenge experiments (figure 3C) did not display increased blood NK cell counts following the challenge ($p>0.05$; unpaired t-test at day 15 following inoculation: average percentage 10 (+/- 1.4)). This indicates that the identified NK cell expansion is MIC treatment-dependent. Furthermore, this suggests that the protective immunological memory observed in figure 3C is not dependent on NK cells. Instead, we found circulating melanoma antigen-specific CD8+ T cells in the peripheral blood of these MIC-treated, tumor-free surviving mice (figure 4B). To this end, peripheral blood CD8+ T cells were tested for their recognition of the H2-$K_b$/TRP-$2_{180\text{-}188}$-antigen. This is an

**Table 1. Statistics of *in vivo* tumor experiments.**

| Exp. | Treatment | # mice | Days after inoculation[1] | Average tumor size ($mm^2$) (+/- SEM) | p-value tumor size* (vs. untreated) | TFS during treatment (# mice) (% of group) | Median survival (days) | p-value survival** | TFS >100 days (# mice) (% of group) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | untreated | 7 | 22 | 64.8 (14.3) | nt | 0 | nt | nt | nt |
| | monobenzone | 7 | 22 | 27.1 (7.5) | <0.04 | 0 | nt | nt | nt |
| | MIC | 7 | 22 | 3.1 (1.9) | <0.0011 | 4 (57%) | nt | nt | nt |
| 2 | untreated | 5 | 18 | 70.6 (18.0) | nt | 0 | nt | nt | nt |
| | monobenzone | 5 | 18 | 50.0 (29.7) | ns | 0 | nt | nt | nt |
| | CpG | 5 | 18 | 14.4 (3.5) | <0.02 | 0 | nt | nt | nt |
| | imiquimod | 5 | 18 | 61.6 (7.2) | ns | 0 | nt | nt | nt |
| | CI | 5 | 18 | 9.0 (4.5) | <0.02 | 0 | nt | nt | nt |
| | MIC | 5 | 18 | 5.6 (3.0) | <0.008 | 2 (40%) | nt | nt | nt |
| 3 | untreated | 11 | 27 | 81.6 (20.2) | nt | 0 | 40 | nt | 0 |
| | MIC | 11 | 27 | 0.4 (0.2) | <0.0006 | 8 (72%) | >100 | <0.0001 | 7 (64%) |
| 4 | untreated | 7 | 27 | 187.9 (13.4) | nt | 0 | 27 | nt | 0 |
| | monobenzone | 7 | 27 | 88.9 (35.3) | <0.03 | 1 (14%) | 34 | <0.03 | 1 (14%) |
| | CpG | 7 | 27 | 71.1 (30.0) | <0.004 | 0 | 35 | <0.005 | 0 |
| | imiquimod | 7 | 27 | 102.9 (31.9) | <0.04 | 0 | 34 | <0.03 | 0 |
| | CI | 7 | 27 | 13.0 (5.3) | <0.0001 | 1 (14%) | 36 | <0.0003 | 1 (14%) |
| | MIC | 7 | 27 | 6.0 (6.0) | <0.0001 | 6 (85%) | >200 | <0.0003 | 4 (57%) |

**ns**: not significant (considered if p>0.05); **nt**: not tested; *: Unpaired *t*-test; **: Logrank test for survival (endpoint tumor size max 200 $mm^2$); **[1]**:Day of tumor size comparison (last day on which experimental animals were all alive); For Exp. 2 see Fig. 1A/B, for Exp. 3 see Fig. 3C (upper panel), for Exp. 4 see Fig. 3A/B and C (lower panel); **Exp**.: experiment; **TFS**: tumor-free survival; **CI**: CpG & imiquimod; **MIC**: monobenzone, imiquimod & CpG

immunodominant CD8+ T cell epitope of B16 melanoma. Figure 4B shows that a significant population of TRP-2 antigen-specific CD8+ T cells is present in tumor-free surviving mice, 120 days following the first melanoma inoculation and 85 days after MIC treatment cessation. Recognition of the control H2-$K_b$/$OVA_{257-264}$-tetramer by peripheral blood T cells of these mice was negative (data not shown).

Taken together, these data demonstrate that the MIC treatment effect is likely mediated by activated NK cells and CD8+ T cells. Importantly, when treatment is stopped melanoma antigen-specific CD8+ T cells remain active in tumor-free surviving mice, suggesting that these T cells are responsible for the MIC therapy-induced tumor protective immunological memory.

## CD8+ T cell- and NK cell depletion prior to- and during MIC therapy abrogates therapeutic effect

To verify whether NK cells and/or CD8+ T cells are essential for the therapeutic effect of the MIC therapy, we performed MIC treatment in C57BL/6 *wildtype* mice (n=5/

group) inoculated with B16.F10 melanoma, following either a CD8+ T cell- or NK-cell depleting pre-conditioning monoclonal antibody (mAb) regimen. Control groups either received the MIC treatment following an isotype control mAb regimen, or were left untreated. Depletion of CD8+ T cells and NK-cells was confirmed to be >98% and >95% respectively, as measured by flowcytometry (data not shown).

As shown in figure 5A, MIC treatment combined with CD8+ T cell-depletion (*black lines*) resulted in melanoma development in 100% of the mice. In contrast, MIC-treated mice receiving isotype control mAb (*grey lines*) showed reduced or no tumor outgrowth comparable to previous experiments (table 1), while melanomas grew out rapidly in untreated mice (*dashed lines*). Importantly, tumor outgrowth for CD8+ T cell-depleted mice was significantly faster than in mice treated with MIC therapy and the isotype control, nonetheless still significantly slower than in untreated mice. These

**Table 2. Statistics of *in vivo* peripheral blood NK cell counts.**

| Exp. | Treatment | # mice | Days after inoculation[1] | average % NK1.1+/CD3- PBL (+/- SEM) | p-value* |
|---|---|---|---|---|---|
| 2 | untreated | 5 | 15 | 7.3 (0.4) | nt |
| | monobenzone | 5 | 15 | 6.3 (0.2) | ns |
| | CpG | 5 | 15 | 8.2 (1.1) | ns |
| | imiquimod | 5 | 15 | 8.2 (0.3) | ns |
| | CI | 5 | 15 | 12.3 (1.9) | <0.04 |
| | MIC | 5 | 15 | 13.3 (2.2) | <0.03 |
| 4 | untreated | 7 | 8 | 6.65 (0.7) | nt |
| | monobenzone | 7 | 8 | 5.91 (0.2) | ns |
| | CpG | 7 | 8 | 11.42 (1.5) | <0.02 |
| | imiquimod | 7 | 8 | 9.91 (0.7) | <0.01 |
| | CI | 7 | 8 | 17.29 (1.0) | <0.0001 |
| | MIC | 7 | 8 | 17.43 (1.6) | <0.0001 |
| | untreated | 7 | 23 | 8.38 (1.2) | nt |
| | monobenzone | 7 | 23 | 7.03 (0.7) | ns |
| | CpG | 7 | 23 | 6.17 (0.7) | ns |
| | imiquimod | 7 | 23 | 3.81 (0.1) | ns |
| | CI | 7 | 23 | 5.95 (0.5) | ns |
| | MIC | 7 | 23 | 13.74 (0.7) | <0.01 |
| 7 | untreated | 7 | 2 | 6.7 (0.4) | nt |
| | MIC | 7 | 2 | 16.6 (2.2) | <0.001 |
| | untreated | 7 | 8 | 4.9 (0.3) | nt |
| | MIC | 7 | 8 | 12.6 (1.1) | <0.0001 |
| | untreated | 7 | 15 | 5.9 (0.8) | nt |
| | MIC | 7 | 15 | 12.8 (0.3) | <0.002 |

**Exp.**: experiment; **nt**: not tested; **ns**: not significant (considered if p>0.05); *: Unpaired t-test; [1]:Day after tumor inoculation on which PBL were tested; **CI**: CpG, imiquimod; **MIC**: monobenzone, imiquimod & CpG; For Exp. 4 see Fig. 4A

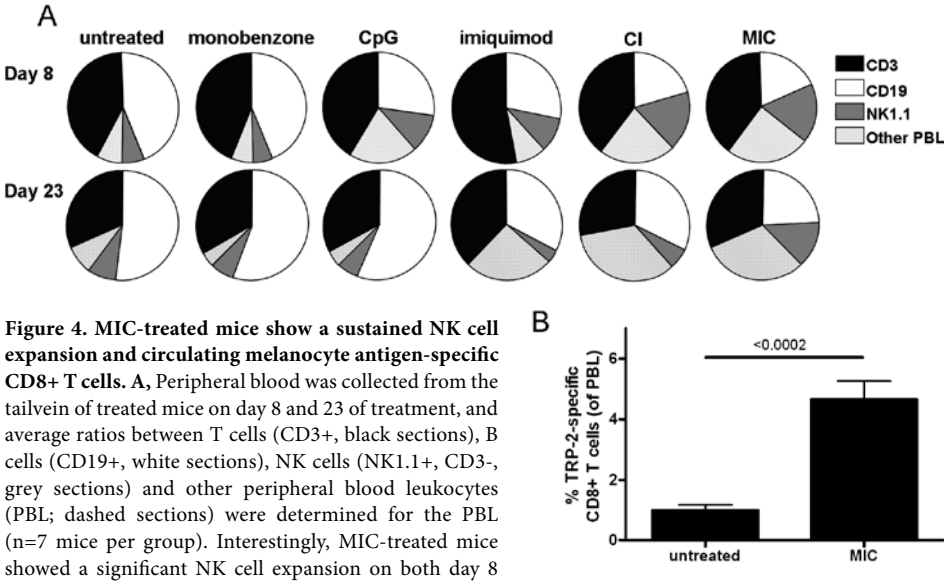


**Figure 4. MIC-treated mice show a sustained NK cell expansion and circulating melanocyte antigen-specific CD8+ T cells. A,** Peripheral blood was collected from the tailvein of treated mice on day 8 and 23 of treatment, and average ratios between T cells (CD3+, black sections), B cells (CD19+, white sections), NK cells (NK1.1+, CD3-, grey sections) and other peripheral blood leukocytes (PBL; dashed sections) were determined for the PBL (n=7 mice per group). Interestingly, MIC-treated mice showed a significant NK cell expansion on both day 8 and 23 (see table 2, "Exp. 4"). This expansion of NK cells was also found in CpG-, imiquimod- and CI-treated mice, although for these animals this reaction was only found on day 8. Monobenzone alone did not influence PBL ratios on either time point, comparable to untreated mice. Depicted data is representative of three independent *in vivo* mouse experiments. For the statistical analysis of the *in vivo* differences in NK cell counts in these experiments see table 2. **B,** Peripheral blood CD8+ T cells were tested for binding to H2-$K_b$/TRP-$2_{180\text{-}188}$-tetramers at day 120 following tumor inoculation (day 85 after treatment cessation, n=4). TRP-2 represents one of the immunodominant epitopes of B16.F10 melanoma. Long-term surviving, MIC-treated mice showed a significant population of TRP-2-specific CD8+ T cells circulating in their peripheral blood at day 120, as compared to untreated mice 10 days after tumor inoculation (n=7). Binding to control H2-$K_b$/$OVA_{257\text{-}264}$-tetramer by the tested PBL was negative (data not shown).

results show that the anti-tumor effect of the MIC treatment was largely abolished by the depletion of the CD8+ T cells. The minor but significant tumor growth inhibition by the MIC regimen in the absence of CD8+ T cells suggests that this therapy also effectively involves another cell population, likely the NK cells we observed to be activated in previous experiments. Figure 5B shows that depletion of the NK cell population during MIC therapy resulted in tumor development in 80% of the mice (*black lines*). Importantly, melanoma outgrowth rapidly reached a state of "stable disease" in this situation, with tumors remaining of equal size for extensive periods of time. Taken together, these data imply that the MIC therapy effectively engages both CD8+ T cells and NK cells in its protective therapeutic effect.

## MIC therapy significantly suppresses tumor growth of established melanoma

To determine if MIC treatment has a therapeutic effect on larger established melanoma, mice were subcutaneously inoculated with $1x10^5$ B16.F10 melanoma cells and treatments were started when all tumors had reached the minimum measurable size of 2x2 mm (on average after 3-4 days). Mice were treated with the individual

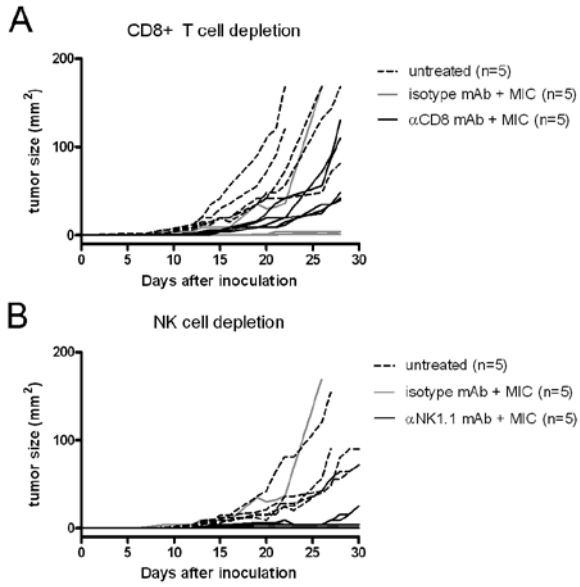


**Figure 5. The therapeutic effect of MIC therapy is abrogated by NK cell- or CD8+ T cell depletion.** Each graph line represents an individual tumor growth curve. **A,** Mice depleted of CD8+ T cells prior to tumor inoculation and throughout the MIC therapy (*black lines*, n=5) all developed a tumor. In these MIC-treated mice tumors grew out significantly faster than in the isotype-control mAb-treated mice (*grey lines*, $p<0.02$, n=5), who displayed tumor growth kinetics similar to MIC-treated animals in previous experiments. Nonetheless, tumor outgrowth in CD8+ T cell depleted mice was still significantly slower than in untreated mice (*dashed lines*; $p<0.02$, n=5). The tumor size of CD8+ T cell depleted animals was statistically compared with isotype-control mAb-treated animals on day 28, and with untreated animals on day 22. **B,** Mice depleted of NK cells prior to tumor inoculation and throughout MIC therapy (*black lines*, n=5) showed tumor establishment in 80% of mice. Tumors in these mice grew out very slowly or remained of equal small size throughout the experiment. In contrast, untreated mice showed rapid tumor development (*dashed lines*, n=5). Mice treated with isotype-control mAb and MIC therapy (*grey lines*, n=5) showed tumor growth similar to mice treated with MIC therapy in previous experiments.

treatment compounds alone, or the CI- or MIC regimen. Additionally, combinations of monobenzone with either CpG or –imiquimod as only additional adjuvant were compared with the CI regimen to verify if monobenzone is the critical component in the MIC therapy. The statistical analyses of the tumor growth kinetics in 4 independent *in vivo* experiments using this established tumor setting are summarized in table 3. All treatments containing monobenzone and at least one adjuvant mediated a significant growth delay of established B16.F10 tumors, whereas the adjuvants imiquimod and CpG alone or combined (CI) did not significantly inhibit tumor growth. Of the treatments containing monobenzone, the combination of all three compounds (MIC treatment) showed the most profound therapeutic effect. Importantly, as shown in table 3 under "Exp. 5", monobenzone combined with either imiquimod or CpG as adjuvants displayed a significantly better therapeutic effect than the CI regimen, when compared to untreated mice. Both exp. 5 and exp. 6 showed that MIC treatment was significantly more effective than CI treatment. This indicates monobenzone to be the pivotal component in mediating the therapeutic effect of the MIC regimen. Besides significantly suppressing tumor growth, MIC therapy was also found to significantly improve the survival of mice bearing established melanoma.

These data illustrate that the MIC regimen also has a significant therapeutic effect in the treatment of established subcutaneous melanoma.

**Table 3. Statistics of *in vivo* tumor experiments inoculating 1x10e5 melanoma cells and starting treatment when all tumors were at least 2x2 mm.**

| Exp. | Treatment | # mice | Days after inoculation[1] | Average tumor size ($mm^2$) (+/- SEM) | p-value tumor size* (vs. untreated) | p-value tumor size* (vs. CI) | Median survival (days) | p-value survival** |
|---|---|---|---|---|---|---|---|---|
| 5 | untreated | 5 | 15 | 98.6 (20.7) | nt | nt | 15 | nt |
| | monobenzone | 5 | 15 | 73.6 (10.2) | ns | nt | 15 | ns |
| | imiquimod | 5 | 15 | 74.4 (5.0) | ns | nt | 15 | ns |
| | CpG | 5 | 15 | 55.0 (5.6) | ns | nt | 19 | <0.05 |
| | CI | 5 | 15 | 57.8 (8.0) | ns | nt | 19 | <0.05 |
| | monobenzone, imiquimod | 5 | 15 | 32.4 (10.3) | <0.03 | ns | 19 | <0.05 |
| | monobenzone, CpG | 5 | 15 | 32.8 (9.6) | <0.03 | ns | 22 | <0.02 |
| | MIC | 5 | 15 | 26.4 (7.5) | <0.02 | <0.03 | 22 | <0.02 |
| 6 | untreated | 3 | 10 | 81.3 (29.4) | nt | nt | 13 | nt |
| | CI | 5 | 10 | 1.4 (0.2) | <0.01 | nt | 28 | <0.007 |
| | MIC | 5 | 10 | 1.0 (0.3) | <0.01 | <0.04 | 24 | <0.007 |
| 7 | untreated | 7 | 16 | 135.6 (24.4) | nt | nt | 18 | nt |
| | MIC | 7 | 16 | 8.9 (3.1) | <0.001 | nt | 28 | <0.002 |
| 8 | untreated | 7 | 16 | 145.4 (15.1) | nt | nt | 16 | nt |
| | MIC | 7 | 16 | 17.6 (6.3) | <0.0001 | nt | 26 | <0.0001 |

**ns**: not significant (considered if p>0.05); **nt**: not tested; [1]:Day of tumor size comparison (last day on which experimental animals were all alive); *: Unpaired *t*-test; **: Logrank test for survival; **Exp.**: experiment; **TFS**: tumor-free survival; **CI**: CpG, imiquimod; **MIC**: monobenzone, imiquimod & CpG

## DISCUSSION

In the present study we demonstrate that the vitiligo-inducing properties of monobenzone cream combined with the immunostimulatory adjuvants CpG and imiquimod synergistically induces potent melanoma antigen-specific immunity and tumor eradication in the B16-B6 mouse model of melanoma. We show that the MIC treatment of subcutaneous B16.F10 melanoma in C57BL/6 *wildtype* mice inhibited the outgrowth of melanoma in up to 85% of the mice. Moreover, 64% or 57% of the mice remained tumor-free for more than 100- or 200 days respectively. The MIC treatment induced melanoma antigen-specific immunological memory, effectively suppressing tumor growth up to 165 days after treatment cessation. Immunomonitoring of the MIC treated mice showed that the regimen induced a systemic B16-specific CD8+ T cell- and NK cell response. Additionally, a melanoma-specific serum IgG response was found in the MIC treated mice. MIC therapy was also effective against larger established tumors, mediating significant tumor growth inhibition and prolonged survival. These data show that MIC therapy triggers a strong melanoma-specific *in vivo* innate- and adaptive immune response.

It has been suggested by Berghöfer *et al.* that the combination imiquimod and CpG is deleterious, since *in vitro* it has been found that triggering of TLR7 and

TLR9 simultaneously on DC or leukocytes will deteriorate the induced interferon-α production by these cells [37]. Importantly, the study by Berghöfer *et al.* concludes that the inhibitory effect of simultaneous TLR7 and -9 triggering only applies to CpG types A and C, while such an effect was not found for CpG type B. Our present study employs CpG 1826, which is a B-type CpG. While Berghöfer *et al.* show that combining B-type CpG with TLR7 triggering was not inhibitory, even showing a slight synergistic trend, we have actually found a functional synergism between CpG 1826 and TLR7-triggering by imiquimod in our present *in vivo* study.

Depletion of CD8+ T cells and NK cells prior to- and during MIC treatment ablated the therapeutic effect, indicating that these cells are imperative in mediating the MIC therapeutic effect. It seems that MIC therapy induces the immune cascade as previously suggested by Liu *et al.* [38], which is characterized by the sequential activation of NK cells, conventional DC and ultimately the formation of effective CD8+ T cell immunity and immunological memory. Importantly, MIC treatment also led to the generation of melanoma-specific antibodies. These antibodies bound to intracellular antigens, selectively in melanoma cells. This indicates they are directed against antigens in the melanosome, an organelle found exclusively in pigmented cells such as melanocytes and melanoma cells. Since the melanosome is the primary source of melanocyte differentiation antigens, the antibodies we identified can enhance the adaptive immune response through opsonisation of antigen liberated from dying pigmented cells. Through the subsequent formation of immune complexes this improves the uptake and successive (cross) presentation of melanoma antigens by DC [39].

Melanoma-specific CD8+ T cells induced by the MIC regimen show a higher functional avidity. As shown in figure 1A, CD8+ T cells in the MIC-treated mice could be activated by non-IFN-γ-primed melanoma cells. These cells have very low levels of MHC class-I (figure 1C), rendering them poorly recognizable to T cells. The occurrence of high avidity T cells recognizing poorly immunogenic melanoma cells suggests that the MIC therapy mediates an avidity maturation process, which is known to occur in T cell populations exposed to constant high levels of antigen [40]. The continuous application of monobenzone on the skin may generate a steady flow of melanocyte-specific antigens, leading to the avidity maturation of the reactive T cell pool. This maturation clearly did not occur during the CI treatment, thereby leading to the absence of high avidity T cells recognizing IFN-γ unprimed melanoma cells in these mice. The CI regimen provides a general immune activating stimulus, which in the presence of melanoma may result in short-term anti-melanoma reactivity. In contrast, the MIC regimen specifically engages melanocyte differentiation antigens in the generation of adaptive immunity via the monobenzone component (discussed below). The therapeutic value of this antigen-targeting by monobenzone is demonstrated by the enhanced ability to induce melanoma-reactive CD8+ T cells and protective anti-melanoma immunity *in vivo*, as compared to the CI regimen.

Concerning the working mechanism of monobenzone, it has a selective- and inactivating interaction with the enzyme tyrosinase which catalyzes pigment synthesis

in the melanosome, an organelle exclusively found in melanocytes [21]. This interaction results in the formation of quinone metabolites which bind to cysteine residues in the protein peptide chain, forming quinone-haptens [31]. These quinone hapten-carrier complexes are known to be potent contact sensitizers which can trigger hapten-specific immune responses [41,42]. Generally, this kind of hapten-induced immunity results in enhanced depigmentation *in vivo*, since quinone-metabolites of phenols and catechols are known to induce more extensive depigmentation than the parental compound [33]. Moreover, the extent of catechol-induced depigmentation depends on quinone formation by the tyrosinase enzyme, including subsequent covalent binding of the quinone to proteins [32]. At high concentrations (250-500 μM for 24 hours) monobenzone can additionally induce non-apoptotic cell death in exposed melanocytes *in vitro* [43]. In contrast, we have investigated that at lower concentrations (20 to 40 μM) monobenzone-exposed pigmented cells provoke robust and specific CD8+ T cell immunity, by the formation of quinone-haptens to the tyrosinase protein and the subsequent activation of local dendritic cells by exposed pigmented cells (Van den Boorn *et al.*, *manuscript in preparation of submission*). Since melanoma cells share many antigens with their normal counterparts, the melanocytes, the melanocyte-specific immunity evoked by monobenzone also acts against melanoma cells (as evidenced by the MIC therapy). Because priming of the anti-melanoma immune response during MIC therapy depends upon the interaction of monobenzone with tyrosinase in skin melanocytes, and the induced immune response encompasses more melanosomal antigens besides tyrosinase (figure 4B and *manuscript in preparation*), our MIC regimen may well be effective against tyrosinase-negative melanoma variants. Moreover, tyrosinase expression appears to be conserved in malignant melanoma cells [44]. Tyrosinase expression in melanoma tissue also provides the possibility of local treatment of cutaneous metastasis with the MIC regimen.

Interestingly, vitiligo-like depigmentation of the fur distant from the monobenzone application site occurred in approximately 50% of the long-term surviving MIC-treated animals (figure 3D), but not in non-responding or control treated mice. This phenomenon illustrates an enduring systemic immune response effective against pigmented cells. However, vitiligo development was expected to directly correlate with the effective anti-tumor immune response observed in all MIC –treated mice, especially since monobenzone is a potent skin depigmenting agent in humans. The vitiligo-like fur depigmentation in mice depends on autoimmune melanocyte destruction in the hair follicle. The modest level of vitiligo may be explained by the fact that hair depigmentation (poliosis) only occurs in advanced cases of vitiligo [45], and is likely related to the hair follicle being an immune-privileged site in both humans [46] and mice [47]. Thereby, fur depigmentation likely underestimates the level of autoimmune activation against pigmented cells. The immune privilege of the hair follicle can thereby explain the modest depigmentation observed in the MIC-treated mice.

In conclusion, we have developed a new form of off-the-shelf melanoma immunotherapy consisting of two creams and four CpG injections. This simple design

makes the MIC therapy easily applicable in the clinic. Moreover, this low-cost regimen does not require stringent patient eligibility criteria such as HLA-haplotype, and does not require elaborate patient-specific *in vitro* cell cultures or non-myeloablative lymphodepletion prior to the start of treatment, reducing patient treatment strain.

## MATERIALS AND METHODS

### Ethics statement

All animal experiments were carried out under protocols approved by the Animal Ethical Committee of the Academic Medical Centre in Amsterdam (DEC code DDE, studies 100452, 100969 and 101291). Mice were obtained from Charles River Labs (Maastricht, The Netherlands). Animals were housed in the Animal Research Institute Amsterdam (ARIA; ABSL-3/DM-II housing level) and cared for by qualified personnel on a daily basis. Food and water were available *ad libitum* and cages contained bedding, shelter and nesting material.

### Cell lines and mice

B16.F10 melanoma cells (a kind gift from Dr. A. Jorritsma, Netherlands Cancer Institute, Amsterdam, The Netherlands) and EL4 thymoma cells (a kind gift from the department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands) were maintained in culture medium consisting of RPMI 1640 (Cambrex bioscience, Verviers, Belgium) with 10% heat-inactivated fetal bovine serum (Hyclone, Erembodegem-Aalst, Belgium), 2mM L-glutamine (Gibco invitrogen, Breda, The Netherlands), 50 U $ml^{-1}$ penicillin and 50 µg $ml^{-1}$ streptomycin (Gibco invitrogen), in a humidified atmosphere at 37 °C and 5% $CO_2$. Female SPF C57BL/6 wildtype mice were used in tumor experiments at 10 weeks of age.

### Tumor inoculation and treatments

Mice were inoculated with $2.5x10^3$ or $1x10^5$ B16.F10 melanoma cells in 50 µl of sterile PBS (Dulbecco's PBS, PAA, Pasching, Austria) subcutaneously in the right flank. Treatments were started 2 days later (for $2.5x10^3$ inoculated melanoma cells) or when all tumors reached a minimum size of 2x2 mm (for $1x10^5$ inoculated melanoma cells on average after 3-4 days). Treatment continued for 33 days. B16.F10 *in vitro* cell culture was standardized for all experiments. Melanoma cells were in the exponential growth phase *in vitro* when used for *in vivo* inoculation, and were confirmed to be >98% viable by trypan blue exclusion. Daily, perpendicular tumor diameters were measured using callipers. Mice were treated with daily applications of 50 µl of 20% monobenzone cream (4-benzyloxyphenol, Sigma-Aldrich, Zwijndrecht, The Netherlands; cream prepared by the Academic Medical Centre pharmacy for use in animal experiments) and/or 50 µl of 5% imiquimod cream (Aldara™, 3M Healthcare, Leicestershire, UK) on Monday, Wednesday and Friday on the shaved abdomen. Creams were completely massaged in using a spatula. Mice received 50 µl of completely phosphorothioated CpG

oligodeoxynucleotides (1 mg $ml^{-1}$ in sterile PBS) injected peritumorally on day 0, 3, 6 and 21 (CpG B 1826, 5'-TCCATGACGTTCCTGACGTT-3', produced as reported previously [48]).

### In vitro splenocyte priming assay

Erythrocytes were removed from fresh splenocyte single cell suspensions by hypotonic lysis, and splenocytes were cultured in RPMI 1640 (Cambrex) with 10% heat-inactivated fetal bovine serum (Hyclone), 2mM L-glutamine (Gibco invitrogen), 50 U $ml^{-1}$ penicillin and 50 µg $ml^{-1}$ streptomycin (Gibco invitrogen), 50 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands), 15 µg $ml^{-1}$ gentamycin (Duchefa, Haarlem, The Netherlands), 20 U $ml^{-1}$ IL-2 (Novartis, Arnhem, The Netherlands) and 5 µg $ml^{-1}$ ConA (Boehringer Mannheim, Mannheim, Germany). ConA blasts were co-cultured on day 10 with B16.F10 and EL4 cells *in vitro* in a 1:1 ratio, in culture medium supplemented with IL-2 and 2-mercaptoethanol for 5 hours at 37 °C and 5% $CO_2$ in the presence of brefeldin-A (1:1000, Golgiplug, BD Bioscience, San Diego, CA) protein transport inhibitor. When applicable B16.F10 cells were 24 hours IFN-γ pre-treated prior to splenocyte co-culture (1000 U $ml^{-1}$, Strathmann, Bergisch Gladbach, Germany). Subsequently cells were stained for intracellular cytokine production.

### Flow cytometry and intracellular cytokine staining

Erythrocyte-free single cell suspensions of splenocytes, ConA blasts or PBL were prepared in FACS buffer (PBS with 1% bovine serum albumin and 0.05% $NaN_3$) and cells were stained in the dark on ice for 20 minutes for surface expression of CD8α (APC-Cy7), CD4 (PerCP-Cy5.5), CD3ε (FITC), CD137 (FITC), CD19 (PerCP-Cy5.5), or NK1.1 (Pe-Cy7; all antibodies BD Bioscience, San Diego, CA). For intracellular staining, cells were permeabilized using the Cytofix/Cytoperm kit (BD Bioscience) according to the manufacturer's protocol, and stained for intracellular cytokines using TNF-α (Pe-Cy7, BD Bioscience), TNF-α (PE, e-Bioscience, San Diego, CA) or IFN-γ (Alexa-700, BD Bioscience). Tetramer analysis was performed by incubating cell suspensions with H2-$K_b$/TRP-$2_{180-188}$-tetramer (SVYDFFVWL, APC-labelled, Sanquin, Amsterdam, The Netherlands) or H2-$K_b$/$OVA_{257-264}$-tetramer (SIINFEKL, PE-labelled, kind gift from K.L. Franken, Leiden University Medical Centre, The Netherlands) in CM for 25 minutes at 37°C and 5% $CO_2$ prior to subsequent surface antibody staining. Surface MHC class-I detection was performed by incubating IFN-γ-primed and –unexposed B16.F10 cells with mouse-anti-mouse MHC class-I antibody (clone 28-14-8, IgG2a, eBioscience, San Diego, CA) followed by incubation with goat-anti-mouse IgG2a-detecting antibody (Alexa 488, Molecular Probes, Invitrogen, Breda, The Netherlands). Samples were measured on a FACS Canto-II flowcytometer (Beckton Dickinson, San Diego, CA). Data was analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

### Serum antibody determination

B16.F10 and EL4 cells were washed in FACS buffer and permeabilized using the Cytofix/Cytoperm kit (BD Bioscience, San Diego, CA) according to the manufacturer's protocol. Cells were incubated with mouse serum diluted 1:200 in Perm/Wash (BD Bioscience) for 1 hour at room temperature. Subsequently, cells were tested for serum-antibody binding by incubation with biotinylated anti-mouse IgG, -IgA and -IgM antibodies (Biolegend, Uithoorn, The Netherlands) for 20 minutes on ice. Binding of biotinylated detection antibodies to target cells was detected by incubation with streptavidin-APC (BD Pharmingen, San Diego, CA) for 20 minutes in the dark on ice, and analyzed by a FACS Canto-II flowcytometer (Beckton Dickinson, San Diego, CA).

### In vivo NK cell- and CD8+ T cell depletion

Mice were injected intraperitoneally on day -2 and 0 prior to tumor inoculation and every following fourth day during the experiment with 100 μg of anti-mouse CD8α or anti-mouse NK1.1 (clone 53-6.7 or PK136 respectively, low-endotoxin azide-free purified, Biolegend, Uithoorn, The Netherlands) or IgG2a κ isotype control (clone RTK2758, low-endotoxin azide-free purified, Biolegend) monoclonal antibodies. Prior to tumor inoculation CD8+ T cell or NK cell depletion was confirmed by flowcytometry.

### Statistical analysis

Analysis comparing values between treatment groups were performed using a two-tailed unpaired t-test (95% CI). Different conditions within the same treatment group were compared using the two-tailed paired t-test (95% CI). Survival data was analysed using the Logrank test (95% CI). *: $p<0.04$, **: $p<0.01$. Analyses carried out with GraphPad Prism 5 software (GraphPad, La Jolla, CA). Graphs depict mean with SEM.

## AUTHOR CONTRIBUTIONS

J.G.B. conducted all experiments and wrote the manuscript, D.K., E.P.M.T., D.I.P., N.J.M. and D.V.F. assisted in performing the in vivo and in vitro experiments, J.P.W.V., J.D.B., C.J.M.M. and R.M.L. supervised this study, R.M.L. is the principal investigator who initiated this study.



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# CHAPTER 8

GENERAL DISCUSSION

## BACKGROUND

Malignant melanoma cells meet with the constant challenge of having to evade host immunity, in order to embody a progressive tumor. From deceiving immune surveillance in the early phases to employing active immune-evasive strategies later on, the tumor cells endure by dodging protective immunity and relying on their melanocytic origin. The initial achievements with Coley's toxins[1] showed that tumor regression can occur when a "foreign" signal is associated with the tumor cells. Many melanoma vaccination regimens since then have been built on a mixture of antigenic tumor cell elements and microbial constituents. The success of anti-melanoma vaccination has been limited[2] as central and peripheral T cell selection mechanisms actively delete and suppress autoreactive T cells, thereby impeding autoimmune responses towards melanocyte differentiation antigens. A situation in which tolerance and autoimmunity are visibly opposed is that of vitiligo and melanoma. While in vitiligo high-avidity T cells progressively eradicate skin melanocytes, melanoma patient-derived T cells with identical melanocyte antigen-specificities display a degenerate functional phenotype[3]. Accordingly, vitiligo development during melanoma treatment is considered to indicate effective T cell immunity against the antigens shared between melanocytes and melanoma cells, and is associated with improved patient survival[4, 5]. The research described in this thesis has focused on actively inducing vitiligo as a treatment for melanoma, by using the strong skin-depigmenting, vitiligo-inducing agent monobenzone. In pursuit of this goal, we have established the active role of CD8+ T cells in the induction and progression of vitiligo vulgaris, thereby revealing its autoimmune basis and providing ground for actively inducing vitiligo as a treatment for melanoma (chapter 3). Subsequently, we have dissected the molecular- and immunological mechanism by which monobenzone induces vitiligo and the associated melanocyte antigen-specific CD8+ T cell response. Moreover, an effective monobenzone-based immunotherapy regimen for melanoma was developed in the B16-B6 model of malignant murine melanoma.

## AN INTEGRATED VIEW OF MONOBENZONE-EVOKED IMMUNITY: THE FORMATION OF QUINONE-HAPTENS

Many phenolic or catecholic substances are able to induce skin depigmentation[6, 7]. Most substances predominantly mediate depigmentation at the site of skin exposure, as they specifically inhibit the enzyme tyrosinase and thereby mediate suppression of melanogenesis in local melanocytes[8, 9]. Additionally, some phenolic derivates, most potently the monobenzyl ether of hydroquinone (monobenzone)[10], have been found to induce progressive skin depigmentation indistinguishable from vitiligo upon prolonged occupational exposure[11]. The exact mode of action of these agents has largely remained obscure. Research on the monomethyl ether of hydroquinone, a skin depigmenting agent structurally closely resembling monobenzone[12], showed that this compound is converted into a highly reactive benzoquinone product by tyrosinase[13]. Benzoquinone

was found to covalently bind to nucleophilic groups in amino acids added to the reaction mixture[14], specifically cysteines[15]. Thereby it forms benzoquinone-haptens to amino acids, which are potent skin sensitizers[16]. Monobenzone is a more effective skin depigmenting agent than the monomethyl ether of hydroquinone, and early clinical studies on monobenzone also report skin sensitization[11, 17, 18]. In trying to explain the progressive vitiligo regularly observed following monobenzone application, some studies postulated that its effect may depend on "a foreign material bearing the mark of monobenzone, becoming attached to the melanin granule"[19]. Thereby, "this possible hapten could initiate a systemic melanocyte-destructive inflammatory response"[20]. These early assumptions were quite in the right direction, since our present findings reveal that monobenzone, like the monomethyl ether of hydroquinone, is converted into a reactive quinone product (4-benzoxy-1,2-benzoquinon) which binds as a quinone-hapten to cysteine residues in the tyrosinase protein (chapter 5). Thereby, monobenzone generates neo-antigens in the tyrosinase peptide chain. Furthermore, we demonstrated that monobenzone-exposed melanoma cells, in contrast to unexposed melanoma cells, were highly immunogenic and able to induce an impressive pigment cell-specific CD8+ T cell response in healthy human donors within 7 days of *in vitro* culture, dependent on cross-presentation by DCs. Although conferring TLR-independent immunogenicity to the antigen they are attached to[21], these quinone-haptens can not solely explain the melanocyte antigen-specific immune response evoked by monobenzone. Indeed, considerable CD8+ T cell reactivity towards non-monobenzone-treated melanoma cells was found, while haptens mainly induce hapten-specific responses[21, 22] (chapter 5). Also, the immune response we found *in vivo* was rapidly effective against a distant, monobenzone-unexposed, subcutaneous melanoma (chapter 7).

## THE GENERATION OF REACTIVE OXYGEN SPECIES, AUTOPHAGY AND EXOSOMES

Monobenzone has been suggested to induce oxidative stress in exposed pigmented cells by producing reactive oxygen species (ROS) such as peroxide upon its catalytic conversion[7]. The generation of ROS upon tyrosinase-dependent conversion has been demonstrated for the closely related phenolic compounds hydroquinone[23] and the monomethyl ether of hydroquinone[24]. It has been found to disrupt melanosomal membranes and melanosome structure[25]. In chapter 5 we have established that monobenzone induces ROS formation in pigmented cells, and furthermore have revealed that monobenzone induces lysosomal degradation of melanosomes by autophagy (chapter 6). Combined with its direct inhibition of melanogenesis by tyrosinase inactivation, these processes mediate the hypopigmented appearance of the exposed melanocytes[19] (chapters 5 and 6). However, monobenzone does not induce vitiligo by direct ROS-related cytotoxicity towards melanocytes, as we have demonstrated that monobenzone-induced cell death is unrelated to cellular pigmentation or tyrosinase expression (chapter 5). Nevertheless, the generation of ROS clarifies the effective spread of immunity beyond the haptenated antigens by the initiation of melanosome autophagy.

Mild levels of cellular oxidative stress, not mediating immediate apoptosis, are known to promote cellular autophagy pathways[26-28]. The lysosomal degradation of damaged organelles by macroautophagy processes antigen for the MHC class-II[29, 30] presentation pathway, as autophagosomes have been found to regularly fuse with MHC class-II-compartments[30]. Importantly, the induction of autophagy in melanoma cells has been shown to confer effective immunogenicity to these cells and exerts exceptional therapeutic efficacy[31]. In chapter 6 we demonstrated that monobenzone-exposed melanocytes and melanoma cells process immunodominant tyrosinase antigens via the lysosomal route into MHC class-II-compartments by melanosome autophagy. In this chapter we furthermore show that tyrosinase is ubiquitinated under influence of monobenzone, which both mediates proteasomal degradation of tyrosinase and can function as an initiating receptor for autophagy[32, 33]. Interestingly, autophagy has also been reported to generate antigens for MHC class-I presentation[34]. By together stimulating antigen processing for the MHC class-I and –II routes, monobenzone effectively elevates the surface presentation of melanosomal antigens. Melanocytes express both MHC class-I and –II on their surface, and have effective antigen-presenting capabilities[35]. Thereby, monobenzone-exposed melanocytes may already by themselves initiate melanocyte antigen-specific T cell responses.

In addition, we have found that monobenzone induces exposed pigmented cells to release exosomes, which contain tyrosinase and MART-1 antigens (chapter 5). Exosome secretion by melanoma cells has been shown to occur under conditions of mild oxidative stress[36], and is important in the communication between tumor- and immune cells[37]. Both ubiquitination and autophagy can generate exosome release. Ubiquitination is a strong sorting signal directing transmembrane proteins like tyrosinase and MART-1 into the internal vesicles of the multivesicular endosome[38-42]. Moreover, the multivesicular endosome can engulf small portions of cytoplasm, containing proteins released from damaged organelles, by micoautophagy[37, 43]. Both processes generate antigen-containing vesicles in the multivesicular endosome, which it can release as exosomes upon fusion with the plasma membrane[44]. Certain cytokine- or oncogenic protein-containing exosomes have been related to tumor immune-escape[36, 45], while tumor antigen-containing exosomes considerably improve the CD8+ T cell response against melanoma antigen *in vivo* by enhancing antigen cross-presentation by DCs[46, 47]. Furthermore, exosomes can express antigen-loaded MHC class-I and –II molecules, directly enhancing antigen presentation by DCs[37]. The melanocyte antigen-containing exosomes we found in our studies help explain why monobenzone-treated intact melanoma cells are immunogenic in our *in vitro* DC-T cell experiments, while intact unexposed melanoma cells were not (chapter 5). By the exosomes monobenzone effectively promotes the transfer of (haptenated) melanosomal antigens from exposed melanocytes to DCs, and thereby the induction of melanocyte antigen-reactive T cell immunity. Moreover, melanosome autophagy and tyrosinase ubiquitination provide the most likely explanation why monobenzone-exposed pigmented cells become immunogenic: monobenzone exposure leads to cellular depigmentation and the

constant engagement of exposed cells in the processing-, presentation- and shedding of melanosome-derived proteins. This augmented presentation of melanosomal antigen can subsequently result in the activation of autoreactive T cells.

## RAPID INNATE IMMUNE RESPONSIVENESS

*In vivo* we have shown that monobenzone, when combined with the immunostimulatory adjuvants imiquimod and CpG (MIC therapy), induces effective immunity against subcutaneous malignant B16.F10 melanoma (chapter 7). In these studies we have found a rapid and persistent innate immune activation, illustrated by a significant melanoma-reactive NK cell expansion being present in the blood as early as 1 day following start of treatment (chapter 7). This rapid NK cell response probably mediated the immediate tumor growth-suppression observed upon starting MIC treatment (unpublished observations), since NK-cell depletion during MIC therapy abolishes its early protective effect (chapter 7). Augmented NK cell activation and -cytotoxicity can be evoked by high levels of interferon-α (IFN-α) and IL-12, produced by plasmacytoid- and conventional DCs becoming activated by PAMPs such as CpG[48, 49] and imiquimod[50]. Although in our *in vivo* work we observed synergism between CpG and imiquimod in inducing an NK cell response (chapter 7), the NK cell response here subsided within approximately 15-20 days. In contrast, during MIC therapy this response was sustained throughout therapy, indicating further synergism between monobenzone and the two adjuvants. This was also reflected in the repression of melanoma outgrowth. The synergism we found in the MIC therapy cannot be completely explained by the generation of the quinone-hapten or the occurrence of exosomes. While these processes enhance the generation of tumor-reactive CD8+ T cells by DCs, they can not account for the immediate synergistic suppression in tumor growth we found in the MIC combination. Therefore, it is likely that monobenzone-exposed skin melanocytes in another way aid the innate immune response, and this may well be connected to triggering of the NALP3 inflammasome in DCs and keratinocytes.

Technically, monobenzone is a contact-sensitizer inducing a typical type IV delayed-type hypersensitivity response[17, 51] against the quinone hapten formed by tyrosinase. In the response to contact-sensitizers, the adaptive immune priming- and effector phase depends on the production of pro-inflammatory cytokines like IL-1β and IL-18 by Langerhans cells[52] or keratinocytes[53], mediating an acute inflammatory response[51, 54]. Recent work shows that contact-sensitizing compounds such as trinitrochlorobenzene can activate the innate immune system via the NALP3 inflammasome, essential components of which are also present in human epidermal keratinocytes[55, 56]. Furthermore, the generation of antigen-specific T cells in response to contact-sensitizers was even found to depend entirely upon NALP3 engagement[55, 57]. Since monobenzone does not induce an inflammatory response in depigmented skin[58] devoid of tyrosinase-expressing melanocytes, and T cell responses to contact sensitizers rely on protein complex formation[14, 15, 59], it is likely that the quinone-hapten generated by monobenzone triggers the NALP3 inflammasome. Via NALP3 activation in local

DCs and keratinocytes, monobenzone can thereby activate the innate immune system. Besides this hapten-mediated activation of NALP3, the mild levels of oxidative stress generated by monobenzone may enhance activation of the NALP3 inflammasome. ROS induced by chemotherapeutic drugs such as anthracyclines[60] are known to mediate adenosine tri-phosphate (ATP) release from exposed cells. Cellular ATP-release is a danger-associated molecular pattern (DAMP) and also induces activation of the metabolic danger-sensing NALP3-inflammasome[61]. These NALP3-dependent processes could account for the DC activation we found intact monobenzone-treated melanoma cells to induce (chapter 5).

Like toll-like receptors, the activated inflammasome triggers robust DC activation[62], pro-inflammatory cytokine production[62], and the initiation of antigen-specific CD8+ T cells[63]. By the potent activation of local innate immune cells, high levels of pro-inflammatory cytokines are produced. These could explain the rapid NK cell response we observed in our *in vivo* work. Importantly, TLR-triggering upregulates the NALP3 inflammasome in DCs[62], and thereby the effective synergism we found in our *in vivo* MIC regimen, may reflect the functional synergism between TLR-7, -9 and NALP3 triggering respectively by imiquimod, CpG and monobenzone. Since the NK cell response only persisted during treatment in the MIC-treated animals, this suggests that the constant application of monobenzone on the skin mediated constant NALP3 activation, which, after initially being boosted by TLR-7 and -9 triggering, provides a constant source of pro-inflammatory cytokine maintaining the NK response.

## THE INTEGRATION THEORY OF MONOBENZONE-INDUCED VITILIGO

Unlike other hapten-directed immune- or skin hypersensitivity responses which are primarily directed against the hapten[21, 22], the monobenzone-induced response spreads to unaltered autoantigens resulting in progressive autoimmunity against the skin melanocyte. To clarify this unusual phenomenon, I here propose an "integration theory" on monobenzone-induced vitiligo. In this view, multiple cellular- and immune processes synergize to mediate melanocyte antigen-specific immunity (figure 1).

In chronological order, when monobenzone is applied to pigmented skin it interacts with tyrosinase in melanocytes, generating quinone-haptens to its protein chain. The conversion of monobenzone by tyrosinase is also associated with ROS generation, which damages the melanosomes. This initiates autophagy pathways and tyrosinase ubiquitination, which elevate the presentation of melanosome-derived antigens in surface MHC class-I and –II molecules. Directly stimulating the development of CD8+ and CD4+ T cell responses, this also leads to the formation of a melanocyte-specific antibody response (like we observed in chapter 7). Furthermore, the mild ROS-levels promote the excretion of exosomes from monobenzone-exposed melanocytes. This greatly enhances melanocyte-specific antigen (cross) presentation by DCs, and the formation of CD8+ T cells responsive to haptenated- and unaltered autoantigens. The quinone-haptens attached to the tyrosinase protein, excreted in the exosomes, may activate the innate immune system via triggering of the NALP3 inflammasome in local

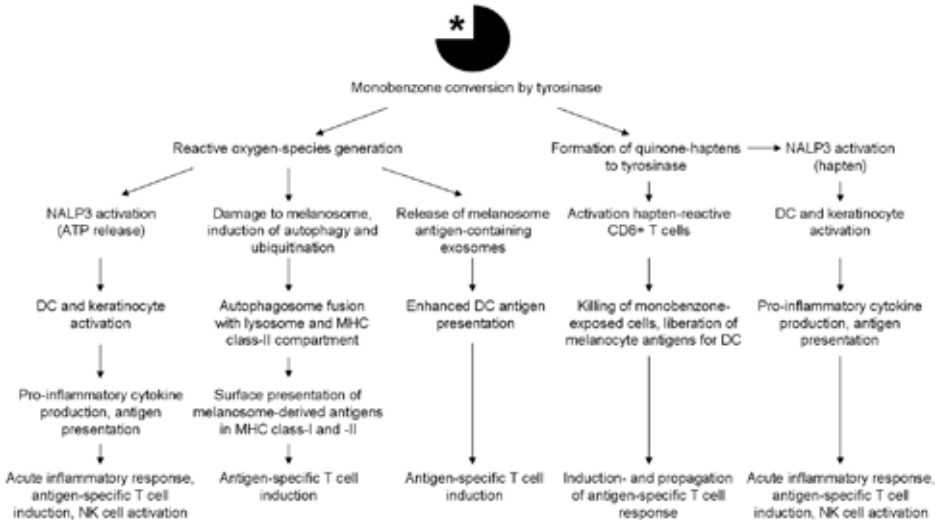


**Figure 1.** Schematic overview of the immunological events and -outcomes related to the enzymatic conversion of monobenzone by the enzyme tyrosinase, as proposed by the integration theory.

DCs and keratinocytes. The innate immune activation can be synergistically enhanced by concomitant activation of TLR-7 and -9 on DCs and TLR-9 on keratinocytes[64] (as in our *in vivo* MIC regimen). Additionally, mild ROS levels may mediate ATP-release from monobenzone-exposed cells, further augmenting local DC activation via the NALP3 inflammasome. The associated production of pro-inflammatory cytokines activates a concurrent NK cell response, which can additionally activate DCs or lyse distant poorly immunogenic, monobenzone-untreated melanoma cells. The quinone-haptens form the sensitization basis of a type IV hypersensitivity reaction, and initiate hapten-specific CD8+ T cell reactivity aided by the innate immune activation. Importantly, the hapten-specific CD8+ T cells, killing monobenzone-exposed melanocytes expressing haptenated antigens on their surface, further liberate antigen for presentation by DCs. Here, antigen uptake of cell-debris from lysed melanocytes may be enhanced by the circulating antigen-specific serum antibodies, through the formation of immune complexes. By the lysis of monobenzone-exposed cells and the release of exosomes in the context of constant innate immune activation, the immune reaction will now spread to non-haptenated melanocyte antigens. Thereby non-monobenzone exposed melanocytes (or melanoma cells) can now be progressively destroyed, leading to progressive vitiligo (or tumor regression).

## THE CHALLENGE OF THE DYNAMIC TUMOR

Malignant melanomas show high tumor cell heterogeneity[65, 66] and especially metastases display cell populations with enhanced tumorigenicity[67, 68]. These aggressive tumor cell

subpopulations have recently been identified as melanoma stem cells (MSC)[69] and are characterized by a highly immune-evasive phenotype[70], constant self-renewal and the ability to form *de novo* melanomas from a single cell[71]. Furthermore, MSC specifically express the trans-membrane transporter ABCB5[69], conferring high multidrug-resistance to these cells. These combined malignant characteristics tellingly put an end to the paradigm that melanoma is a rather homogeneously proliferating tumor cell aggregation. Cancer stem cells have been discovered in many other malignancies[72, 73] and give momentum to the call of developing combinatorial immunotherapy regimens. Targeted eradication of the MSC population using ABCB5-specific antibodies has been shown to abolish melanoma progression[69], and has provided a new therapeutic target for melanoma therapy. Thereby, it is important for effective melanoma immunotherapy to target the bulk- and MSC tumor cells concomitantly.

In employing different immune mechanisms simultaneously (figure 1), our monobenzone-based MIC regimen could be used to target MSC. Interestingly, while expression of other melanoma-associated antigens was found to be abrogated, downregulation of tyrosinase was not identified in ABCB5+ MSC[70]. This preservation of tyrosinase expression seems to be common for metastatic melanoma[74]. Moreover, MSC show intact expression of surface MHC class-II and over-expression of the co-stimulatory molecule molecule B7.2 (CD86)[70]. Thereby it could render them sensitive to monobenzone-induced ROS-dependent effects such as exosome secretion, NALP3-activation and in particular autophagy-induced CD4+ T cell activation through MHC class-II and B7.2. Of course, this would ask for some form of targeted delivery of monobenzone to the MSC, possibly by ABCB5-specific receptor-mediated nanovesicle delivery[75,76]. Otherwise, in the case of tyrosinase antigen-escape, our MIC regimen could also be effectively combined with ABCB5-antibody therapy. Ideally, immunotherapy regimens should enhance tumor-specific immunity while also sequestering tumor-mediated immune suppression. For example, combining the MIC regimen with ABCB5-specific antibodies and classic chemotherapy such as gemcitabine or all-*trans*-retinoic acid would destroy the bulk melanoma- and MSC cells, while also suppressing the activity of myeloid-derived suppressor cells[77, 78]. Multi-targeted immunotherapy will provide the dynamic answer to the pliable character of malignant melanoma.

## BATTLING DISEASE WITH DISEASE

By investigating the consequences of monobenzone-exposure from the molecular level through to the *in vivo* setting, the studies described in this dissertation provide a comprehensive view into the specific immunological effects of monobenzone. Thereby we have established monobenzone-induced depigmentation to be elicited by a distinct set of molecular- and immunological mechanisms, as incorporated into the new integration theory. These distinct mechanisms were found to specifically activate the innate immune system against the monobenzone-exposed melanocyte while also enhancing its immunogenicity for the adaptive system, advancing effective immunity beyond the exposed melanocyte. Using *in vitro* and *in vivo* approaches

we have furthermore shown, that hereby monobenzone readily evokes pigment cell-specific CD8+ T cell immunity in healthy human individuals, and that monobenzone-induced vitiligo is an effective basis for melanoma-specific immunotherapy. The initial observation that melanoma patients who develop vitiligo during treatment have a better prognosis, logically leads to the active induction of vitiligo as an immunotherapy for melanoma. The results of this thesis therefore invite further scrutiny of the exploitation of monobenzone-induced vitiligo for the treatment of melanoma, thereby treating a deadly disease with a much less serious affliction.

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# ADDENDUM

SUMMARY
SAMENVATTING
SAMENVATTING VOOR NIET-INGEWIJDEN
DANKWOORD
LIST OF PUBLICATIONS
CURRICULUM VITAE
USED ABBREVIATIONS

## SUMMARY

### Background

Melanoma is a cancer derived from the pigment cell, the melanocyte. While localized melanoma is surgically well treatable, it tends to metastasize rapidly to distant sites in the body. Metastatic disease is very resistant to standard treatments such as chemotherapy and irradiation. Thereby, the long-term survival prognosis for patients with metastatic melanoma is poor. Nonetheless, melanoma is an immunogenic malignancy, and thus melanoma patients could benefit from immunotherapy. This immunogenicity is illustrated by the observations that melanoma antigen-specific T cells often circulate in the blood of melanoma patients and that infiltration of melanoma tumors by these T cells is associated with an improved prognosis. Also, the primary lesion spontaneously regresses in about 9% of patients with metastatic disease. By its immunogenicity, autoimmune side-effects regularly occur during melanoma immunotherapy, typically vitiligo. While melanoma is a cancer of the melanocytes, vitiligo represents the progressive loss of melanocytes from the skin. The development of vitiligo during melanoma immunotherapy is associated with a superior prognosis, and is considered to represent an active T cell-mediated autoimmune response against the melanocyte differentiation antigens shared between melanocytes and melanoma cells.

Based on this clinical observation, the work described in this dissertation pursued to develop an effective melanoma immunotherapy based on the direct induction of vitiligo. Thereby we first defined the active role of perilesional T cells in the progressive eradication of melanocytes in the vitiligo skin. Aiming for the active induction of vitiligo during melanoma, and the skin-bleaching compound monobenzone being a potent initiator of vitiligo, we advanced to dissect the immunological mechanism by which this compound induces T cell-mediated autoimmunity to pigmented cells. Finally, using the B16-B6 model of malignant murine melanoma, we established an effective monobenzone-based immunotherapeutic regimen for melanoma.

### Findings

An effective immune response against invading micro-organisms or proliferating malignant cells is the result of the intimate interactions between different cell types of the immune system. In chapter 1 we discuss how an effective immune response is initiated, and how deviations in this process may cause autoimmunity. While autoimmunity is mostly considered harmful to the host, cancer immunotherapy depends on effective targeting of autoimmunity against the body's own malignant tissues. The possibilities and challenges of establishing such effective anti-tumor immunity are considered, and the immunological potential between vitiligo and melanoma is highlighted. In particular, attention is paid to actively inducing vitiligo by the skin-bleaching compound monobenzone as an immunotherapeutic tool against melanoma. In this context, current immunological knowledge on monobenzone is discussed.

The generation of effective anti-tumor immunity is often impeded by the tolerance of the peripheral T cell pool towards the body's own tissues. Thereby, any immunotherapeutic regimen is required to break this tolerance in order to accomplish destruction of malignant cells. In contrast, immunotherapy should not exaggerate this process and maintain its "antigenic focus", to prevent extensive multi-organ autoimmunity. A fine balance lies between self-tolerance and autoimmunity, which is mainly governed by central and peripheral selection mechanisms shaping the reactivity pattern of peripheral T cells. By a review of the present literature, chapter 2 shows that these selection mechanisms are not as rigid as suggested by the classic "one T cell-one antigen" paradigm. Instead, the antigen-reactivity of T cells is depends on antigen cross-reactivity and avidity tuning, resulting in a flexible balance between self-tolerance and autoimmunity. This flexibility should be taken into account in the design of more effective cancer immunotherapy regimens.

Autoimmune diseases are afflictions where the balance between self-tolerance and –destruction has tipped in favour of autoimmune tissue destruction. Vitiligo has long been suggested to be caused by autoimmune T cells. In chapter 3 we directly correlate autoreactive CD8+ T cell infiltration in the vitiligo perilesional skin, to local melanocyte destruction using an autologous skin explant model. Moreover, CD8+ T cells obtained from vitiligo perilesional skin biopsies displayed enriched specificity for melanocyte-differentiation antigens and were shown to be reactive to stimulation with these antigens in vitro. These T cells could also infiltrate autologous, normally pigmented, skin explants and mediated cytotoxic melanocyte destruction within the skin microenvironment. Our findings demonstrate that in vitiligo melanocyte antigen-specific perilesional T cells progressively eradicate the skin melanocytes. Thereby we provide evidence for targeted autoimmune tissue-destruction in vitiligo, mediating the characteristic depigmented skin maculae. Importantly this applies momentum to the thought of employing these vitiligo-associated T cells, which recognize the differentiation antigens shared by melanocytes and melanoma cells, in melanoma immunotherapy.

The thought of using vitiligo-derived T cells against melanoma is further encouraged by the observation that perilesional vitiligo skin-derived T cells display superior reactivity against these shared melanocyte differentiation antigens, as compared to T cells derived from melanoma tumors. By a review of the current literature, chapter 4 elaborates on the therapeutic possibility of using vitiligo-derived T cells or their T cell receptors in melanoma immunotherapy.

Since vitiligo development during melanoma immunotherapy indicates targeted T cell-mediated autoimmunity against pigmented cells, it could be most beneficial to actively induce progressive vitiligo in melanoma patients. To initiate vitiligo topical skin-bleaching agents are typically used, the most potent compound being monobenzone. In chapter 5 we address the question how monobenzone induces progressive autoimmune vitiligo. Here we show that monobenzone confers immunogenicity to pigmented cells. In particular, we have demonstrated that upon enzymatic conversion of monobenzone

by the enzyme tyrosinase, quinone-haptens were formed to cysteine residues in tyrosinase. Moreover, the conversion of monobenzone generated reactive oxygen species and mediated the release of tyrosinase- and MART-1 antigen-containing exosomes from exposed pigmented cells. Using autologous healthy human donor DC-T cell stimulations, we established that these exposed pigmented cells activated DCs and induced a robust melanoma-reactive CD8+ T cell response in vitro within 7 days. CD8+ T cell clones were generated from these stimulations, and 78% of these were found to recognize monobenzone-exposed and –unexposed melanoma cells equally well. Together, these findings demonstrate that by its specific effects on the enzyme tyrosinase, monobenzone induces CD8+ T cell immunity towards autoantigens expressed by melanocytes and melanoma cells. This makes monobenzone a powerful immunotherapeutic tool against melanoma.

In chapter 6 we advanced to investigate the catabolic response to monobenzone exposure in pigmented cells, in order to establish the mechanistic basis for the augmented immunogenicity of these cells we observed in chapter 5. Using electron microscopy we revealed that monobenzone induced autophagy of the melanosome in human melanoma cells. Furthermore, confocal laser scanning microscopy experiments in melanocytes and melanoma cells demonstrated that this melanosome autophagy is a lysosomal degradation process resulting in the targeting of tyrosinase to MHC class-II compartments. Moreover, monobenzone exposure mediated the ubiquitination of tyrosinase in melanoma cells. Together these processes can lead to the enhanced presentation of melanosome-derived antigens in MHC class-I and –II. Since monobenzone-exposed pigmented cells are constantly engaged in the degradation-, processing- and presentation of melanosomal proteins via autophagy and ubiquitination, the findings in this chapter provide the most likely explanation for the elevated immunogenicity of monobenzone-exposed pigmented cells.

Finally, by combining our findings from the previous chapters, a new effective melanoma immunotherapy regimen based on topical monobenzone application was established in chapter 7. Hereby we made use of the B16-B6 model of malignant murine melanoma. We combined the vitiligo-inducing effect of monobenzone with the immunostimulatory adjuvants CpG and imiquimod, in the MIC-immunotherapy regimen. This approach abolished subcutaneous B16.F10 melanoma growth in 85% of C57BL/6 wildtype mice during treatment, and induced over 100 days of tumor-free survival in 60% of the mice on average. Importantly, the MIC-regimen induced a robust anti-melanoma immune response involving the activation of melanoma antigen-specific CD8+ T cells, melanoma-reactive NK cells and the generation of a melanoma-reactive IgG response. Also, MIC-therapy instigated effective immunological memory which forcefully suppressed melanoma growth upon re-challenge up to 165 days after treatment cessation. The simple to use MIC regimen thereby provides a high-yield and low-cost therapy for melanoma, readily applicable in the clinic. Moreover, the MIC regimen establishes that the active induction of vitiligo using monobenzone is an effective immunotherapeutic approach against melanoma.

Chapter 8 integrates the molecular-, *in vitro*- and *in vivo* findings obtained in the individual chapters, into a central 'integration theory' of monobenzone-induced vitiligo. Moreover this integration theory is discussed in the light of current knowledge and literature, and the possibilities and challenges for future melanoma immunotherapy are interpreted.

## SAMENVATTING

Melanoom is een kanker van de pigment cel, de melanocyt. Terwijl gelokaliseerde melanoom chirurgisch goed te behandelen is, metastaseert deze kanker betrekkelijk snel naar andere plekken in het lichaam. Gemetastaseerd melanoom is zeer slecht te behandelen middels standaard therapieën zoals bestraling of chemotherapie. Daarmee is de lange-termijn prognose voor patiënten met gemetastaseerd melanoom zeer ongunstig. Desalniettemin is melanoom een immunogene tumor, en daarmee zouden melanoom patiënten baat kunnen hebben bij immuuntherapie. De immunogeniciteit van melanoom wordt geïllustreerd door de observaties dat melanoom antigeen-specifieke T cellen vaak in het bloed van melanoom patiënten gevonden worden, en dat infiltratie van de tumor door deze T cellen gepaard gaat met een verbeterde prognose. Daarnaast, gaat bij ongeveer 9% van de patiënten met gemetastaseerd melanoom de primaire laesie in spontane regressie. Dankzij deze immunogeniciteit worden autoimmuun neven-effecten vaak waargenomen tijdens melanoom immuuntherapie, voornamelijk vitiligo. Terwijl melanoom een kanker van de melanocyt is, verdwijnen bij vitiligo juist de melanocyten uit de huid. Het ontwikkelen van vitiligo tijdens het ondergaan van melanoom immuntherapie is geassocieerd met een superieure prognose, en wordt gezien als het resultaat van een actieve T cel-gemedieerde autoimmuun respons gericht tegen de melanocyt differentiatie antigenen welke tot expressie worden gebracht door zowel melanocyten als melanoom cellen.

Gebaseerd op deze klinische observatie, heeft het werk beschreven in deze dissertatie tot doel het ontwikkelen van een effectieve melanoom immuuntherapie gebaseerd op de directe inductie van vitiligo. Hierbij hebben we als eerste de actieve rol van perilesionale T cellen gedefinieerd in de progressieve destructie van melanocyten in de vitiligo huid. Met het doel actief vitiligo te induceren bij melanoom, en de huid-blekende stof monobenzon zijnde een sterke initiator van vitiligo, hebben wij hierna onderzocht welk immunologisch mechanisme ten grondslag ligt aan de inductie van T cel-gemedieerde autoimmuniteit tegen gepigmenteerde cellen door deze stof. Uiteindelijk hebben we een effectieve melanoom immuuntherapie ontwikkeld op basis van monobenzon, in het B16-B6 muismodel voor maligne melanoom.

### Bevindingen

Een effectieve immuun respons tegen binnendringende micro-organismen of maligne cellen is het resultaat van de intieme interacties tussen verschillende celtypen van het immuunsysteem. In hoofdstuk 1 bespreken we hoe een effectieve immuun respons wordt opgebouwd, en hoe afwijkingen in dit proces autoimmuniteit kunnen veroorzaken. Terwijl autoimmuniteit vaak wordt gezien als schadelijk voor het eigen lichaam, is kanker immuuntherapie afhankelijk van gerichte autoimmuniteit tegen de lichaamseigen kwaadaardige weefsels. De mogelijkheden en obstakels voor het induceren van een dergelijke respons worden besproken, en de immunologische mogelijkheden omtrent vitiligo en melanoom worden uitgelicht. In het bijzonder wordt aandacht geschonken aan het actief induceren van vitiligo middels de huid-blekende

stof monobenzon als een immuuntherapie voor melanoom. In deze context wordt de huidige immunologische kennis betreffende monobenzon besproken.

Effectieve anti-tumor immuniteit wordt vaak belemmerd door de zelf-tolerantie aanwezig in de circulerende T cel populatie. Om destructie van kankercellen teweeg te brengen, zal iedere immuuntherapie deze tolerantie moeten doorbreken. Echter, dit proces moet gedoseerd en gericht plaatsvinden om te voorkomen dat wijdverspreide autoimmuniteit ontstaat gericht tegen uiteenlopende weefsels. Er bestaat een fijne balans tussen zelf-tolerantie en autoimmuniteit, welke voornamelijk geregeld wordt door centrale en perifere selectie mechanismen welke het reactiviteitspatroon van perifere T cellen vorm geven. Door de recente literatuur te bespreken, laat hoofdstuk 2 zien dat deze selectie mechanismen echter niet zo strikt zijn als wordt verondersteld door de klassieke aanname van het "een T cell-een antigeen" dogma. In tegendeel, de antigeen-reactiviteit van T cellen hangt af van antigeen kruis-reactiviteit en aviditeits tuning, wat ertoe leidt dat er een flexibele balans bestaat tussen zelf-tolerantie en autoimmuniteit. Deze kennis zou in ogenschouw moeten worden genomen bij het ontwerpen van meer effectieve anti-tumor immuuntherapieën in de toekomst.

Autoimmuun ziekten zijn aandoeningen waarbij de balans tussen zelf-tolerantie en –autoimmuniteit in het voordeel van de autoimmuniteit is uitgevallen. Vitiligo is lang gedacht veroorzaakt te worden door autoimmuun T cellen. In hoofdstuk 3 correleren we de infiltratie van autoreactieve CD8+ T cellen in de perilesionale vitiligo huid direct aan lokale melanocyt destructie, gebruik makende van een autoloog huid explant model. CD8+ T cellen verkregen uit perilesionale vitiligo huid biopten, waren in specificiteit verrijkt voor het herkennen van melanocyt differentiatie antigenen en konden in vitro geactiveerd worden door middel van stimulatie met deze antigenen. Bovendien konden deze T cellen autologe, normaal gepigmenteerde, huid explants infiltreren alwaar zij in de huidstructuur zelf cytotoxische destructie van melanocyten teweeg brachten. Onze bevindingen laten zien dat bij vitiligo melanocyt antigeen-specifieke T cellen de melanocyten in de huid vernietigen. Daarmee leveren we bewijs voor gerichte autoimmuun weefsel-destructie bij vitiligo, waardoor de karakteristieke witte huidvlekken ontstaan. Deze resultaten geven een sterke impuls aan de gedachte om deze vitiligo-geassocieerde T cellen, welke de differentiatie antigenen herkennen die gedeeld tot expressie worden gebracht door zowel melanocyten als melanoom cellen, te gebruiken in melanoom immuuntherapie.

De gedachte om vitiligo-geassocieerde T cellen tegen melanoom te gebruiken wordt verder versterkt door de observatie dat T cellen aanwezig in de perilesionale vitiligo huid een verhoogde reactiviteit vertonen tegen deze gedeelde melanocyt differentiatie antigenen, in vergelijking met de T cellen die gevonden worden in melanomen. Aan de hand van de huidige literatuur bespreekt hoofdstuk 4 de mogelijkheid om vitiligo-geassocieerde T cellen of hun T cel receptoren te gebruiken bij melanoom immuuntherapie.

Omdat vitiligo ontwikkeling tijdens melanoom immuuntherapie wijst op gerichte T cel-gemedieerde autoimmuniteit tegen gepigmenteerde cellen, zou het zeer gunstig

kunnen zijn om actief vitiligo op te wekken bij melanoom patiënten. Om vitiligo te induceren worden vaak topicale huid-blekende stoffen toegepast, de sterkste hiervan is monobenzon. In hoofdstuk 5 bestuderen we de vraag hoe de huid-blekende stof monobenzon progressieve autoimmuun vitiligo veroorzaakt. We laten hier zien dat monobenzon de immunogeniciteit van gepigmenteerde cellen verhoogd. In het bijzonder tonen we aan dat wanneer monobenzon door tyrosinase enzymatisch werd omgezet, zich quinon-haptenen vormden aan cysteïne-residuen in tyrosinase. Daarnaast laten we zien dat de conversie van monobenzon door tyrosinase gepaard ging met het genereren van reactieve zuurstof radicalen, en dat daarnaast tyrosinase- en MART-1-bevattende exosomen werden losgelaten door blootgestelde gepigmenteerde cellen. Door gebruik te maken van autologe DC-T cel stimulaties, demonstreren we bovendien dat monobenzon-blootgestelde gepigmenteerde cellen DCs kunnen activeren en dat zij binnen 7 dagen in vitro een sterke melanoom-reactieve CD8+ T cel respons kunnen opwekken. Van de CD8+ T cel klonen geïsoleerd uit deze stimulaties, was 78% in staat zowel monobenzon-blootgestelde als niet blootgestelde melanoom cellen te herkennen. Gezamenlijk laten deze resultaten zien dat door de specifieke effecten van monobenzon op tyrosinase, monobenzon CD8+ T cel immuniteit tegen de specifieke zelf-antigenen van melanocyten en melanoom cellen kan opwekken. Dit maakt monobenzon tot een krachtig nieuw immuuntherapeutisch middel tegen melanoom

In hoofdstuk 6 gaan we dieper in op de directe effecten van monobenzon op de gepigmenteerde cel, om zo een mechanistische basis te vormen aangaande de verhoogde immunogeniciteit van de pigmentcellen zoals we die hebben gezien in hoofdstuk 5. Door middel van electronen microscopie laten we zien dat monobenzon autofagie van het melanosoom induceerde in melanoom cellen. Daarnaast, middels confocale laser scanning microscopie experimenten met melanocyten en melanoom cellen, demonstreren we dat deze autofagie een lysosomale afbraak route vertegenwoordigd welke leidt tot het dirigeren van tyrosinase naar het MHC klasse-II compartiment. Monobenzon blootgestelde melanoom cellen vertoonden bovendien ook ubiquitinatie van tyrosinase. Tezamen kunnen deze twee processen leiden tot de verhoogde presentatie van melanosoom-specifieke antigenen in MHC klasse-I en –II. Gezien monobenzon behandelde gepigmenteerde cellen constant verwikkeld zijn in het afbreken-, verwerken-, en presenteren van melanosoom eiwitten via autofagie en ubiquitinatie, vormen de resultaten van dit hoofdstuk de meest waarschijnlijke verklaring voor de verhoogde immunogeniciteit van monobenzon-blootgestelde gepigmenteerde cellen.

Uiteindelijk, door de bevindingen uit de eerdere hoofdstukken te combineren, is er een nieuwe effectieve melanoom immuuntherapie ontwikkeld in hoofdstuk 7, gebaseerd op topicale monobenzon applicatie. Hierbij is gebruik gemaakt van het B16-B6 model voor maligne melanoom bij de muis. We hebben de vitiligo-inducerende werking van monobenzon gecombineerd met de immuunstimulerende effecten van de adjuvants CpG en imiquimod, gezamenlijk als MIC-immuuntherapie. Deze aanpak

belette de groei van B16.F10 melanoom tijdens therapie in 85% van de C57BL/6 wildtype muizen, en induceerde een tumor-vrije overleving van meer dan 100 dagen in gemiddeld 60% van de muizen. De MIC-therapie induceerde ook een sterke anti-melanoom immuun respons, gekenmerkt door de activatie van melanoom antigeen-specifieke CD8+ T cellen, melanoom-reactieve NK cellen en het initiëren van een melanoom-reactieve IgG respons. Daarnaast bracht de MIC therapie beschermende immuniteit teweeg, welke 165 dagen na het stoppen van de behandeling melanoom uitgroei sterk wist te onderdrukken, wanneer de tumor opnieuw werd ingespoten. De simpel te gebruiken MIC therapie vertegenwoordigd daarmee een effectieve en goedkope therapie, welke direct toepasbaar is in de kliniek. Bovendien laat de MIC therapie zien dat de actieve inductie van vitiligo door middel van monobenzon een effectieve immuuntherapeutische aanpak is tegen melanoom.

Hoofdstuk 8 integreert de moleculaire-, in vitro- en in vivo bevindingen uit de individuele hoofdstukken in een centrale 'integratie theorie' betreffende monobenzon-geïnduceerde vitiligo. Bovendien wordt deze integratie theorie besproken in het licht van de huidige kennis en literatuur, en worden de mogelijkheden en obstakels voor toekomstige melanoom immuuntherapie geïnterpreteerd.

# SAMENVATTING VOOR NIET-INGEWIJDEN

## Achtergrond

De huid is het grootste orgaan van ons lichaam en is van vitaal belang voor onze overleving. Middels de ondoordringbare buitenste hoornlaag (stratum corneum) beschermt het ons onder andere tegen invloeden van buitenaf zoals infecties en schadelijke stoffen. Bovendien bevinden zich in de onderste laag van de opperhuid pigmentcellen (melanocyten). Via het pigment dat zij produceren, bieden zij bescherming tegen de schadelijke effecten van ultraviolette straling uit bijvoorbeeld zonlicht of zonnebank-apparatuur. Dit pigment wordt door de melanocyten afgegeven aan andere cellen in de opperhuid, waardoor een egale huidpigmentatie ontstaat. Bij een bruinende huid wordt dit proces tijdelijk geïntensiveerd door de melanocyten.

In de gezonde huid zijn de melanocyten netjes gerangschikt als één enkele cellaag op de onderste laag van de opperhuid. Bepaalde ziekten gaan echter gepaard met afwijkingingen in het aantal melanocyten in de huid. Zo kan er bijvoorbeeld een kanker ontstaan van de melanocyten, dit heet een melanoom. Een melanoom (letterlijk: zwart gezwel) kan zich oppervlakkig verspreiden maar zal over het algemeen snel in de diepere huidlagen doorgroeien. Wanneer dit gebeurd, zaait een melanoom vaak snel uit naar lokale lymfeknopen en andere delen van het lichaam. Uitgezaaid melanoom is bijzonder slecht te behandelen omdat het zeer ongevoelig is voor chemotherapie en bestraling. De beste optie is dan ook om het melanoom weg te snijden vóórdat het is uitgezaaid. Naast deze woekering van melanocyten bij melanoom, is er ook een aandoening waarbij de melanocyten juist verdwijnen uit de huid: vitiligo. Bij vitiligo ontstaan hierdoor witte vlekken in de huid, die zich vaak in een symmetrisch patroon over het lichaam uitbreiden. Doordat in deze vlekken geen melanocyten en pigment meer aanwezig zijn, verbrandt deze huid bijzonder snel door zonblootstelling. Bovendien is vitiligo een misvormende aandoening, de psychologische belasting voor vitiligo patienten is hoog, zeker wanneer de witte vlekken zich in het gelaat bevinden. Onderzoek beschreven in deze dissertatie bewijst dat afweercellen van het lichaamseigen afweersysteem de melanocyten opruimen bij vitiligo. Dit brengt niet alleen het vinden van een genezing voor vitiligo een stap dichterbij, het geeft ook een sterke impuls aan de gedachte om deze afweercellen te gebruiken tegen melanoom.

Naast de overduidelijke tegenstelling tussen melanoom en vitiligo wat betreft het overmatig aanwezig- of juist afwezig zijn van melanocyten, is er namenlijk nog een zeer interessante tegenstelling tussen de twee aandoeningen betreffende het afweersysteem. Bij melanoom is een kanker ontstaan uit de melanocyten, waar de afweercellen geen grip meer op hebben. Er is dus "te weinig afweer" tegen de kwaadaardige melanocyten, waardoor de melanoom zich kan verspreiden en uitzaaien. Aan de andere kant is er vitiligo, waarbij hoogstwaarschijnlijk de afweercellen te actief zijn tegen de melanocyten. Er is "te veel afweer" tegen de melanocyten, en dit leidt vervolgens tot het verdwijnen van de melanocyten uit de huid. Ook al is melanoom ongevoelig voor bestraling en chemotherapie, de melanoomcellen zijn wel redelijk makkelijk te herkennen door het afweersysteem. Hierdoor kunnen patiënten met uitgezaaid melanoom baat hebben bij

een aanpak waarbij lichaamseigen afweercellen specifiek geactiveerd worden om de melanoom aan te vallen, dit heet immuuntherapie. Een interessant gegeven is dat bij sommige melanoom patiënten die immuuntherapie ondergaan, ook vitiligo optreedt. Dit komt doordat de geactiveerde afweercellen slecht onderscheid kunnen maken tussen de kwaadaardige melanocyten (melanoom cellen) en de gewone melanocyten in de huid. Het optreden van vitiligo tijdens melanoom immuuntherapie is gerelateerd aan een verbeterde prognose, en is een teken van een actieve afweerrespons tegen de melanoom.

### Doelstellingen

Gebaseerd op de observatie dat vitiligo ontwikkeling bij melanoom gunstig is, is de centrale gedachte in dit proefschrift dat een immuuntherapie die meteen vitiligo induceert wel eens bijzonder effectief zou kunnen zijn tegen melanoom. In dit proefschrift is dan ook de toepasbaarheid van een melanoom immuuntherapie gebaseerd op de actieve inductie van vitiligo onderzocht. Hiervoor hebben wij als eerste bestudeerd welke rol specifieke afweercellen (de T lymfocyten) spelen bij de vernietiging van melanocyten in de vitiligo huid. Sommige chemische stoffen kunnen direct vitiligo induceren en de stof met het sterkste huid-blekende effect is monobenzon. We hebben daarom vervolgens onderzocht hoe monobenzon het afweersysteem stimuleert om de melanocyten in de huid op te ruimen. Uiteindelijk hebben we een immuuntherapie voor melanoom ontwikkeld in het B16-B6 muismodel voor melanoom.

### Bevindingen

Een effectieve immuun respons tegen binnendringende micro-organismen of kwaadaardige cellen is het resultaat van de intieme interacties tussen verschillende celtypen van het afweersysteem. Hoofdstuk 1 geeft een algemene introductie over hoe een effectieve afweerreactie wordt opgebouwd door de cellen van het afweersysteem. Bovendien wordt besproken hoe afwijkingen in deze opbouw kunnen leiden tot autoimmuniteit (het aanvallen van lichaamseigen cellen of weefsels door het afweersysteem). Terwijl autoimmuniteit vaak wordt gezien als schadelijk voor het eigen lichaam, is kanker immuuntherapie juist afhankelijk van gerichte autoimmuniteit tegen de lichaamseigen kwaadaardige weefsels. In de context van de recente literatuur worden de mogelijkheden omtrent anti-kanker immuuntherapie uiteen gezet, en worden de tegenstellingen en mogelijkeden betreffende vitiligo en melanoom besproken. In het bijzonder wordt de mogelijkheid om vitiligo op te wekken als therapie voor melanoom besproken, en daarbij wordt de huidige kennis over monobenzon-geïnduceerde vitiligo uiteen gezet.

Een effectieve afweerreactie tegen een tumor wordt vaak belemmerd doordat T lymfocyten tolerant zijn voor lichaamseigen cellen en weefsels. Om destructie van kankercellen teweeg te brengen, zal iedere immuuntherapie deze tolerantie moeten doorbreken. Echter, dit proces moet gedoseerd en gericht plaatsvinden om te

voorkomen dat wijdverspreide autoimmuniteit ontstaat gericht tegen uiteenlopende weefsels. Er bestaat een fijne balans tussen zelf-tolerantie en autoimmuniteit, welke voornamelijk geregeld wordt door centrale en perifere selectie mechanismen welke het reactiviteitspatroon van perifere T cellen vorm geven. Door de recente literatuur te bespreken, laat hoofdstuk 2 zien dat deze selectie echter niet zo strikt is als wordt verondersteld door de klassieke aanname van het "één T cell-één antigeen" dogma. Hierbij zou één T lymfocyt slechts één specifiek onderdeeltje (een zogenaamd antigeen) van een cel of mirco-organisme kunnen herkennen. Om beschermd te zijn tegen alle mogelijke infecties en kankers zou je dus ook een nagenoeg eindeloos aantal T lymfocyten in je lichaam moeten hebben. In tegendeel blijkt dat T lymfocyten meerdere verschillende antigenen kunnen herkennen, en dat de antigeen-reactiviteit van T lymfocyten juist af hangt van de regulatie omtrent de gevoeligheid voor zijn antigenen. Dit heet aviditeits tuning, en leidt ertoe dat er een flexibele balans bestaat tussen zelf-tolerantie en autoimmuniteit. Deze kennis zou in ogenschouw moeten worden genomen bij het ontwerpen van meer effectieve anti-tumor immuuntherapieën in de toekomst.

Autoimmuun ziekten zijn aandoeningen waarbij de balans tussen zelf-tolerantie en –autoimmuniteit in het voordeel van de autoimmuniteit is uitgevallen. Vitiligo is lang gedacht veroorzaakt te worden door autoimmuun T cellen. Hoofdstuk 3 correleert de destructie van melanocyten in de vitiligo huid direct aan de infiltratie van autoreactieve CD8+ T lymfocyten. De CD8+ T lymfocyten zijn cellen die andere geïnfecteerde of kwaadaardige cellen kunnen doden. CD8+ T lymfocyten gekweekt uit huid biopten die waren afgenomen van de gepigmenteerde huid direct rondom een witte vitiligo vlek, waren verrijkt voor het herkennen van melanocyt antigenen en konden in kweek geactiveerd worden door middel van stimulatie met deze antigenen. Bovendien konden deze T lymfocyten normaal gepigmenteerde huid biopten weer binnendringen alwaar zij in de huidstructuur zelf destructie van melanocyten teweeg brachten. Onze bevindingen laten zien dat bij vitiligo deze T lymfocyten verantwoordelijk zijn voor het verdwijnen van de melanocyten uit de huid, waardoor de karakteristieke witte huid vlekken ontstaan. Daarmee leveren we bewijs dat bij vitiligo autoimmuniteit optreedt tegen de melanocyten, en dat deze actief worden vernietigd door de T lymocyten. Deze resultaten geven een sterke impuls aan de gedachte om deze vitiligo-geassocieerde T lymfocyten, welke de antigenen herkennen die gedeeld aanwezig zijn in zowel melanocyten als melanoom cellen, te gebruiken in melanoom immuuntherapie.

De gedachte om vitiligo-geassocieerde T lymfocyten tegen melanoom te gebruiken wordt verder versterkt door de observatie dat T lymfocyten aanwezig in de vitiligo huid een verhoogde reactiviteit vertonen tegen deze gedeelde melanocyt antigenen, in vergelijking met de T cellen die gevonden worden in melanomen. Aan de hand van de huidige literatuur, bespreekt hoofdstuk 4 de mogelijkheid om vitiligo-geassocieerde T lymfocyten of hun specifieke antigeen-herkennende oppervlakte receptoren te gebruiken bij melanoom immuuntherapie.

Omdat vitiligo ontwikkeling tijdens melanoom immuuntherapie wijst op gerichte autoimmuniteit van T lymfocyten tegen melanocyten én kwaadaardige

melanoom cellen, zou het zeer gunstig kunnen zijn om actief vitiligo op te wekken bij melanoom patiënten. Om vitiligo te induceren worden vaak topicale huid-blekende stoffen toegepast, de sterkste hiervan is monobenzon. In hoofdstuk 5 bestuderen we de vraag hoe de huid-blekende stof monobenzon vitiligo veroorzaakt. We laten hier zien dat monobenzon de immunologische herkenbaarheid van gepigmenteerde cellen, zoals melanocyten en melanoomcellen, verhoogd. De vorming van pigment in gepigmenteerde cellen is vooral afhankelijk van het enzym tyrosinase, wat ook alleen gevonden wordt in gepigmenteerde cellen. Monobenzon kan een specifieke interactie aangaan met tyrosinase. We tonen hier aan dat wanneer monobenzon door tyrosinase enzymatisch werd omgezet, een zeer reactieve stof wordt gevormd. Deze stof verbind zich vervolgens als hapteen aan het tyrosinase enzym waardoor het net is omgezet. Een hapteen is een zeer klein molecuul wat door zijn verbinding aan een eiwit, dit eiwit er "vreemd" uit laat zien voor het afweersysteem. De T lymfocyten zullen antigenen afkomstig van dit gehapteneerde eiwit hierdoor sneller herkennen. Daarnaast laten we zien dat de omzetting van monobenzon door tyrosinase gepaard ging met het genereren van reactieve zuurstof radicalen, en dat daarnaast tyrosinase- en MART-1 antigeen-bevattende exosomen werden losgelaten door monobenzon-blootgestelde gepigmenteerde cellen. Exosomen zijn zeer kleine blaasjes die snel kunnen worden opgenomen door dendritische cellen (DC). Deze DC zijn van groot belang voor het activeren van de T lymfocyten. Middels de antigeen-bevattende exosomen, kunnnen de DC dus makkelijk T lymfocyten activeren die de antigenen aanwezig in de exosomen herkennen. Vervolgens, door in kweek T lymfocyten met DC te stimuleren, demonstreren we dat monobenzon-blootgestelde gepigmenteerde cellen de DC activeren en dat zij binnen 7 dagen in kweek een sterke melanoom-reactieve CD8+ T lymfocyt respons kunnen opwekken. Bovendien laten we zien dat van de melanoom-reactieve CD8+ T lymfocyten opgewekt met monobenzon, 78% in staat is niet monobenzon-blootgestelde melanoom cellen te herkennen. De resterende 22% van de T lymfocyten herkend waarschijnlijk het hapteen, wat niet aanwezig zal zijn in niet monobenzone-blootgestelde cellen. Gezamenlijk laten deze resultaten zien dat door de specifieke effecten van monobenzon op tyrosinase, monobenzon CD8+ T lymfocyten gericht tegen de antigenen van melanocyten en melanoom cellen kan opwekken.

In hoofdstuk 6 gaan we dieper in op de directe effecten van monobenzon op de gepigmenteerde cel, om zo duidelijk inzicht te krijgen in de oorzaak van de verhoogde immunologische herkenbaarheid van de pigmentcellen, zoals we die hebben gezien in hoofdstuk 5. Door middel van electronen microscopie laten we zien dat monobenzon autofagie van het melanosoom induceerde in melanoom cellen. Autofagie (letterlijk: zelf-opeten) is een proces waarbij een cel een beschadigd onderdeel actief begint af te breken. Hierdoor worden zeer veel antigenen gegenereerd, waardoor de cel beter herkenbaar wordt voor het afweersysteem. In het geval van monobenzon, treedt melanosoom autofagie op. Het melanosoom is de pigmentkorrel in de melanocyt waar de pigment vorming plaats heeft, hierin zitten ook alle antigenen die het mogelijk maken voor het afweersysteem om specifiek melanocyten te herkennen. Omdat

monobenzon autofagie van melanosomen veroorzaakt, verhoogd het dus daarmee de herkenbaarheid van de blootgestelde melanocyt. Daarnaast, middels confocale laser scanning microscopie experimenten met melanocyten en melanoom cellen, demonstreren we dat deze autofagie een afbraak route vertegenwoordigd via lysosomen naar het MHC klasse-II compartiment. Monobenzon blootgestelde melanoom cellen vertoonden bovendien ook ubiquitinatie van tyrosinase. Tezamen kunnen deze twee processen leiden tot de verhoogde presentatie van melanosoom-specifieke antigenen op het oppervlak van de melanocyt, waardoor deze cel makkelijker T lymfocyten kan activeren. Gezien monobenzon behandelde gepigmenteerde cellen via deze autofagie constant verwikkeld zijn in het afbreken-, verwerken-, en presenteren van melanosoom antigenen, vormen de resultaten van dit hoofdstuk de meest waarschijnlijke verklaring voor de verhoogde immunologische herkenbaarheid van monobenzon-blootgestelde gepigmenteerde cellen.

Uiteindelijk, door de bevindingen uit de eerdere hoofdstukken te combineren, is er een nieuwe effectieve melanoom immuuntherapie ontwikkeld in hoofdstuk 7, waarbij gebruik is gemaakt van het B16-B6 muismodel voor melanoom. We hebben de vitiligo-inducerende werking van monobenzon gecombineerd met de afweersysteem-stimulerende effecten van de stoffen CpG en imiquimod, gezamenlijk genoemd "MIC-therapie". Deze aanpak belette de groei van melanoom tijdens therapie in 85% van de muizen, en medieerde een tumor-vrije overleving van meer dan 100 dagen in gemiddeld 60% van de muizen. MIC-therapie induceerde ook een sterke anti-melanoom reactie van het afweersysteem, gekenmerkt door de activatie van verschillende cel typen van het afweersysteem. Met name melanoom-specifieke CD8+ T lymfocyten (welke de melanoomcellen kunnen doden na herkenning van antigeen op het oppervlak van deze cellen) en melanoom-reactieve NK cellen (Natural Killer cellen, deze kunnen melanoom cellen doden die expres geen antigenen op hun oppervlak laten zien). Daarnaast zorgde MIC therapie ook voor een antilichaam respons gericht tegen het melanoom. Deze antilichamen kunnen de immuun respons versterken door de opname van antigenen door DC te bevorderen, welke op hun beurt weer T lymfocyten kunnen activeren. Daarnaast bracht MIC therapie beschermende lange-termijn afweer teweeg, welke zelfs 165 dagen na het stoppen van de behandeling melanoom uitgroei sterk wist te onderdrukken. Dit hebben we getest door de tumor opnieuw in te spuiten. MIC therapie vertegenwoordigt daarmee een effectieve en realitief goedkope therapie, welke direct toepasbaar is in de kliniek. Bovendien laat MIC therapie zien dat het actief veroorzaken van vitiligo door middel van monobenzon een effectieve aanpak is tegen melanoom.

Hoofdstuk 8 integreert de bevindingen uit de individuele hoofdstukken in een centrale 'integratie theorie' aangaande de vraag hoe monobenzon autoimmuun vitiligo veroorzaakt. Bovendien wordt deze integratie theorie besproken in het licht van de huidige kennis en literatuur, en worden de mogelijkheden en obstakels voor toekomstige melanoom immuuntherapie geïnterpreteerd.

# DANKWOORD

"Op een dag is het proefschrift klaar, ga je zitten, en schrijf je het dankwoord". Ook al lijkt dit magische moment in het begin vaak oneindig ver weg, het is nu dan toch echt gekomen. Als hekkensluiter van het proefschrift, sluit het daarbij ook een hele bijzondere en leerzame periode af.

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## CURRICULUM VITAE

Jasper Guido van den Boorn was born in Leiderdorp on August 12, 1980. After completing the Gymnasium at the Rijnlands Lyceum in Oegstgeest in 1999, he started the study of Biology at the Vrije Universiteit in Amsterdam. After obtaining his 'propaedeuse' exam after one year, his professional interest shifted from all things living to bio-medical studies, in particular the oncology research field. For his Bachelor of Biomedical Sciences and Master Programme of Oncology he performed his undergraduate research at the department of Pathology at the Vrije Universiteit of Amsterdam, under supervision of prof. dr. R. Scheper and dr. G. Scheffer. This project focussed on multidrug-resistance in breast cancer cells and aimed to modulate the expression of the 'breast cancer resistance protein' (BCRP) using a hammerhead ribozyme. His second research training period he completed under supervision of dr. J. Haanen and dr. W. Overwijk at the Netherlands Cancer Institute - Antoni van Leeuwenhoek hospital in Amsterdam, which dealt with the microarray analysis of IL-23-stimulated tumor-specific CD8+ T cells. Before he obtained his Master of Science-degree in Oncology in 2004, his final internship took place in the lab prof. dr. C. Melief and dr. F. Ossendorp at the department of Immunohematology and Bloodtransfusion of the Leiden University Medical Center. During this period he investigated the role of CD30 and 4-1BB in T cell-mediated immunity against retrovirus-induced sarcoma in mice. In 2005, Jasper started his PhD research project under supervision of prof. dr. J.D. Bos, prof. dr. C. Melief and dr. R. Luiten at the Academic Medical Center of the University of Amsterdam. The results of this work are described in this dissertation. He will now continue his research on melanoma immunotherapy as a post-doc at the Universitätsklinikum in Bonn, Germany, in the research team of prof. dr. G. Hartmann in collaboration with prof. dr. T. Tüting.

## ABBREVIATIONS USED IN THIS THESIS

| | |
|---|---|
| AB | human serum type AB |
| ABCB5 | ATP-binding cassette subfamily B member 5 |
| αCD40 | anti-CD40 |
| ADCC | antibody-dependent cellular cytotoxicity |
| AEC | aminoethylcarbazole |
| AIRE | autoimmune regulatory element |
| AL | autolysosome |
| αMSH | melanocyte stimulating hormone |
| AP | autophagosome |
| APC | antigen-presenting cell |
| APC | allophycocyanin |
| APL | altered peptide ligand |
| ATP | adenosine tri-phosphate |
| BSA | bovine serum albumin |
| CD | cluster of differentiation |
| CFSE | carboxyfluorescein succinimidyl ester |
| CI | CpG and imiquimod |
| CLSM | confocal laser scanning microscopy |
| CM | culture medium |
| ConA | concanavalin-A |
| CpG | cytosine-guanine oligodeoxynucleotides |
| CTL | cytotoxic T lymphocyte |
| CTLA-4 | cytotoxic T lymphocyte associated antigen-4 |
| DAMP | danger-associated molecular pattern |
| DAPI | 4',6-diamidino-2-phenylindole |
| DC | dendritic cell |
| DHI | 5,6-dihydroxyindole |
| DMSO | dimethyl sulfoxide |
| EBV-B | eppstein-barr virus-transformed B cell |
| EEA1 | early endosome antigen-1 |
| ELISA | enzyme linked immunosorbent assay |
| EM | electron microscopy |
| FACS | fluorescence-activated cell sorting |
| FBS | fetal bovine serum |
| Fc | fragment crystallizable |
| FITC | fluorescein isothiocyanate |
| Flu | influenza virus |
| Glow o/u | glow over/under |
| GM-CSF | granulocyte monocyte colony stimulating factor |
| H&E | hematoxylin and eosin staining |

| | |
|---|---|
| HLA | human leukocyte antigen |
| HMGB1 | high mobility group box-1 protein |
| HRP | horse radish peroxidase |
| IBMX | isobutylmethylxanthine |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| IMDM | iscove's modified dulbecco's medium |
| IS | immunological synapse |
| JY | EBV-B cell line JY |
| L | lysosome-like vesicle |
| LAMP1 | lysosome-associated membrane protein-1 |
| L-DOPA | L-3,4-dihydroxyphenylalanine |
| mAb | monoclonal antibody |
| MART-1 | melanoma-associated antigen recognized by T cells |
| MBEH | monobenzylether of hydroquinone, monobenzone |
| MCR1 | melanocortin-1 receptor |
| MHC | major histocompatibility compex |
| MIC | monobenzone, imiquimod and CpG |
| MSC | melanoma stem cell |
| MTT | 3,(4,5-dimethylthiazol-2-yl)2,5diphenyl-tetrazolium bromide |
| NALP3 | nacht domain-, leucine-rich repeat-, and pyrin domain-containing protein 3 |
| NHS | normal human serum |
| NK | natural killer |
| NMR | nuclear magnetic resonance |
| NOD | non-obese diabetic |
| OD | optical density |
| OVA | ovalbumin |
| PAMP | pathogen-associated molecular pattern |
| PBL | peripheral blood leukocytes |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate buffered saline |
| PE | phycoerythrin |
| PerCP | peridinin chlorophyll protein complex |
| PL | perilesional |
| PMA | phorbol 12-myristate 13-acetate |
| Poly I:C | polyinosinic:polycytidylic acid |
| PRR | pattern-recognition receptor |
| PTK | protein tyrosine kinase |
| PTPase | protein tyrosine phosphatase |
| ROS | reactive oxygen species |

| | |
|---|---|
| rpHPLC | reversed phase high performance liquid chromatography |
| RPMI | roswell park memorial institute medium |
| RT | room temperature |
| SMAC | supramolecular activation cluster |
| TBS | tris-buffered saline |
| TCR | T cell receptor |
| TD | tumor development |
| TFS | tumor-free survival |
| TLR | toll-like receptor |
| TNF | tumor necrosis factor |
| TNFR | tumor necrosis factor receptor family members |
| Treg | regulatory T cell |
| TRP-2 | tyrosinase-related protein-2 |
| U | units |
| UM | unmelanized melanosome |
| UV | ultraviolet |
| UVB | ultraviolet B |