Molecular basics for T cell resistance of malignant melanoma

Molekulare Grundlagen für T Zellresistenz im malignen Melanom

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1. List of content

2. Preface

The herein presented thesis consists of a selection of three original articles that are published or prepared for submission.

The work presented in this thesis was carried out between October 2012 and May 2016 and was supervised by principal investigator PD Dr. Annette Paschen in the Dermatology Department of the University Hospital Essen, Group ‘Molecular Tumor Immunology’.

3. Summary

Melanoma is one of the most aggressive cancers due to an early dissemination of tumor cells and thus, a high metastatic rate leading to death. Simultaneously, melanoma cells exhibit a high intrinsic immunogenicity that is currently exploited successfully in different immunotherapeutic approaches including antibody based immune checkpoint blockade and adoptive T cell therapy, marking an impressive breakthrough in the treatment of this otherwise treatment-resistant disease. Nonetheless, only a subset of patients shows responsiveness to immunotherapy, pointing to tumor immune escape mechanisms as described for many other solid malignancies. A better understanding of the evolution of melanoma escape variants in the course of disease progression and the underlying molecular mechanisms should allow improving immune therapeutic strategies for melanoma patients, also with regard to personalized medicine. The aim of this thesis was (I) to gain deeper insight into the mechanisms underlying melanoma escape from $CD8^+$ T cell recognition and (II) to study the recognition and killing of melanoma cells by autologous $CD4^+$ T cells as alternative anti-tumor effector cells apart from $CD8^+$ T cells. To achieve this, studies on several melanoma model systems were performed consisting of tissue, corresponding cell lines and autologous T cells.

The first two manuscripts included in this thesis characterize the $CD8^+$ T cell immunogenicity of melanoma cells obtained from consecutive metastases of two patient models. In one patient system, a progressive loss of immunogenicity and finally complete T cell resistance of melanoma cells were observed. The latter was due to a MHC class I negative phenotype of the tumor cells caused by alterations in the *B2M* gene. In the second patient system, different resistance mechanisms were detectable in different metastases. Tumor cells from one lesion lost expression of specific T cell antigens due to dedifferentiation whereas melanoma cells from additional three metastases showed either haplotype loss or a complete loss of MHC class I expression, rendering cells invisible to $CD8^+$ T cells.

The third manuscript then addresses the expression of MHC class II molecules on melanoma cells as a prerequisite for the development of $CD4^+$ T cell based therapies. A strong heterogeneity of MHC class II expression was observed for melanoma tissues and cell lines from different patients but also among cell lines from consecutive metastases of one patient. In the presence of constitutive or IFNγ induced MHC class II expression, autologous $CD4^+$ T cells were basically capable of recognizing melanoma cells and some of these T cells showed cytolytic activity. However,

melanoma cells could also escape from $CD4^+$ T cell recognition by acquired genetic defects in the IFN signaling pathway and epigenetic silencing of the MHC class II transcriptional regulator CIITA, allowing melanoma cells to maintain their MHC class II negative phenotype in the presence of IFNγ.

Multiple studies have accumulated evidences for immune escape mechanisms of melanoma cells. The presented studies demonstrated the evolution of $CD8^+$ T cell resistant metastases within different patient models, presumably allowing escape from tumor latency and progression of disease. Additionally, it was demonstrated that a subgroup of $CD4^+$ T cells can exert cytolytic activity against autologous melanoma cells and to some extend contribute to direct anti-tumor immunity. However, also in this case melanoma cells can evolve to escape from $CD4^+$ T cell recognition. Thus, monitoring of immune-related alterations in melanoma cells, as presented in this work, could be of value for treatment decision.

4. Zusammenfassung

Das maligne Melanom ist aufgrund seiner frühen Ablösung von Krebszellen und der daraus folgenden hohen Metastasierungsrate eine der tödlichsten Krebsarten. Gleichzeitig besitzt das Melanom eine hohe intrinsische Immunogenität, die die Grundlage für diverse immuntherapeutische Ansätze bildet, wie beispielsweise die Antikörper-basierte Blockade immun-inhibitorischer Signale oder der Transfer tumorspezifischer T Zellen. Bei der Behandlung des ansonsten therapieresistenten Melanoms stellen diese Ansätze einen großen Durchbruch dar, jedoch spricht nur ein Teil der Patienten auf die Immuntherapie an. Untersuchungen im Melanom und anderen Tumorentitäten weisen darauf hin, dass unter dem Druck des Immunsystems Varianten von Krebszellen entstehen, die sich der Kontrolle durch Immunzellen entziehen. Ein besseres Verständnis der molekularen Mechanismen sowie der Evolution solcher „immune escape“ Varianten während des Krankheitsverlaufs ist elementar, um die Verbesserung immuntherapeutischer Strategien zu ermöglichen. Das Ziel dieser Arbeit war (I) die Charakterisierung der dem Melanom zugrundeliegende Mechanismen des „immune escapes“ gegenüber $CD8^+$ T Zellen während des Krankheitsverlaufs und (II) die Untersuchung der Erkennung und des Tötens von Melanomzellen durch autologe $CD4^+$ T Zellen als alternative Effektorzellen neben $CD8^+$ T Zellen. Um einen Einblick in molekulare Mechanismen zu bekommen, die zu einem Verlust der Immunogenität von Tumoren eines Patienten führen, wurden mehrere Patientensysteme benutzt, die aus Gewebe, Zelllinien verschiedener Metastasen und autologen T Zellen bestehen.

Die ersten beiden Manuskripte befassen sich mit Immun-Verlust-Varianten konsekutiver Metastasen zweier Melanompatienten. In einem Patientensystem ist ein progressiver Verlust der Immunogenität zu beobachten, der zu einer kompletten $CD8^+$ T Zellresistenz einer metastatischen Läsion aufgrund eines MHC Klasse I negativen Phänotyps führt, hervorgerufen durch graduelle genetische Veränderungen des *B2M* Gens. Im zweiten Patientensystem sind unterschiedliche Resistenzmechanismen in verschiedenen Metastasen zu beobachten. Während eine Metastase den Verlust von Antigenen durch Dedifferenzierung aufweist, zeigen die anderen Metastasen einen Haplotyp-Verlust von MHC Klasse I oder einen kompletten Verlust der MHC Klasse I Expression, resultierend in einer partiellen oder kompletten Resistenz gegenüber $CD8^+$ T Zellen.

Das dritte Manuskript charakterisiert die Expression von MHC Klasse II Molekülen auf Melanomzellen als Voraussetzung für die Entwicklung $CD4^+$ T Zell-basierter

Therapien. Untersuchungen in Tumorgewebe und an Zelllinien zeigen, dass MHC Klasse II heterogen innerhalb eines Tumors und zwischen verschiedenen Metastasen eines Patienten exprimiert wird. *In vitro* expandierte tumor-spezifische $CD4^+$ T Zellen erkennen Melanomzellen mit konstitutiver als auch IFNγ induzierter MHC Klasse II Expression und einige dieser $CD4^+$ T Zellen zeigen einen cytotoxischen Phänotyp. Allerdings wird diese Tumorerkennung durch genetische Defekte im IFNγ Signalweg sowie durch epigenetische Veränderung des MHCII-Transaktivators CIITA limitiert, da diese Melanomzellen einen MHC Klasse II negativen Phänotypen auch in Anwesenheit von IFNγ behalten.

Zahlreiche Publikationen konnten zeigen, dass „immune-escape“ Mechanismen Melanomzellen gegenüber cytotoxischen $CD8^+$ T Zellen unsichtbar werden lassen. Die ersten beiden Studien dieser Arbeit zeigen deutlich die Evolution von $CD8^+$ T Zell-Resistenzmechanismen zwischen Metastasen eines Melanompatienten, die vermutlich zum Ende der Tumor-Latenz und letztendlich zum Fortschreiten der Erkrankung führen. Zusätzlich konnte gezeigt werden, dass eine Subgruppe von $CD4^+$ T Zellen eine cytotoxische Aktivität gegenüber autologen Melanomzellen aufweisen können und somit zu einer direkten Antitumor-Immunität beitragen können. Allerdings können Melanomzellen auch in diesem Fall Resistenzmechanismen gegenüber $CD4^+$ T Zellen entwickeln. Dementsprechend kann das Beobachten immunologischer Charakteristika, wie in diesem Manuskript beschrieben, wertvoll für Therapieentscheidungen von Krebspatienten sein.

5. Introduction

5.1. Malignant melanoma

With an incidence of 15-25/100.000, melanoma is not the most common but one of the most aggressive forms among various types of skin cancer (Miller and Mihm, 2006). Because of rapid dissemination of tumor cells, even in early stages, and following metastasis formation in lymph nodes and distant organs, melanoma is a very fatal disease. The median survival of a stage IV melanoma patient ranges seven to nine month and a long term survival of five years is only achieved in 5-10% of the patients (Eigentler et al., 2003; Schadendorf et al., 2014).

5.1.1. Evolution of malignant melanoma

Melanoma arises from a malignant transformation of the pigment producing melanocytes in the skin, eye or mucosa (Liubinas et al., 2010). Under normal conditions, melanocytes produce the pigment melanin that is transferred to keratinocytes to protect the skin against UV mediated DNA damages (Gilchrest et al., 1999). Continuous and intense exposure to UV light, however, leads to irreversible DNA damages that can subsequently drive malignant transformation of melanocytes. Known risk factors for the evolution of melanocytes to melanoma cells are, beside UV exposure, the number of melanocytic or dysplastic nevi, genetic predisposition, light skin and hair color and an age above 50 years (Psaty et al., 2010). Each of these risk factors contributes to melanoma genesis, whose exact interplay is complex and not fully understood.

Histologically and genetically, development of melanoma is a gradual process. According to the histopathological model of Clark and Elder, the first step of melanoma progression is the formation of a melanocytic nevus, formed by proliferating melanocytes. Transformation proceeds via a melanoma *in situ*, a primary malignant melanoma in radial-growth phase (RGP melanoma), which does not possess the ability to invade into surrounding tissue but only shows strong signs of dysplasia and intraepidermal proliferation. Transformation into a vertical-growth phase melanoma (VGP melanoma) allows melanoma cells to grow vertically into the skin, the first step of invasion. The last stage in melanoma progression is the metastasized melanoma, which has spread into surrounding tissue and formed tumors in adjacent skin, sentinel lymph nodes or distant metastases in lung, liver or brain. Prognostic factors for disease progression are mitotic rate of the tumor, the number of tumor-infiltrating lymphocytes

as well as tumor thickness. Certainly, progression stages do not have to follow those steps linearly, skipping of a step is possible and a RGP melanoma does not have to progress to invasive disease in any cases (Clark et al., 1984). Of note, integration of histological, pathological and genetic observations led to the conclusion that there are several different types of melanoma, dependent on their origin, grade of UV-induced damage, type of precursor lesion as well as the pattern of somatic mutations (Shain and Bastian, 2016).

5.1.2. Molecular mechanisms

At a more molecular level, a variety of genetic changes contribute to the formation of melanoma cells. The majority of melanoma patients, approximately 50-60%, exhibit an amino acid substitution from Valine to Glutamate at the position 600 within the BRAF protein (*BRAF*^{V600E}) (Brose et al., 2002) Whereas this mutation in the mitogen-activated protein kinase (MAPK) signaling pathway presumably leads to senescence of melanocytic nevi cells, the acquisition of other oncogenic mutations then can stimulate malignant transformation into melanoma cells.

In general, the BRAF^{V600E} mutant is constitutively active, which then activates the downstream factors MEK and ERK. As a consequence, expression of genes responsible for cell proliferation and survival is permanently activated. Besides, the G-protein NRAS is frequently mutated in melanoma tumors. Approximately 15-20% of melanoma patients harbor activating mutations of this gene (*NRAS*^{Q61K}, *NRAS*^{Q61R}). Interestingly, mutations in this gene are only rarely found in line with the BRAF^{V600E} mutation (Flaherty, 2012; van 't Veer et al., 1989). Further common mutations are those which play a role in the PI3K-AKT pathway. Mutations affecting the PTEN tumor suppressor are described in 10-30% of all melanoma and presumably coincide with BRAF mutations (Daniotti et al., 2004; Tsao et al., 2004; Wu et al., 2003). Because PTEN is a negative regulator of the PI3K-AKT pathway, its mutational inactivation leads to the constitutive activation of the signaling cascade and therefore to increase of proliferation and invasion of melanocytes (Li et al., 2002). In 25-40% of familial melanoma, a loss of the tumor suppressor and cell cycle regulator p16 (*INK4A*) is described. Loss of p16 is caused by mutation, deletions or transcriptional silencing of its encoding locus CDKN2A and subsequently helps melanocytes to overcome cell cycle arrest (Thompson et al., 2005; Yang et al., 2005).

The transcription factor MITF (microphtalmia associated transcription factor), that has been defined as the master regulator of melanocytic differentiation, development and

pigmentation genes, is found to be amplificated in 15% of melanomas (Garraway et al., 2005). Other, less frequent, detected genetic alterations are the amplifications of Cyclin D1 (*CCND1*) or mutations in the Cyclin dependent kinase 4 (*CDK4*) (Smalley et al., 2008).

5.1.3. Therapeutic approaches

Until 2010, the 3-year survival rate of stage IV melanoma patients was approximately 6-12% (Korn et al., 2008). Back then, the only treatment option was the repeated administration of Dacarbazine (DTIC), a cytostatic agent. However, the given therapeutic options were almost unable to benefit melanoma patients with an overall response rate of 10-20% and no prolonging effect on overall survival (Avril et al., 2004; Middleton et al., 2000). In the last couple of years, a variety of promising therapeutic alternatives have emerged, including molecular targeted therapies, immunotherapies and immune checkpoint blockade.

Progress in the understanding melanoma driver mutations in genes such as BRAF and NRAS and their role in cell growth and survival led to the development of targeted therapies with specific inhibitors. BRAF inhibitors Dabrafenib and Vemurafenib target the special mutation BRAFV600E that occurs in 50-60% of all melanoma patients (Davies et al., 2002). Since it specifically binds to the mutant form of the RAF protein, dividing non-tumor cells are not attacked and side effects are limited. One reported side effect is the appearance of Squamous Cell Carcinoma (SCC) in 22% of Vemurafenib treated patients due to paradoxical activation of RAF signaling in BRAF wildtype cells (Hatzivassiliou et al., 2010; Heidorn et al., 2010).

In randomized phase III clinical trials, both inhibitors showed a prolonged median progression free survival (PFS) of 5.3 months (Vemurafenib) and 5.1 months (Dabrafenib) compared to 1.6 (2.7) months for the Dacarbazine-treated group (Chapman et al., 2011; Hauschild et al., 2012). This tremendous success led to FDA approval of Vemurafenib in 2011 (Zhu et al., 2015). Due to the fact that resistance to BRAF inhibitors occurs in most of the patients, other strategies emerged (Sullivan and Flaherty, 2013). Targeting the downstream protein MEK in the MAPK signaling pathway with specific inhibitors Trametinib and Cobimetinib also prolonged patients PFS but the problem of drug-resistance persists. Combinatorial targeting of multiple molecular targets within the MAPK pathway further enhanced tumor eradication and delayed acquired resistance in recent phase III clinical studies (Long et al., 2014).

5.2. Cancer Immunogenicity

Burnet and Thomas first summarized in 1957 observations of immune control of malignant cells and called the interaction between host immune cells and malignant cells 'immune surveillance' (Burnet, 1957). Basis of this theory was the observation that immune cells presumably recognize distinct antigens on the cell surface of malignant cells and are thus able to distinguish between normal and transformed cells (Old and Boyse, 1964). Testing this hypothesis though was a problem due to the fact that the, at this time, immunodeficient considered athymic nude mouse still exhibits immune cells and is, thus, not completely immune-compromised (Dunn et al., 2004; Maleckar and Sherman, 1987). Not until the establishment of the gene-targeted RAG-$2^{-/-}$ mouse (recombination-activation gene-2), Interferon γ (IFNγ) and lymphocyte dependent immunosurveillance of malignant cells has been experimentally approved (Shinkai et al., 1992). These mice are completely immunodeficient without impairment of non-lymphoid cells. Challenge of these mice with the carcinogen Methylcholanthrene (MCA) led to the formation of tumors in 30/52 RAG-$2^{-/-}$ compared to 11/57 in the control group (Shankaran et al., 2001). Also in these mice, work was performed on the role of IFNγ and/or Perforin as mediators in limiting the growth of tumor cells. Knock-out of either IFNγ or Perforin also led to an increase in MCA-induced tumor formation in mice, pointing to an important role of these immune molecules in tumor control (Street et al., 2001; Street et al., 2002). Several similar studies with IFNγ-insensitive tumor cells emphasized a direct role of immune cell-derived IFNγ on tumor cells, for example the capacity of enhancing expression of Major Histocompatibility Class I (MHC class I) and MHC class II antigen processing and presentation that leads to a higher immunogenicity and was sufficient for tumor rejection (Kaplan et al., 1998). In addition, the anti-proliferative, pro-apoptotic and anti-angiogenic activity of IFNγ might be responsible for tumor rejection (Bromberg et al., 1996; Chin et al., 1997; Strieter et al., 1995; Xu et al., 1998).

Several early observations led to the conclusion that immunosurveillance also must play a role in human cancer control. For instance, the risk for developing a tumor is much higher in transplant patients than in the general population, as shown for a variety of cancers such as colon, pancreas, lung and malignant melanoma (Sheil, 1986). In addition, a fourfold higher incidence of malignant melanoma was observed after organ transplantation (Sheil, 2001). Also spontaneous regression of tumors like melanoma has been observed, pointing to the inherent possibility of recognition of tumor antigens

by endogenous innate or adaptive immunity in human. Additionally, the existence of tumor-infiltrating lymphocytes (TILs) and its association with a better prognosis of these patients has been proven for several solid tumors like melanoma, non-small lung cancer and ovarian cancer (Al-Shibli et al., 2008; Tuthill et al., 2002). Furthermore, the fact that antibodies blocking immune-inhibitory signals on immune cells regularly leads to constant remission in a subset of cancer-patients, clearly supported the hypothesis that an immune response against malignant cells exists (Page et al., 2013).

5.3. Immunotherapeutic approaches in melanoma

First attempts of using the patient's immune system have been made with using IFNα, a type I IFN cytokine that shows immune-modulatory and anti-angiogenic effects and leads to a delay in relapse-free survival of melanoma patients (Kirkwood et al., 1996; Moschos et al., 2006). Unfortunately, patients showed no benefit in overall survival and high cytotoxicity (Eggermont et al., 2012).

Many tumor antigens that are possibly recognized by $CD8^+$ cells have been described. Differentiation antigens are expressed during cell development, e.g. melanocyte differentiation antigens such as Melan-A, gp-100 or tyrosinase and occur in normal cells as well as in the majority of melanoma cells. Other antigen groups such as mutated antigens (e.g. mutated p53), overexpressed or amplified antigens or viral antigens can also play a role in tumor recognition. Another important group of cancer antigens are Cancer-Testis antigens as their expression normally not occurs in adult normal tissue but is restricted to germ cells and interestingly also occurs in many tumors (Boon and van der Bruggen, 1996; Rosenberg, 1999; Scanlan et al., 2002).

Table 1: Overview of selected known tumor antigens. Source: cancerimmunity.org

differentiation antigens	• tyrosinase • tyrosinase related protein-1 (TRP-1) • tyrosinase related protein-2 (TRP2) • gp100/Pmel17 • melanoma antigen recognized by T-cells (Melan-A)
onkofetal antigens	• Melanoma-Antigen (MAGE) Familie • B Melanom Antigen (BAGE) Familie • G Antigen (GAGE) Familie • NY-ESO
overexpressed antigens	• preferentially expressed antigen of melanoma (PRAME) • Survivin
mutated antigens, tumor specific	• N-RAS • CDK4 • β-Catenin

Therefore, antigen-specific therapy attempts have been developed, as there is the vaccine approach to boost the patient's immune system in order to attack cancer cells. Thereof, most common vaccine strategies focus on $CD8^+$ T cells/MHC class I restricted epitopes of tumor antigens (Butterfield, 2015). Peptide-based vaccination uses computer prediction of epitopes possibly presented on MHC molecules, whereas antigen-presenting-cell (APC)-based vaccination makes use of autologous dendritic cells (DC) and B cells presenting tumor antigens to $CD4^+$ and $CD8^+$ T cells, which can trigger the immune system more complex and effectively (Slingluff et al., 2013; Zhao et al., 2012). But the clinical response of vaccination has been limited. Peptide vaccination in combination with Interleukin-2 (IL-2) for example lead to prolonged progression free survival of one month, compared to IL-2 alone, respectively (Schwartzentruber et al., 2011).

Another promising field of immunotherapy is the administration of *ex vivo* expanded tumor specific T cells. Adoptive T cells transfer (ACT) protocols for melanoma have first been designed by the group of Steven Rosenberg in 1988 (Rosenberg et al., 1988). In this, meanwhile advanced, protocol TILs, classically $CD8^+$ T cells, are isolated from the patient's tumor. Tumor specific T cells are enriched and grown *ex vivo* by addition of IL-2 and then re-infused into the host. This protocol allows the enrichment and growth of high-avidity lymphocytes that are highly specific for the tumor cells.

Additionally, it allows the growth of a high number (up to 10^{11}) of these lymphocytes without any *in vivo* acting immunosuppressive mechanisms (Dudley et al., 2002; Dudley et al., 2005). Combination of ACT with lympho-depletion greatly enhanced tumor immunogenicity after TIL reinfusion by abolishing immunosuppressive cells in the tumor microenvironment (Dudley et al., 2002; Dudley et al., 2008). Current ACT protocols lead to an objective response in 40-50% of the patients, whereof 20% showed complete remission (Rosenberg et al., 2011). Additional to lympho-depletion, studies in mice showed that combinational immunotherapy applying both ACT and αCTLA-4 blockade promotes optimal immunity against melanoma compared to monotherapy alone (Mahvi et al., 2015). The effect of combining αCTLA-4 and ACT in human has to be evaluated in the future.

Another approach to restore antitumor activity of T cells is the blockade of immunosuppressive signaling pathways with monoclonal antibodies (mAb). T cell mediated antitumor activity signals via a T cell receptor (TCR)- antigen interaction and the binding of the costimulatory molecule CD28 on the T cell to B7-1 on antigen presenting cells (Chen and Flies, 2013; Pardoll, 2012). Cytotoxic T lymphocyte–associated protein 4 (CTLA-4), an immunoglobulin superfamily receptor expressed on $CD4^+$ and $CD8^+$ T cells after initial activation, acts as co-inhibitory signaling molecule by binding with greater affinity to B7-1 on APCs. This binding then prevents over-activation and autoimmunity (Pentcheva-Hoang et al., 2009; Rudd, 2009). Application of the αCTLA-4 mAb Ipilimumab enhances activation, proliferation and effector functions of T cells and thereby leads to prolonged overall survival in patients with advanced melanoma (Hodi et al., 2010).

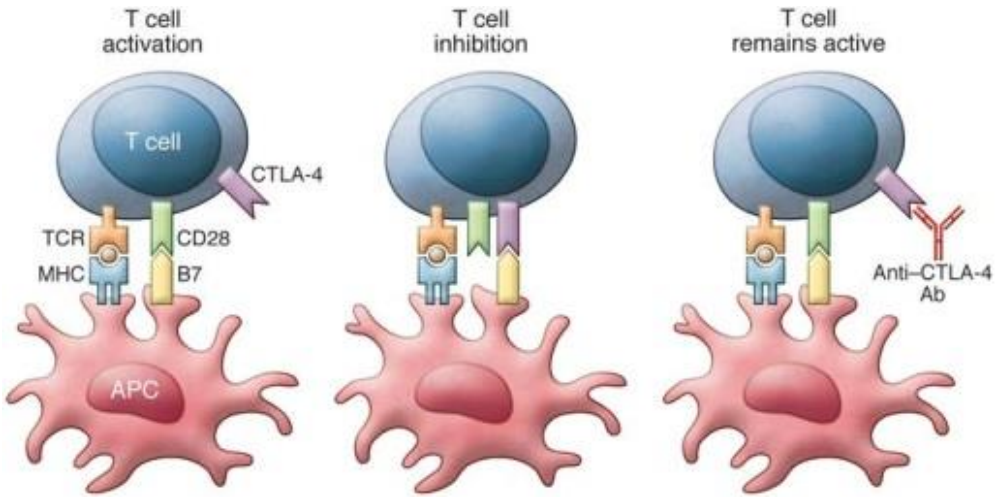

Figure 1: CTLA-4 on T cells binds to B7 on APCs and prevents T cell activation. Administration of a CTLA-4 antibody blocks this interaction and leads to activation of the T cell (Buchbinder and Hodi, 2015).

Another inhibitory receptor expressed on T cells, B cells and NK cells is Programmed cell Death-1 (PD-1) (Fanoni et al., 2011; Terme et al., 2011). T cells can become tolerant by binding of PD-1 on T cells to the ligands Programmed Cell Death ligand-1 (PD-L1) or Programmed Cell Death Ligand-2 (PD-L2) expressed on tumor cells (Blank et al., 2005; Okazaki and Honjo, 2007). Treatment of melanoma patients with the αPD-1 mAb Pembrolizumab in clinical studies showed a prolonged progression-free survival and prolonged overall survival even compared to the treatment with Ipilimumab (Robert et al., 2015). Therapeutic approaches combining αPD-1 and αCTLA-4 antibodies resulted in PFS of 12 months in 55% of the patients, which is an increase of 32% compared to αCTLA-4 monotherapy (Postow et al., 2015). Because immune checkpoint antibodies increase immune response, auto-immune related side effects such as colitis, rash or pruritus occur in 60% of the patients treated with Ipilimumab (Hodi et al., 2010; Postow, 2015). Furthermore, only a subset of patients, approximately one third, shows a response to immune checkpoint blockade (Zhu et al., 2015), pointing to mechanisms of the tumor cells to circumvent surveillance by the immune system and highlighting the importance of finding biomarkers that can predict response to immunotherapy.

5.4. Effector cells of Cancer Immunogenicity

The process of cancer immunogenicity is a complex interplay of cells of the innate and adaptive immune system. Are cancer cells beginning to grow invasively, they are disrupting the surrounding tissue, leading to inflammatory signals recruiting first cells

of the innate immune system like Natural Killer (NK) cells, Natural Killer T (NKT) cells, γδ T cells, macrophages and DCs (Girardi et al., 2001). Exemplarily, stress-induced ligands on tumor cells play an important role in recognition by cells of the innate immune response. MHC class I chain-related proteins A and B (MICA/B) are bound by complementary receptors present on the cell surface of NK-, NKT- or γδT cells, leading to a perforin-dependent killing of tumor cells (Bauer et al., 1999). Besides important effector cells of the innate immune system, $CD4^+$ and $CD8^+$ T cells are considered to be the main actors of cancer immune surveillance, because their depletion caused MCA- induced cutaneous malignancies in mice and in a model for spontaneous lymphoma depleted for T cell derived cytotoxic Perforin, mice were more prone to cancer development (Girardi et al., 2003; Girardi et al., 2001; Koebel et al., 2007; Smyth et al., 2000).

5.4.1. $CD8^+$ T cells

$CD8^+$ T cells are responsible for the recognition and elimination of infected and transformed cells. Basis is the presentation of peptides by MHC class I molecules that are expressed on virtually all nucleated cells. Also tumor cells normally express MHC class I-peptide complexes, eventually presenting their distinct antigen-repertoire on the surface where it can be recognized by the TCR of $CD8^+$ T cells, leading to lysis of the tumor cells.

Does a $CD8^+$ T cell carry the MHC class I/peptide complementary TCR, activation of the T cell is initiated. One of the mechanisms that are activated upon recognition of a target cell is the granula-dependent exocytosis. Thereby, granules are released, containing cytolytic molecules such as Perforines and Granzymes. Perforines form pores in the plasma membrane for Granzymes to enter the cell and enable apoptotic pathways subsequently leading to caspase-dependent or –independent apoptosis of the target cell (Chavez-Galan et al., 2009; Liu et al., 1995; Shen et al., 2006; Waterhouse et al., 2006). Another activated mechanism is the Fas-FasL dependent apoptosis pathway, whereby FasL expressed on T cells binds to Fas on the target cells, subsequently leading to induction of caspase-dependent apoptosis (Luo et al., 1998).

5.4.2. $CD4^+$ T cells

The second important mediators of action of the adaptive immune system are the $CD4^+$ T cells. In contrast to $CD8^+$ T cells, their TCR recognizes antigens that are bound to MHC class II molecules and presented on the cell surface.

Activation of $CD4^+$ T cells by binding of their specific TCR to the complement MHC class II-antigen complex leads to proliferation and to differentiation into highly diverse T helper (Th) cell subsets. This differentiation is additionally influenced by cytokines, chemokines, the affinity of peptide/TCR interactions and costimulatory interactions (O'Garra and Arai, 2000). Upon activation of naïve $CD4^+$ T cells by interactions with MHC class II/peptide molecules on DCs or macrophages, additional signals via cytokines lead to the differentiation of $CD4^+$ T cells into different subsets, including Th1, Th2, Th9, Th17, Th25 or Tregs. For example, stimulation of $CD4^+$ T cells with IL-12, leading to the activation of STAT-4, results in the induction of the lineage-specific transcription factor T-bet (Szabo et al., 2000). T-bet leads to the production of Th1 signature cytokines such as IFNγ, TNFα and IL-2 and negatively regulates genes that are specific for a differentiation towards the Th2 or Th17 (Zhu et al., 2012; Zhu et al., 2010).

In contrast, IL-4 induces the transcription factor GATA3 which in turn leads to differentiation into Th2 cells, mainly producing IL-4, IL-5 and IL-13 (Amsen et al., 2009). The role of Th2 cells in anti-tumor immunity remains under investigation, as they have been linked to both indirect anti-tumor effects and cancer progression (Mattes et al., 2003; Ochi et al., 2012). Th17 cells differentiate in the presence of TGF-β and IL-6 and mainly produce IL-12 and IL-17 (Caza and Landas, 2015). This novel type of $CD4^+$ T cells has been linked to certain infections and to autoimmunity (Bettelli et al., 2007; Weaver et al., 2007). Additionally, the Th17-produced cytokines were shown to act proinflammatory and pro-tumorigenic (Langowski et al., 2006; Tartour et al., 1999). In contrast, Th17 cells can also have anti-tumorigenic effects in an antigen-specific manner after adoptive transfer into mice (Muranski et al., 2008).

Thus, among the different $CD4^+$ Th subsets, Th1 $CD4^+$ T cells are considered to be the most important cells in anti-tumor immunity as the production of IFNγ leads to the activation of DCs close to the tumor, therefore to priming and maturation of CTLs, activation of NK cells and macrophages and to enhanced expression of the chemokine receptor CXCR3 and its ligands, subsequently attracting T cells to the side of the tumor (Corthay et al., 2005; Hung et al., 1998; Janssen et al., 2003; Palucka and Banchereau, 2012; Rotondi et al., 2003; Sun et al., 2004).

Thus, adoptive T cell transfer was applied for autologous $CD4^+$ T cells specific for the tumor associated antigen NY-ESO-1, resulting in clinical remission in one melanoma patient, even of tumor cells not expressing NY-ESO-1, most likely due to antigen-spreading (Hunder et al., 2008). In this mouse study, tumors were rejected due to transfer of tumor specific $CD4^+$ T cells, even of tumors that have been resistant to the clearance by $CD8^+$ T cells. $CD4^+$ T cells worked independent of $CD8^+$ T cells but partnered with NK cells and MHC class II expression was not necessarily needed, pointing to indirect effects of the $CD4^+$ T cells. Also in this study, $CD4^+$ T cells outperformed $CD8^+$ T cells in rejecting the tumor (Hunder et al., 2008; Perez-Diez et al., 2007). These indirect anti-tumor effects are important in the case of MHC class $II^{negative}$ tumors. But on many cells, MHC class II is inducible by IFNγ or even constitutively expressed rendering them accessible for the direct recognition by $CD4^+$ T cells.

Besides the known, cytokine producing subsets of $CD4^+$ T cells, cytotoxic activity in a Perforin-/Granzyme-dependent manner has been described e.g. for cutaneous T cell lymphoma, Friend-virus-induced FBL-tumors and mouse-melanoma models (Akhmetzyanova et al., 2016; Echchakir et al., 2000; Johnson et al., 2015; Qui et al., 2011). For some tumor models, depletion of $CD8^+$ T cells and/or treatment with CD137 (4-1BB) and/or CD134 (OX-40) agonist antibody was necessary to induce the cytotoxic phenotype of $CD4^+$ T cells (Akhmetzyanova et al., 2016; Hirschhorn-Cymerman et al., 2012; Qui et al., 2011). Furthermore, transfer of naïve NY-ESO-1 specific $CD4^+$ T cells into lymphopenic mice led to differentiation into cytolytic T cells, exhibiting Th1 profile with additional expression of Perforin and Granzymes depending on the transcription factor Eomesodermin (EOMES) (Xie et al., 2010). In a simultaneously published study, a generated NY-ESO specific $CD4^+$ T cell line of a melanoma patient showed upregulation of Granzyme B and Perforin and directly lysed autologous melanoma in a MHC class II-restriced manner (Quezada et al., 2010). A similar type of cytotoxic $CD4^+$ T cell was detectable in the peripheral blood of melanoma patients, where the treatment with αCTLA-4 even enhanced the expression of cytolytic granules via induction of EOMES (Kitano et al., 2013).

5.4.3. Antigen presentation on MHC class I and II

MHC molecules are generally presenting cell intrinsic proteins to immune cells and are in humans referred to as HLA molecules (*human leucocyte antigen*). Most of the proteins within a tumor are processed and presented via the MHC class I-related

pathway (Boon et al., 2006). For peptide presentation on the cell surface, the antigen-MHC class I presentation has taken advantage of the Ubiquitin-Proteasome system (UPS) within every cell. Thus, ubiquitinylated, for degradation tagged, proteins are targeted to the cytosolic 26S proteasome, which consists of a 20S and two 19S caps and is responsible for the first degradation of the proteins into peptide precursors (Rock and Goldberg, 1999; Strehl et al., 2008). These epitope precursors are then further processed by cytosolic peptidases into fragments of a length of 8-12 amino acids (aa). Alternatively, fragments of 9-16 as in length can be further processed via ER peptidases ERAP1 and ERAP 2 in the ER (Saric et al., 2002; van Endert, 2011). Epitopes carrying the right C-terminus that serves as anchor for the MHC class I molecule, are then transported via TAP1 and TAP2 transporter (transporter associated with antigen processing) into the lumen of the endoplasmatic reticulum (ER) (van Endert et al., 1994). There, the loading of MHC class I molecules with the peptide fragments takes place, involving the chaperone Tapasin that initiates the peptide-loading complex (PLC) (Bangia et al., 1999; Ortmann et al., 1997). MHC class I molecules are heterodimers built by a constant light chain, called β-2-microglobulin (β2m) and a varying transmembrane heavy α-chain. In humans there are different genes in the MHC complex, referred to as human-leukocyte antigen A, B and C (York et al., 1999). In the ER, the heavy α-chain and the β2m molecule assemble with the help of chaperones and additional binding of the peptide fragments then stabilizes the complex (Rammensee et al., 1993). Interestingly, IFNγ, Type I IFN (IFNα, IFNβ) as well as TNFα can induce the transcription of variants of the proteasome, namely iβ1 (LMP-1), iβ5 (LMP-2) and iβ2 (MECL-1), then forming the so called immunoproteasome (Groll et al., 1997). Peptides generated by this immunoproteasome differ from the ones formed by the normal proteasome and some peptides are differentially processed by the immunoproteasome than by the standard proteasome, leading to differences in T cell recognition (Schwarz et al., 2000; Shin et al., 2006; Van den Eynde and Morel, 2001). Via a process called cross-presentation, antigen-presenting cells are also able to present exogenous peptides on MHC class I molecules to $CD8^+$ T cells which is particularly of importance for loading of DCs in the course of vaccination. Different pathways are involved in this process (Ma et al., 2016). MHC class II expression is normally restricted to professional APCs such as DCs or B cells, whereon they capture antigens from viruses, bacteria and tumors and are thereby the starting point for the adaptive immunity by $CD4^+$ and $CD8^+$ T cells (Banchereau et al., 2000). MHC class II molecules consist of the two transmembrane

glycoproteins referred to as subunit α and subunit β, both forming a peptide binding groove at the membrane-distal region and presenting peptides of 13-17 aa in length (Rudensky et al., 1991). Endocytosed proteins, internalized and degraded by lysosomal proteolysis in early endosomes, late endosomes and lysosomes, as well as phagocytosed proteins, that are degraded in phagolysosomes, represent the main source for peptides presented on MHC class II molecules (Huotari and Helenius, 2011). Clathrin-mediated endocytosis leads to the internalization of cell surface-ligand complexes. Besides viruses, bacteria or fungi, APCs are also able to take up self-proteins by internalization of necrotic or apoptotic cells by phagocytosis, which causes the presentation of a wide range of self-antigens, leading to self-tolerance (Steinman et al., 2000). Furthermore, the non-specific uptake of extracellular material by macropinocytosis is an important mechanism for the uptake of self- and non-self-proteins (Lim and Gleeson, 2011). Also macroautophagy leads to the presentation of a large amount of cytolytic antigens on MHC class II molecules (Crotzer and Blum, 2010). Once translocated into the ER, MHC class II molecules assemble with the invariant chain (I chain) CD74, which serves as a chaperone for peptide binding (Roche et al., 1991). Conserved di-leucine-rich motifs within the I chain target the complex with MHC class II to late endosomal compartments, where binding of peptides takes place (Zhong et al., 1997).

On the molecular level, MHC class II expression depends on its transcriptional regulator Class II transactivator (CIITA). Its deficiency is linked to the type II bare-lymphocyte syndrome and to cancer (Steimle et al., 1993). Induction of MHC class II molecules is initiated by protein-protein interactions with a multiprotein-complex containing the regulatory-factor X (RFX), activating transcription factor (ATF)/cAMP-responsive element binding protein (CREB) and NF-Y, that can bind to the SXY regulatory element of the MHC class II promoter region (Gobin et al., 2001; Masternak et al., 2000). Upon recruitment to the MHC class II promoter, it does not bind directly to the DNA but acts as a scaffold protein for stabilization of the MHC-II-enhanceosome and subsequently activates transcription (Masternak et al., 2000). Expression of CIITA is driven by four different tissue-specific promoters, whereof pI is active in DCs, pIII in B cells and PIV is inducible by IFNγ in various cell types including cancer cells (Krawczyk and Reith, 2006).

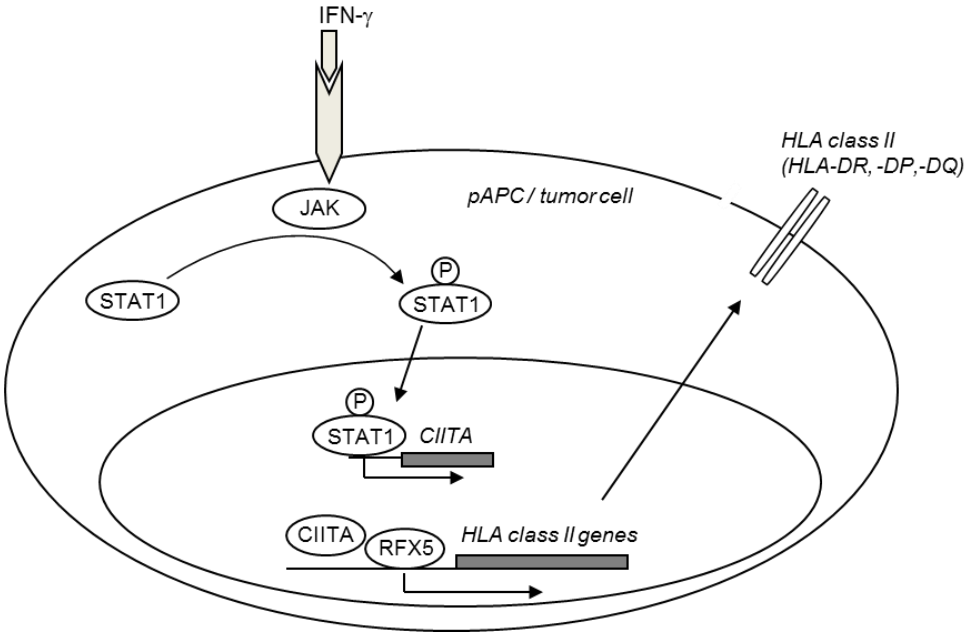

Figure 2: IFNγ dependent induction of HLA class II and its transcriptional regulator CIITA. Upon binding of IFNγ to the corresponding receptor, activation of the JAK/STAT1 pathway leads to transcriptional activation of CIITA. CIITA in turn activates transcription of HLA class II genes that present antigens on the cell surface.

5.4.4. MHC class II expression on melanoma cells

As mentioned before, MHC class II expression is normally restricted to only a subset of immune cells, including B cells, macrophages, DCs, thymic epithelial cells and activated T cells (Glimcher and Kara, 1992). Upon treatment with IFNγ, also most cells are able to express MHC class II *de novo* (Steimle et al., 1994). Despite this possible induction by IFNγ, immunohistochemical analysis showed that melanoma cells are able to express MHC class II constitutively (van Duinen et al., 1984).

Constitutive surface expression of MHC class II molecules on melanoma cells has mostly been associated with an unfavorable outcome for the patient because it has been linked to cancer progression. While melanocytes and normal or dysplastic nevi do not express MHC class II, its expression increases with cancer progression as expression is visible in around 80% of local metastatic lesions and 40-70% of primary melanoma (Brocker et al., 1985; Elder et al., 1989; Ruiter et al., 1984; van Duinen et al., 1984). Constitutive expression of HLA-DR is more common than expression of HLA-DP or HLA-DQ, and its expression has been linked to a more aggressive phenotype and a high risk of metastasis (Lopez-Nevot et al., 1988).

In melanoma cell lines, constitutive expression is also a frequent event (Houghton et al., 1982). Cell lines that do not express MHC class II are mostly inducible by IFNγ, but some remain negative (Houghton et al., 1984; Lee et al., 1999).

On the molecular level, it has been shown that constitutive MHC class II expression in melanoma cells depends on CIITA in most cases and is driven by CIITA via its B-cell specific promoter III (Deffrennes et al., 2001; van der Stoep et al., 2007). Responsible might be a 4kb enhancer upstream of this promoter III, which has also been described as an IFN-responsive sequence (Deffrennes et al., 2001). Of note, the constitutive expression is not linked to a general activation of the IFN signaling pathway, but has been shown to be associated to a trans-acting factor that has not been identified yet (Goodwin et al., 2001). The MAPK pathway that is frequently deregulated in melanoma cells might activate expression of CIITA, in combination with AP-1 acting on an AP-1-responsive element in pIII of the CIITA gene (Martins et al., 2007).

In line with the data to MHC class II expression, also activation of $CD4^+$ Tregs might occur by presenting antigens on MHC class II molecules. In addition, MHC class II related signaling into the tumor cell has been shown to protect melanoma cells against apoptosis (Aoudjit et al., 2004; Hemon et al., 2011; Wang et al., 2005). But although MHC class II expression on melanoma cells has been linked to disease progression, it still renders melanoma cells possibly recognizable to $CD4^+$ T cells.

5.5. Immune evasion

Besides the existence of a, at least, initial surveillance of malignant transformation by the innate and adaptive immune system, tumor cells still develop, even in fully immune competent hosts. This is pointing towards mechanisms that counteract elimination of tumor cells, leading to a refinement of the “cancer surveillance theory” into a more complex theory by Schreiber et al., called “cancer immune-editing”, in which three different steps of cancer development have been proposed, namely elimination, equilibrium and escape (Mittal et al., 2014).

Transplant experiments showed that tumor cells developed in the absence of an intact immune system, like in RAG-$2^{-/-}$ mice, are more immunogenic and are rejected when transplanted into immune-competent mice compared to tumors that were formed in wild-type mice at first (Engel et al., 1997; Shankaran et al., 2001; Svane et al., 1996). These experiments showed that less immunogenic variants of tumor cells must be selected by the pressure of immune cells.

After the first elimination of immunogenic tumor cell variants by the complex interplay of innate and adaptive immunity, a state of equilibrium arises that is dominated by the balance of tumor eliminating/tumor promoting mechanisms and subsequently leads to

tumor escape (Mittal et al., 2014). Important factors are tumor-promoting cytokines such as IL-10 and IL-23 in contrast to anti-tumor cytokines like IL-12 and IFNγ (Mittal et al., 2014). In addition, immunosuppression can occur by the recruitment of immune suppressors such as myeloid derived suppressor cells (MDSCs) or $FOXP3^+$ regulatory T cells (Tregs) expressing immunosuppressive molecules and cytokines such as IL-10, Transforming Growth Factor-β and PD-L1 (Sakaguchi et al., 2008; Schilling et al., 2013; Weide et al., 2014). The tumor cells themselves escape T cell recognition by the loss of tumor antigens, insensitivity to IFNγ or the expression of antiapoptotic molecules (Ikeda et al., 1997; Jager et al., 1996; Yamshchikov et al., 2005). Furthermore, loss of MHC class I antigen and presentation machinery has been described multiple times as a frequent event in human cancers reducing immunogenicity of these tumor variants (Garrido et al., 2010; Marincola et al., 1994; Paschen et al., 2006; Seliger et al., 1997). Additionally, it has been shown that tumor cells can actively suppress immunogenicity by expressing e.g. PD-L1 and IDO, or actively shape their environment by promoting angiogenesis by expressing VEGF, IL-6 or M-CSF (Brown et al., 2006; Dong and Chen, 2003; Nomura and Sakaguchi, 2005; Uyttenhove et al., 2003). Witnessing immune evasion mechanisms during cancer development or after immunotherapy has led to technologies identifying immune signatures predicting the likelihood of responding to immunotherapy in various types of cancer. Underlying characteristic is the so called “immune score”, thus infiltration of tumors with $CD3^+$, $CD8^+$ or $CD45RO^+$ (activated) T cells, or activated Th1 signatures expressed within a tumor, including IFNγ, STAT1, T-bet, Perforin etc. (Angell and Galon, 2013; Galon et al., 2013).

6. Objective

Due to achievements of basic and translational research, immunotherapeutic approaches for solid malignancies have progressed rapidly over the last years, representing a huge success for cancer patients.

Nevertheless, resistance mechanisms are an obstacle to overcome, both in targeted therapy with specific inhibitors as well as in immunotherapy. Concerning immunotherapeutic approaches, cancer cells eventually manage to evade surveillance by the immune system, leading to progression of the disease. The aim of this study is to outline resistance mechanisms towards both traditional anti-tumor $CD8^+$ T cells as well as to $CD4^+$ T cells to allow deeper insight into development of such resistance mechanisms and to contribute valuable characteristics to monitor during cancer therapy.

7. Manuscripts

I. Genetic evolution of T-cell resistance in the course of melanoma progression.

Antje Sucker, Fang Zhao, Birgit Real, **Christina Heeke**, Nicola Bielefeld, Maβen S, Susanne Horn, Iris Moll, Raffaela Maltaner, Peter A. Horn, Bastian Schilling, Sabbatino F, Volker Lennerz, Matthias Kloor, Soldano Ferrone, Dirk Schadendorf, Christiane S. Falk, Klaus Griewank, Annette Paschen

Published in: Clinical Cancer Research, December 2014

II. Melanoma lesions independently acquire T-cell resistance during metastatic latency.

Fang Zhao, Antje Sucker, Susanne Horn, **Christina Heeke**, Nicola Bielefeld, Barbara Schrörs, Anne Bicker, Monika Lindemann, Alexander Roesch, Gustav Gaudernack, Mathias Stiller, Jürgen C. Becker, Volker Lennerz, Thomas Wölfel, Dirk Schadendorf, Klaus Griewank, Annette Paschen

Published in: Cancer Research, June 2016

III. Development of stable HLA class II-deficient melanoma phenotypes in the course of disease progression leads to total T cell resistance in the context of HLA class I deficiency

Christina Heeke, Fang Zhao, Antje Sucker, Nicola Bielefeld, Raffaela Maltaner, Ulf Dittmer, Soldano Ferrone, Klaus Griewank, Dirk Schadendorf, Matthias Kloor, Annette Paschen

Prepared for submission

7.1. Article I

Clinical Cancer Research

Biology of Human Tumors

Genetic Evolution of T-cell Resistance in the Course of Melanoma Progression

Antje Sucker[1,2], Fang Zhao[1,2], Birgit Real[1,2], Christina Heeke[1,2], Nicola Bielefeld[1,2], Stefan Maβen[3], Susanne Horn[1,2], Iris Moll[1,2], Raffaela Maltaner[1,2], Peter A. Horn[2,4], Bastian Schilling[1,2], Francesco Sabbatino[5], Volker Lennerz[6], Matthias Kloor[7], Soldano Ferrone[5], Dirk Schadendorf[1,2], Christine S. Falk[3], Klaus Griewank[1,2], and Annette Paschen[1,2]

Abstract

Purpose: $CD8^+$ T lymphocytes can kill autologous melanoma cells, but their activity is impaired when poorly immunogenic tumor phenotypes evolve in the course of disease progression. Here, we analyzed three consecutive melanoma lesions obtained within one year of developing stage IV disease for their recognition by autologous T cells.

Experimental Design: One skin (Ma-Mel-48a) and two lymph node (Ma-Mel-48b, Ma-Mel-48c) metastases were analyzed for T-cell infiltration. Melanoma cell lines established from the respective lesions were characterized, determining the T-cell–stimulatory capacity, expression of surface molecules involved in T-cell activation, and specific genetic alterations affecting the tumor–T-cell interaction.

Results: Metastases Ma-Mel-48a and Ma-Mel-48b, in contrast with Ma-Mel-48c, were infiltrated by T cells. The T-cell–stimulatory capacity was found to be strong for Ma-Mel-48a, lower for Ma-Mel-48b, and completely abrogated for Ma-Mel-48c cells. The latter proved to be HLA class I–negative due to an inactivating mutation in one allele of the beta-2-microglobulin (*B2M*) gene and concomitant loss of the other allele by a deletion on chromosome 15q. The same deletion was already present in Ma-Mel-48a and Ma-Mel-48b cells, pointing to an early acquired genetic event predisposing to development of β2m deficiency. Notably, the same chronology of genetic alterations was also observed in a second β2m-deficient melanoma model.

Conclusion: Our study reveals a progressive loss in melanoma immunogenicity during the course of metastatic disease. The genetic evolvement of T-cell resistance suggests screening tumors for genetic alterations affecting immunogenicity could be clinically relevant in terms of predicting patient responses to T-cell–based immunotherapy. *Clin Cancer Res; 20(24); 6593–604. ©2014 AACR.*

Introduction

$CD8^+$ T lymphocytes (CTL) can exert potent *in vivo* cytotoxicity against autologous melanoma cells. This is demonstrated by the remarkable clinical responses observed in adoptive cellular therapy with autologous tumor-reactive T cells and therapy with immune-modulatory antibodies that release T cells from suppressive signals (1–5). The efficacy of these treatment regimens is based on the high intrinsic immunogenicity of melanoma cells that allows CTL to respond to multiple HLA class I–restricted tumor antigens (6). However, only a subgroup of patients receiving immunotherapy experiences clinical benefit, whereas others do not respond at all or after initial responses show progressive disease. In particular, the coexistence of therapy-responding and nonresponding metastases in individual patients suggests heterogeneous T-cell responsiveness of the different lesions (1). The underlying mechanisms therefore are most likely multifactorial. An immunosuppressive tumor microenvironment generated by regulatory T cells, myeloid-derived suppressor cells, or other immune regulators can restrict T-cell effectiveness (7–9). Furthermore T-cell activity can be hampered by specific genetic alterations that affect tumor immunogenicity. Interestingly, the antitumor activity of the T cells is considered a driving force that selectively favors the outgrowth of low-immunogenic melanoma phenotypes. Thus, besides eliminating tumor cells, T cells shape the immunogenicity of malignant cells,

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Metastatic melanoma is an aggressive, frequently deadly disease. In recent years, immunotherapies with anti–CTLA-4 and anti–PD-1 antibodies, exploiting the capacity of $CD8^+$ T cells to kill immunogenic tumor cells, have shown great clinical promise. However, not all patients benefit from immunotherapy and currently biomarkers predicting responses to treatment are lacking. In our study, a progressive loss in immunogenicity was observed for melanoma cells derived from three consecutive patient metastases. This culminated in complete T-cell resistance of the tumor cells due to loss of HLA class I expression. Genetic analysis revealed an early acquired chromosomal deletion and subsequent inactivating gene mutation leading to β2m deficiency and the observed HLA class I–negative phenotype. Our findings suggest that tumors can genetically evolve to avoid being recognized by the immune system. Detecting genetic alterations affecting tumor immunogenicity could be of significant value in determining which patients are likely to benefit from melanoma immunotherapy.

thereby supporting the escape of tumors from immune surveillance. This so-called T-cell–mediated immunoediting of tumors, initially defined by Schreiber and colleagues (10), has been observed in several mouse model studies, and evidences that this process also takes place in patients with cancer have been provided. By investigating metastatic cell lines and in some cases tissue samples, several genetic alterations affecting the tumor cells' immunogenicity have been defined, including the loss of single or multiple *HLA* alleles and *HLA* haplotypes as well as the total lack of HLA class I antigen expression (11–15). So far, only a few studies followed the course of melanoma immunogenicity during disease progression due to the poor availability of tissue from sequential tumor metastases, corresponding cell lines, and autologous peripheral blood T lymphocytes. Coulie and colleagues (6) were the first to examine T-cell recognition of autologous tumor cells derived from two sequential metastatic melanoma lesions. They observed that T-cell responses toward melanoma cells from the second metastases were impaired as these cells expressed only one of the six HLA class I alleles (16). In 2005, Yamshchikov and colleagues (14) demonstrated that recognition of tumor cells from two consecutive melanoma lesions by autologous differentiation antigen-specific $CD8^+$ T cells was compromised by an HLA haplotype loss in tumor cells from the first metastasis and by a mixed HLA-low and HLA-negative tumor cell phenotype in the second metastasis. Hence, as a basis for improving melanoma immunotherapy, more information is needed about the type and the sequence of genetic alterations in the tumor cells with potential impact on their recognition by cognate T cells.

Here, we monitored the immunogenicity of three consecutive melanoma metastases from patient Ma-Mel-48 obtained within one year of developing stage IV disease. We observed a gradual loss in immunogenicity culminating in complete T-cell resistance of the tumor cells caused by an irreversible HLA class I–negative phenotype. This originated from two different types of genetic alterations, a deletion on chromosome 15q where the *B2M* gene maps and an inactivating *B2M* gene mutation. Notably, tumor cells from all metastases of patient Ma-Mel-48 carried the same deletion on chromosome 15q, identifying this alteration as an early predisposing genetic event in the development of β2m deficiency. The same chronology of genetic alterations was also observed in a second patient model of β2m-deficient melanoma.

Materials and Methods

Patient samples

Patient Ma-Mel-48 presented with melanoma stage II in December 2000 at the age of 85 years. Progression to stages III and IV was diagnosed in October 2001 and June 2002, respectively. The patient was treated with temozolomide at the beginning of 2003 but never received immunotherapy. In September 2003, the patient died. Patient Ma-Mel-100 presented with melanoma stage II in January 1998 at the age of 72 years. Progression to stage III was diagnosed in September 2002. From February 1998 until September 2002, the patient received adjuvant IFNα therapy. In September 2005, the patient died.

Samples including tumor tissues and peripheral blood mononuclear cells (PBMC) were collected after approval by the institutional review board and patient informed written consent. Tissues were mechanically divided for cryopreservation and generation of the corresponding cell lines. Small tissue pieces were distributed in cell culture dishes, and outgrowing cells were split for the first time at 90% cell confluence. Melanoma cell lines were cultured in RPMI-1640 medium supplemented with glutamine, 10% FCS, and penicillin/streptomycin. Cells were cultured at 37°C in a 5% CO_2 atmosphere. Cell lines were authenticated by genetic profiling at the Institute for Forensic Medicine (University Hospital Essen) using the AmpFLSTR-Profiler Plus Kit (Applied Biosystems) and routinely tested every 6 months.

Antibodies

The following murine mAbs were used for IHC: W6/32 to detect HLA class I antigens (Dianova); bbm.1 to stain for β2m (kindly provided by G. Moldenhauer, German Cancer Research Center, Heidelberg, Germany); anti–HMB-45 (Dako) to detect melanoma cells; anti-CD3 (BD Pharmingen) to stain for T cells.

For flow cytometry, the mouse mAbs were as follows: anti–HLA-ABC-APC (eBiosciences), anti–CD54-PE and anti–HLA-DR-PECy7 (Beckmann Coulter), anti–PD-L1-PE and anti–PD-L2 (Biolegend), anti–B7-H3 (R&D Systems), anti–B7-H4 (eBiosciences); L243 was used for detection of HLA-DR molecules (17) and HC10 for labelling of β_2m-free HLA heavy chains (18, 19).

The following antibodies were used for Western blot analysis: mouse anti–Melan-A/MART-1 (Zytomed), mouse anti-Tyrosinase and anti-MITF (Santa Cruz Biotechnology), rabbit anti-DCT/TRP2 (kindly provided by V. Hearing, National Cancer Institute, NIH, Bethesda); mouse anti-STAT1, rabbit anti-phospho(p)STAT1, rabbit anti-JAK1, rabbit anti-GAPDH (Cell Signaling Technology); rabbit anti-IRF1 and mouse anti-β2m (Santa Cruz Biotechnology); rabbit anti-β2m (Sigma); mouse anti-TAP1 (NOB-1) and mouse anti-tapasin (TO-3; ref. 20); mouse mAb HC10 (18, 19).

Mixed lymphocyte-tumor cell culture

Anti–CD8-Microbeads (Miltenyi Biotec) were used for positive selection of $CD8^+$ T cells from PBMC. Selected T cells were cocultured in 24-well plates at 10^6 cells per well with irradiated (100 Gray) autologous tumor cells at 10^5 cells per well in 2 mL AIM-V medium (GIBCO-BRL) supplemented with 10% human AB serum (complete medium). IL2 was added on day 3 at 250 IU/mL (Chiron Corporation). Lymphocytes (10^6 cells/well) were restimulated weekly with 10^5 irradiated tumor cells in IL2-supplemented complete medium.

Intracellular cytokine staining

For detection of intracellular IFNγ and TNFα, lymphocytes were stimulated for 4 hours with the indicated tumor cells (effector-to-target ratio of 1:1 or 1:2) in AIM-V complete medium containing 10 μg/mL Brefeldin A (Sigma-Aldrich). Then cells were stained with anti-human CD3-PE/Cy7 and CD8-APC-Alexafluor700 antibodies (Beckman Coulter) followed by fixation and permeabilization using the Fixation/Permeabilization Concentrate and Diluent Kit (eBioscience) and addition of an anti-IFNγ–FITC antibody (Beckman Coulter) or anti-TNFα–Pacific Blue antibody (Biolegend). Cells were analyzed in a Gallios flow cytometer, and the Kaluza software was used for data analysis (Beckman Coulter). Where indicated, antibody W6/32 (50 μg/mL; purified from hybridoma supernatant, kindly provided by M. Fatho, Mainz) and anti–PD-L1 (10 μg/mL; Biolegend) were added to block the TCR/HLA class I and PD-1/PD-L1 interactions, respectively. Mouse monoclonal IgG1 (mIgG; R&D Systems) was used as control antibody.

SNP array analysis

Genomic DNA was isolated from melanoma cell lines of patients Ma-Ma-48 and Ma-Mel-100 and from nonfixed peripheral blood cells (available only for patient Ma-Mel-48) using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. SNP arrays were performed using CytoScan HD Array from Affymetrix. Hybridization was done according to the manufacturer's protocol, and data analysis was performed applying the program Chromosome Analysis Suite from Affymetrix. SNP array data files are accessible at the NCBI GEO database (accession number: GSE60218).

To infer the phylogenetic relationship of Ma-Mel-48 cells, genotype calls were extracted from the CytoScan HD Array data using the linux shell and R (21). As an outgroup genotype, calls from the autologous blood sample (166) were used. We identified 111,029 variable sites among the cell lines and blood sample. Of those, 64,023 were parsimony informative. We then used the maximum parsimony criterion, minimizing the total number of evolutionary steps required to explain the relationship of the tested samples. We ran maximum parsimony in MEGA (22) using 500 bootstrap replications and complete deletion of missing data, as well as subtree pruning–regrafting as a search method.

Additional information for Materials and Methods is provided in Supplementary Methods.

Results

Consecutive metastases from patient Ma-Mel-48 show heterogeneous T-cell infiltration and HLA class I antigen expression

To determine the development of melanoma immunogenicity during disease progression, we collected tumor tissues from three consecutive metastases of patient Ma-Mel-48. The first Ma-Mel-48a, a skin metastasis, was excised in July 2002, a few weeks after diagnosis of stage IV disease. The second and the third lesions Ma-Mel-48b and Ma-Mel-48c, lymph node metastases, were surgically removed in January 2003 and July 2003, respectively (Fig. 1A).

Serial tissue sections of the three metastases were first analyzed by IHC for their infiltration by $CD3^+$ T lymphocytes. T cells accumulated in the periphery and center of metastases Ma-Mel-48a and Ma-Mel-48b pointing to a close tumor-T cell interaction (Fig. 1B and Supplementary Fig. S1). In contrast, no T-cell infiltrate was detected in metastasis Ma-Mel-48c (Fig. 1B). The presence of T cells correlated with the expression of HLA class I molecules, stained for with the antibody W6/32 binding a structural epitope formed by complexes of the β2m light chain and the HLA heavy chains. HMB-45–positive melanoma cells expressed HLA class I molecules in metastasis Ma-Mel-48a but not in Ma-Mel-48c (Fig. 1B).

Next, cell lines established from the respective metastatic lesions were analyzed for their HLA class I surface expression by flow cytometry. Ma-Mel-48a as well as Ma-Mel-48b cells were MHC class I–positive, whereas Ma-Mel-48c cells proved to be negative (Fig. 1C). Thus, the data generated by flow cytometry were in agreement with the results obtained by IHC.

Progressive loss in T-cell recognition of the different melanoma cell lines

On the basis of the HLA class I expression pattern, we analyzed the T-cell–stimulatory capacity of Ma-Mel-48a and Ma-Mel-48b cells. Mixed lymphocyte-tumor cell cultures (MLTC) were set up, consisting of purified autologous peripheral blood $CD8^+$ T cells and Ma-Mel-48a or Ma-Mel-48b tumor cells. $CD8^+$ T cells were stimulated in

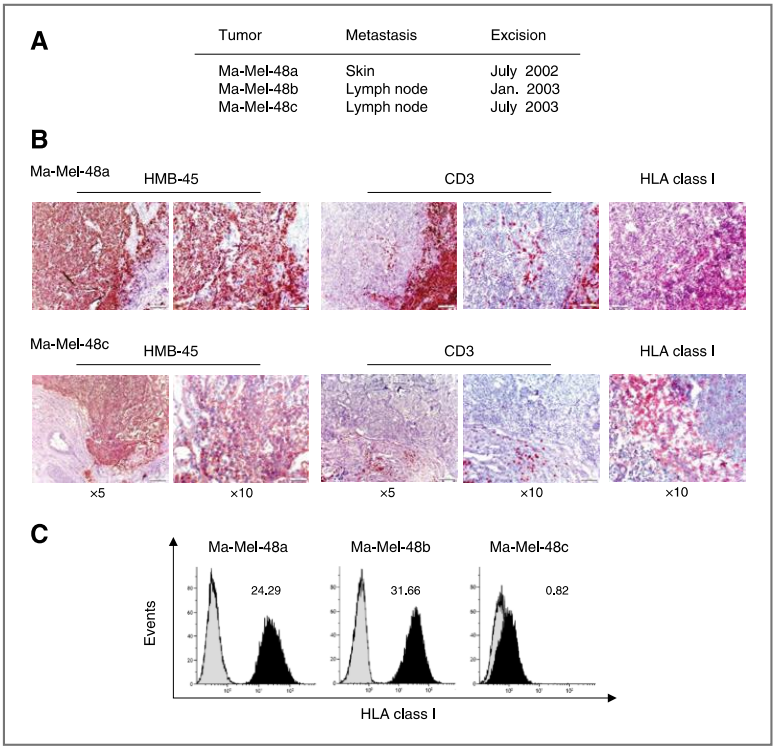

Figure 1. Heterogeneous T-cell infiltration and HLA class I antigen expression in different metastases from patient Ma-Mel-48. A, localization and excision date of three sequential metastases from patient Ma-Mel-48. B, serial sections from cryopreserved Ma-Mel-48a and -48c metastatic lesions were analyzed for the expression of HMB-45 (melanoma cells), CD3 (T cells), and HLA class I antigens by IHC. Red staining indicates positive cells. C, flow cytometric analysis of total HLA class I antigen expression on melanoma cell lines established from the different metastases of patient Ma-Mel-48. Cells were stained with mAb W6/32. Histograms from one representative of three independent experiments are shown. Numbers indicate mean fluorescence intensity (MFI).

weekly intervals with the indicated irradiated tumor cells to increase the frequency of tumor-reactive T lymphocytes. After two stimulations, $CD8^+$ T cells were harvested and analyzed for their responsiveness toward the tumor cells. As shown in Fig. 2A, approximately 9% of $CD8^+$ T cells from MLTC with Ma-Mel-48a cells (MLTC-48a) responded to Ma-Mel-48a cells with IFNγ production, whereas only around 2% of the $CD8^+$ T cells reacted against Ma-Mel-48b cells. Interestingly, $CD8^+$ T cells harvested from MLTC-48b showed limited responsiveness to Ma-Mel-48b as well as Ma-Mel-48a cells (Fig. 2B). Approximately 2% of the T cells recognized Ma-Mel-48b cells, and comparable reactivity toward Ma-Mel-48a cells was observed. These results pointed to a lower T-cell–stimulatory capacity of Ma-Mel-48b cells as compared with Ma-Mel-48a cells. In either case, production of $CD8^+$ T-cell cytokines proved to be HLA class I–dependent (Fig. 2C), thus no T-cell reactivity toward the HLA class I–negative Ma-Mel-48c cells was observed (Fig. 2A and B).

In contrast with $CD8^+$ T cells, autologous $CD4^+$ T cells did not react toward any of the Ma-Mel-48 cell lines that lacked HLA class II expression even in the presence of IFNγ, due to epigenetic silencing of the transcriptional regulator CIITA (Supplementary Fig. S2 and data not shown).

Low immunogenic phenotype of Ma-Mel-48b cells is not due to altered expression of HLA alleles or immunomodulatory B7 molecules

To define the molecular mechanisms underlying differential T-cell recognition, we first analyzed Ma-Mel-48a and Ma-Mel-48b cells for potential alterations in HLA allele expression. HLA genotype analysis demonstrated that the HLA-A*0101, -B*0801, -C*0701 homozygous haplotype of autologous peripheral blood cells was conserved in the cell lines, both expressing considerable amounts of *HLA-A*–, *HLA-B*–, and *HLA-C*–specific mRNA (Fig. 3A). Data generated by qRT-PCR pointed to elevated *HLA-B* mRNA levels in Ma-Mel-48b cells as compared with Ma-Mel-48a cells. Accordingly, staining of Ma-Mel-48b cells for surface expression of HLA class I antigens and β2m-free HLA heavy chains was more intense compared with Ma-Mel-48a cells (Figs. 1C and 3A). Not expecting this shift in HLA class I allele expression to be responsible for the lower immunogenic phenotype of Ma-Mel-48b cells, we studied the surface expression of additional molecules involved in tumor–T-cell interaction. As shown in Fig. 3B, both cell lines expressed similar levels of the immunomodulatory B7 molecules PD-L1 (B7-H1), PD-L2 (B7-DC), and B7-H3, whereas no expression of B7-H4 was noted in either case. Though inhibitory PD-L1 was clearly detectable on Ma-Mel-

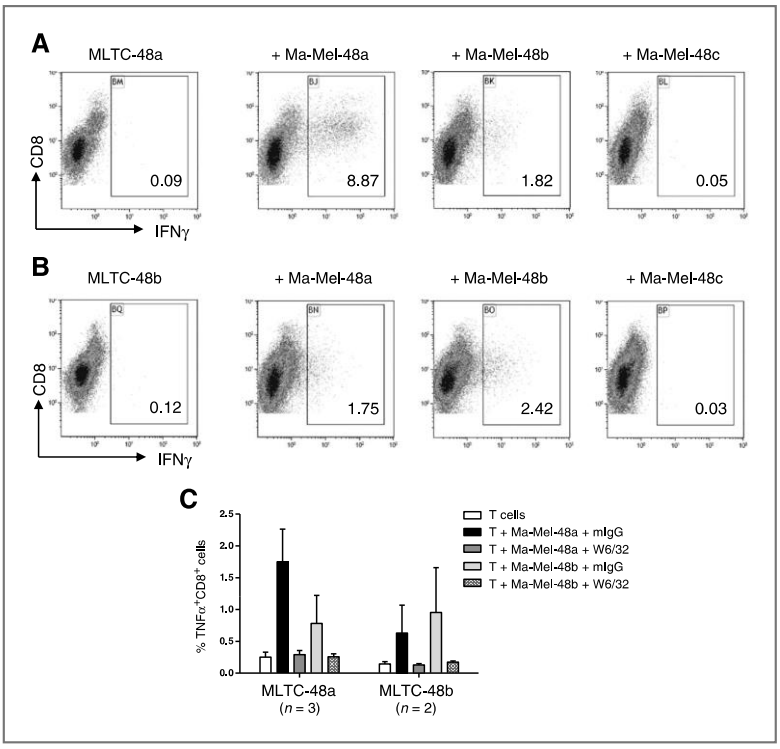

Figure 2. Tumor cells from sequential lesions differ in their T-cell–stimulatory capacity. A–C, analysis of the T-cell–stimulatory capacity of the different Ma-Mel-48 tumor cell lines. In autologous MLTC, isolated $CD8^+$ T cells were stimulated twice with Ma-Mel-48a (MLTC-48a; A, C) or Ma-Mel-48b (MLTC-48b; B, C) cells and subsequently analyzed for their response toward different target cells by intracellular staining for IFNγ or TNFα. A, left, first dot plot: spontaneous IFNγ production by $CD8^+$ T cells from MLTC-48a; second to fourth dot plot: production of IFNγ in response to the different target cells indicated above. B, left, first dot plot: spontaneous IFNγ production by $CD8^+$ T cells from MLTC-48b; second to fourth dot plot: production of IFNγ in response to the different target cells indicated above. Results from one representative of two independent experiments are presented, numbers in dot plots indicate % $IFN\gamma^+CD8^+$ T cells within $CD8^+CD3^+$ T cells. C, to demonstrate HLA class I–dependent production of T-cell cytokines, tumor cells were incubated with blocking mAb W6/32 before addition of T cells. Control cells were incubated with mouse IgG1 (mIgG). The mean % $TNF\alpha^+CD8^+$ T cells within $CD8^+CD3^+$ T cells of $n = 2$ or $n = 3$ independent experiments is presented.

48a and Ma-Mel-48b cells, its blockade did not enhance T-cell activities in our *in vitro* settings (Supplementary Fig. S3). Both melanoma cell lines also expressed similar levels of CD54 (ICAM-1), of importance for T-cell adhesion to the tumor cells (Fig. 3C).

Although the analysis of specific surface molecules did not reveal significant differences between Ma-Mel-48a and Ma-Mel-48b cells, we postulated that variations in antigen expression levels could be responsible for the differential immunogenicity. Although the specificity of tumor-reactive T cells could not be studied due to limited sample material, we investigated the expression of melanoma differentiation antigens (MDA) in the tumor cells. As shown in Fig. 3D, Melan-A, Tyrosinase, and DCT/TRP2 as well as their transcriptional regulator MITF were decreased in Ma-Mel-48b as compared with Ma-Mel-48a cells, suggesting MDA down-regulation in Ma-Mel-48b cells could contribute to their lower T-cell–stimulatory capacity. Interestingly, Ma-Mel-48b dedifferentiation was not associated with a slow proliferative phenotype, as frequently observed for MITF-low melanoma cells (Supplementary Fig. S4; ref. 23).

The HLA class I–negative phenotype of Ma-Mel-48c cells is caused by β2m deficiency

To determine whether the HLA class I–negative phenotype of Ma-Mel-48c cells was reversible, cells were treated with type I and type II IFN. As shown in Fig. 4A, surface levels of HLA class I antigens were enhanced on Ma-Mel-48a and Ma-Mel-48b cells in response to IFN treatment. However, HLA class I antigen expression on Ma-Mel-48c cells could not be restored (Fig. 4A), although basic expression of interferon pathway components in Ma-Mel-48c cells was similar to the other cell lines and also the upregulation of STAT1, pSTAT1, and IRF1 in response to interferon

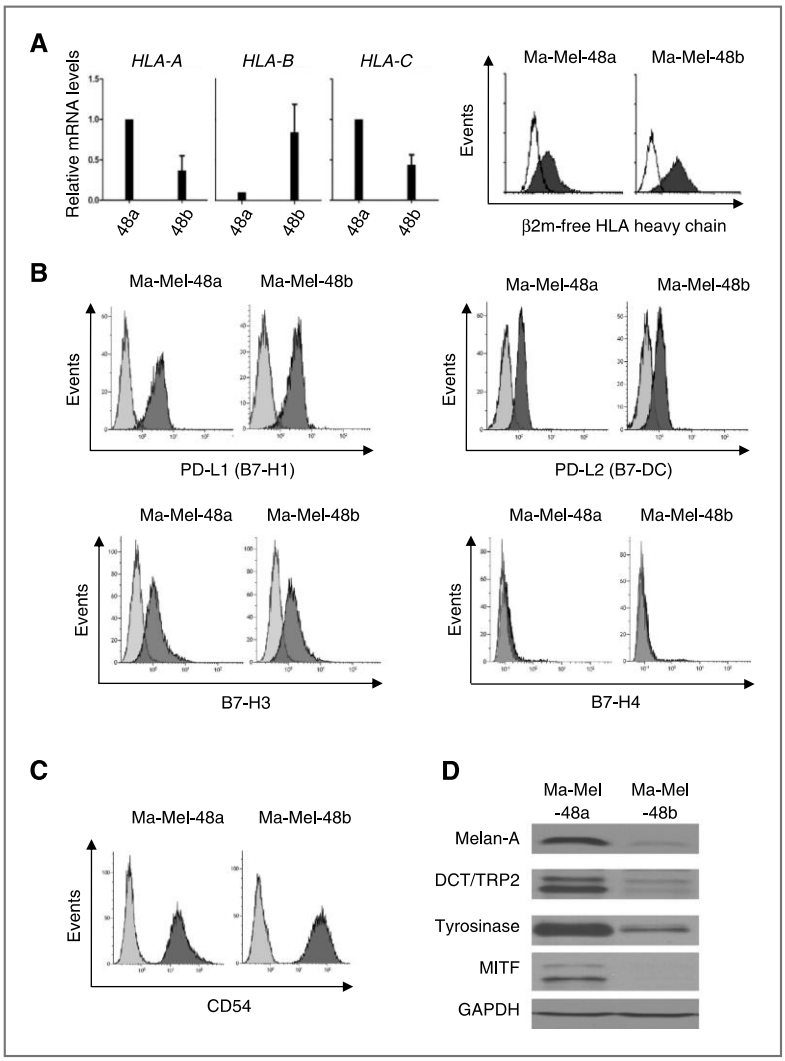

Figure 3. Conserved HLA phenotype but altered antigen expression in Ma-Mel-48b cells. A, left, mRNA levels of *HLA-A*, *HLA-B*, *HLA-C* in Ma-Mel-48a and Ma-Mel-48b cells were quantified by qRT-PCR and normalized to endogenous *GAPDH* mRNA. Expression levels, given as mean (+ SEM) of three independent experiments, are depicted relative to the expression in Ma-Mel-48a cells. A, right, flow cytometric analysis of β2m-free HLA heavy chain expression on Ma-Mel-48a and Ma-Mel-48b cells with mAb HC10. B, C, analysis of PD-L1 (B7-H1), PD-L2 (B7-DC), B7-H3, B7-H4, and CD54 surface expression on melanoma cell lines Ma-Mel-48a and Ma-Mel-48b by flow cytometry. Histograms from one representative of three independent experiments are shown. D, expression of MDA (Melan-A, Tyrosinase, DCT/TRP2) and its transcriptional regulator MITF in Ma-Mel-48a and Ma-Mel-48b cells was determined by Western blot. GAPDH served as a loading control. Data from one representative of three independent experiments are presented.

treatment was comparable (Fig. 4B; Supplementary Fig. S5A). Analysis of several components of the antigen processing and presentation machinery (APM) by Western blot revealed expression of HLA heavy chains, TAP1, and tapasin in all cell lines, whereas β2m protein was expressed in Ma-Mel-48a and Ma-Mel-48b but not in Ma-Mel-48c cells, even when cells were treated with IFN (Fig. 4B). Also, mRNA expression of specific APM components was comparable between the three melanoma cell lines and was detectable in similar amounts also in melanocytes, but only Ma-Mel-48c cells lacked *B2M* mRNA (Fig. 4C; Supplementary Fig. S5B). These findings suggested the HLA class I–negative phenotype of Ma-Mel-48c cells was caused by the lack of *B2M* gene expression. Although *B2M* mRNA was not detectable by qRT-PCR, we obtained a shortened *B2M*-specific product by RT-PCR only for Ma-Mel-48c cells with primers amplifying the coding region from the start to the stop codon (Fig. 5A). Sequence analysis of this PCR product identified a 60-bp deletion starting in codon 96 (Exon II) of the *B2M* gene, leading to a C-terminally altered gene product (Fig. 5B). Notably, this *B2M* deletion variant could not be detected by qRT-PCR (Fig. 4C), as the assay primers were located in the *B2M* Exon II–III boundary. To confirm that the loss in HLA class I antigen expression was solely due to β2m deficiency,

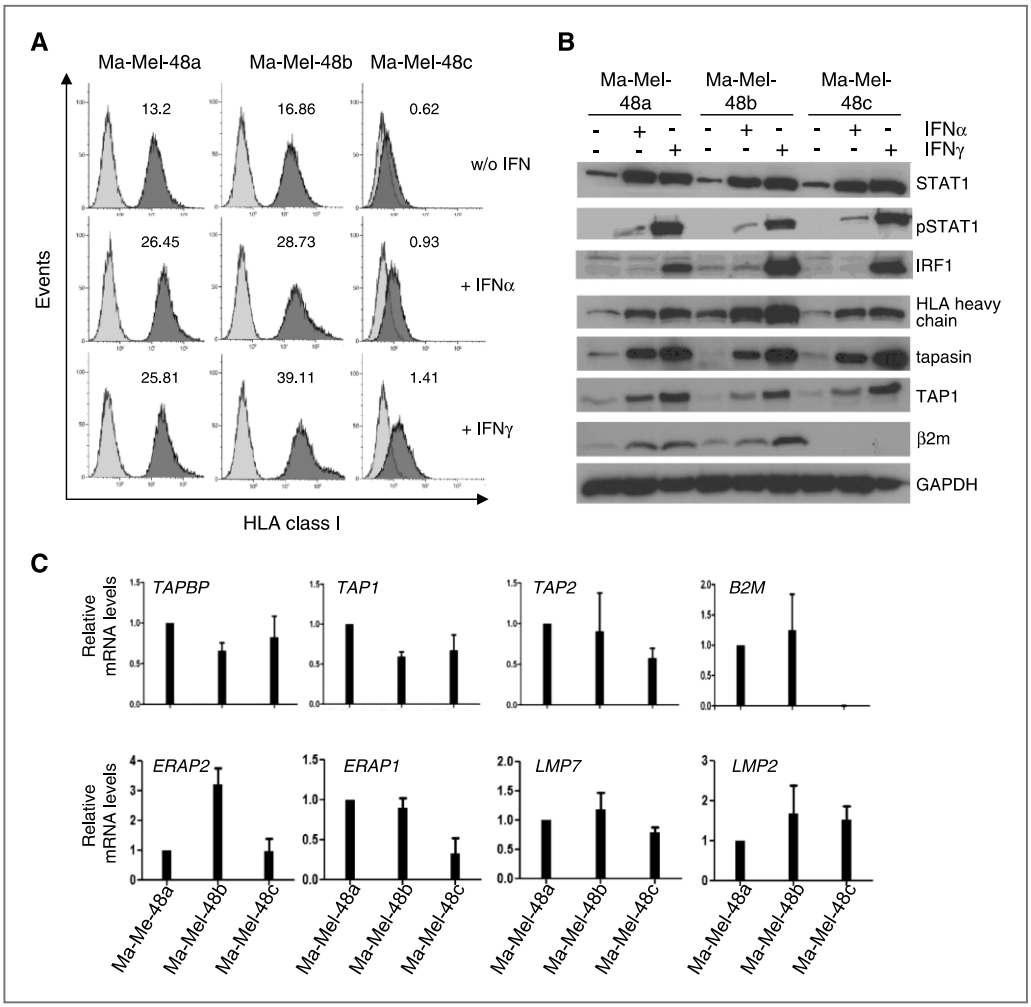

Figure 4. Interferon treatment of Ma-Mel-48c cells does not restore HLA class I antigen surface expression. The different Ma-Mel-48 cell lines were treated with IFNα (1000 U/mL) or IFNγ (500 U/mL) for 48 hours, controls were left untreated. A, cells were stained with mAb W6/32 for detection of HLA class I antigen expression. Data from one representative of three independent experiments are depicted, numbers indicate MFI values. B, cell lysates were analyzed by Western blot for the protein levels of interferon pathway components (STAT1, pSTAT1, IRF1) or APM components (HLA heavy chains, tapasin, TAP1, β2m). GAPDH served as loading control. One representative of three independent experiments is depicted. C, mRNA levels of different APM components were quantified by qRT-PCR and normalized to endogenous *GAPDH* mRNA. Expression levels, given as mean (+SEM) of three independent experiments, are depicted relative to the expression in Ma-Mel-48a cells.

Ma-Mel-48c cells were transfected with a *B2M* expression plasmid. As shown in Fig. 5C, induction of HLA class I antigen expression was detectable on a population of transiently *B2M*-transfected cells. In agreement with the results obtained for the cell line, β2m was not detected in tumor cells in the corresponding metastatic lesion while it was expressed by normal cells (Fig. 5D).

B2M allele loss is an early predisposing genetic event in the development of β2m deficiency

Previously, we reported that β2m deficiency is caused by the coincidence of a *B2M* gene mutation and allelic *B2M* loss. The *B2M* gene maps to chromosome 15q21.1 and can be lost as a result of chromosome 15q aberrations (12, 24). To define whether this was also the case in Ma-Mel-48c cells,

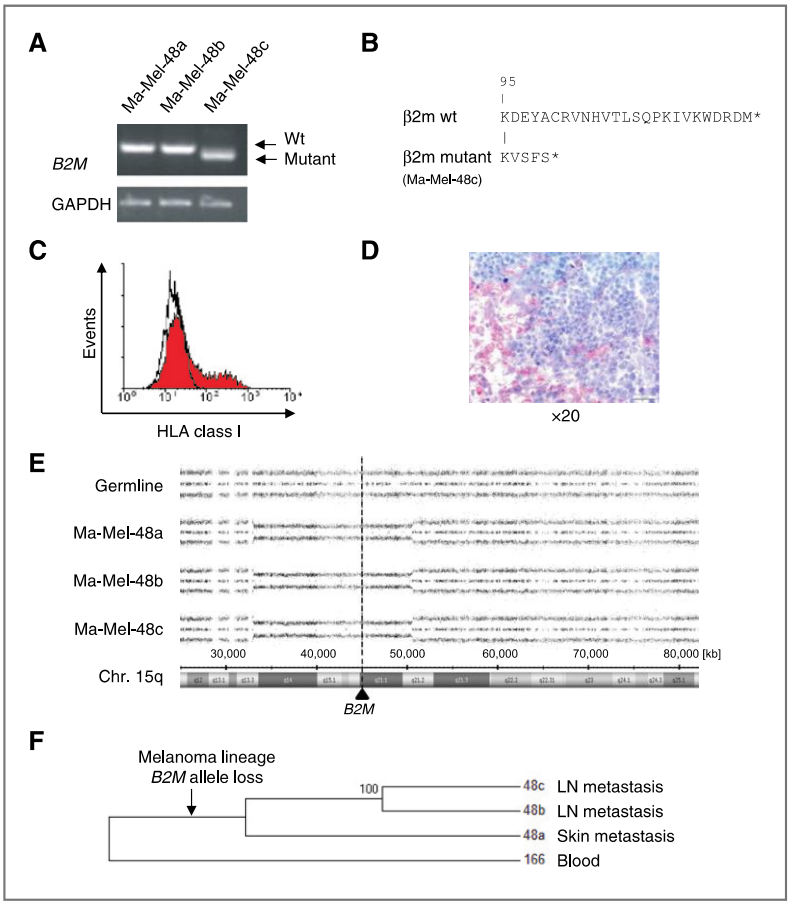

Figure 5. β2m deficiency of Ma-Mel-48c cells is caused by *B2M* gene mutation subsequent to *B2M* allele loss. A, semiquantitative analysis of *B2M* mRNA expression by RT-PCR. Ma-Mel-48c cells express a shortened *B2M* mRNA, a PCR product in the size of wild-type (wt) mRNA was only detectable in Ma-Mel-48a and Ma-Mel-48b cells. B, comparison of the C-terminal amino acid sequence (aa) of mutant β2m, as expressed in Ma-Mel-48c cells, and wt β2m. Capital letters indicate the aa sequence. The first 95 aa are identical in wt and mutant proteins. Stars indicate stop codons. C, Ma-Mel-48c cells were transfected with a *B2M* expression plasmid. Expression of HLA class I antigens on transient transfectants was determined by flow cytometry. D, analysis of β2m expression in metastatic lesion Ma-Mel-48c by IHC. Tumor cells were negative for β2m and did not stain red. E, SNP results given as allelic distribution of chromosome 15q are shown for DNA obtained from autologous PBMC and melanoma cells. All Ma-Mel-48 cell lines show loss of one chromosomal allele in the region 15q13.3 to 15q21.2 (Chr.15: 33,045,756-50,579,508; hg19). The location of *B2M* at 15q21.1 (Chr.15: 45,003,675-45,011,075) is shown by the dashed line. F, maximum parsimony tree showing the phylogenetic relationship of the melanoma cell lines and the blood sample (166) used as outgroup. Within the patient, a melanoma lineage leading to the studied cell lines evolved and was genetically divergent from the blood sample. A *B2M* loss evolved on this lineage. Then, a lineage leading to Ma-Mel-48a diverged from the melanoma ancestor and cumulated genotypic differences specific to this lineage. Later the lineages of Ma-Mel-48b and Ma-Mel-48c diverged and cumulated their specific genotypic differences. Hundred percent of the bootstrap replicates showed this grouping.

SNP array analyses were performed on DNA obtained from the various tumor cell lines and autologous peripheral blood cells as a constitutive, normal control. Indeed, a partial deletion on chromosome 15q was observed in Ma-Mel-48c cells, encompassing the region 15q13.3 to 15q21.2 (Fig. 5E). Interestingly, the same portion of chromosome 15q was also found deleted in Ma-Mel-48a and Ma-Mel-48b cells.

From the genome-wide SNP genotype data, we therefore inferred the phylogenetic relationship of the melanoma cell lines and the corresponding autologous blood sample. A phylogenetic tree based on maximum parsimony showed that Ma-Mel-48b and Ma-Mel-48c cells were more closely related to each other than to Ma-Mel-48a cells, indicating that a lineage leading to Ma-Mel-48a evolved first. The grouping was present in all 500 bootstrap replicates of the

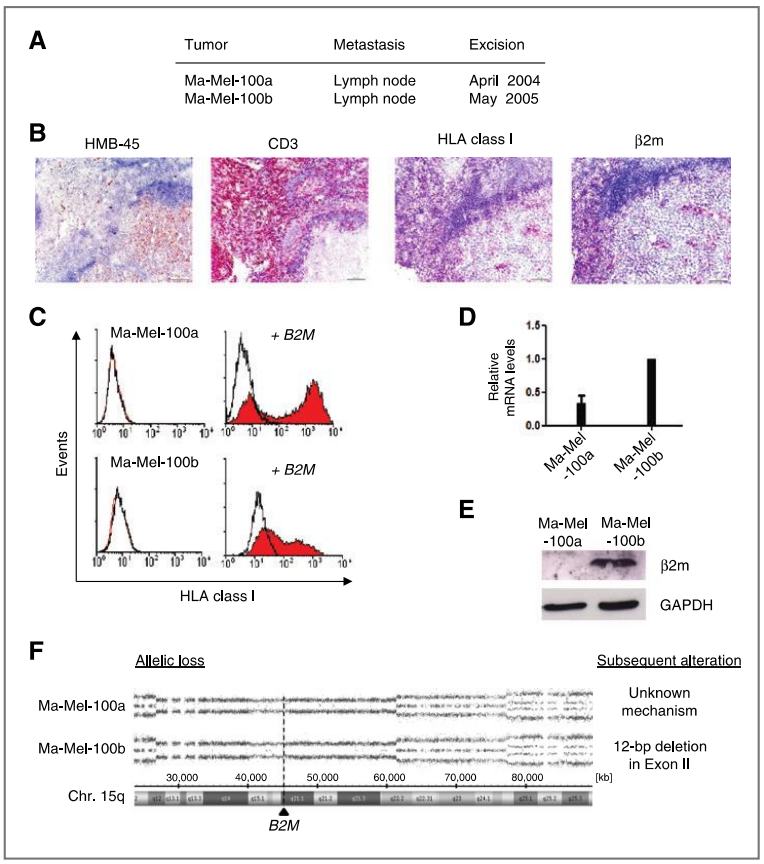

Figure 6. *B2M* allele loss is also the initial genetic alteration in β2m-deficient melanoma cells from patient Ma-Mel-100. A, location and excision date of two sequential metastases from patient Ma-Mel-100. B, serial sections from cryopreserved metastasis Ma-Mel-100a were analyzed for the expression of HMB-45 (melanoma cells), CD3 (T cells), HLA class I antigens, and β2m by IHC. Red staining indicates positive cells. C, flow cytometric analysis of total HLA class I antigens on cell lines established from metastases Ma-Mel-100a and Ma-Mel-100b before and after transient transfection with a *B2M* expression plasmid. Cells were stained with mAb W6/32. Representative histograms from one of three independent experiments are shown. D, *B2M* mRNA levels were quantified by qRT-PCR and normalized to endogenous *GAPDH* mRNA. Expression levels, given as mean (+ SEM) of three independent experiments, are depicted relative to the expression in Ma-Mel-100b cells. E, β2m protein levels in cell lysates were determined by Western blot. GAPDH served as loading control. Representative results from one of three independent experiments are depicted. F, SNP array results showing the allelic distribution of chromosome 15q from DNA obtained from Ma-Mel-100a and Ma-Mel-100b cells. One chromosomal allele in the region 15q12 to 15q22.2 (Chr.15: 26,840,997-61,436,881; hg19) is lost in both Ma-Mel-100 cell lines. The location of *B2M* at 15q21.1 is shown by the dashed line. A subsequent *B2M* gene mutation was only detected in Ma-Mel-100b cells, identified as 12-bp deletion in Exon II.

analysis (Fig. 5F). Overall, these results demonstrated that the allelic loss of *B2M* occurred early in the course of disease and that a subsequent 60-bp deletion in the remaining *B2M* allele resulted in β2m deficiency in Ma-Mel-48c cells.

β2m-deficient melanoma cells from patient Ma-Mel-100 show the same chronology of genetic alterations

In addition to patient Ma-Mel-48, we observed loss of HLA class I antigen expression on melanoma cells from patient Ma-Mel-100 (Fig. 6A). Tumor cells from the two regional lymph node metastases Ma-Mel-100a and Ma-Mel-100b excised in April 2004 and May 2005, respectively, could not be stained with mAb W6/32 (Fig. 6B; Supplementary Fig. S6A). Consistent with the lack of HLA class I expression, T cells did not infiltrate the tumor lesion but were localized in the periphery (Fig. 6B). Also, cell lines established from the metastases showed an HLA class I–negative phenotype, which remained stable in the presence of type I and II interferons (Fig. 6C, data not shown). But HLA class I surface expression on Ma-Mel-100a and Ma-Mel-100b cells could be induced upon transfection of a *B2M* expression plasmid (Fig. 6C). Both cell lines also expressed

similar amounts of *B2M*-specific mRNA (Fig. 6D); however, only Ma-Mel-100a cells lacked β2m expression *in situ* and *in vitro* (Fig. 6B and E), while the protein was detected in lysates from Ma-Mel-100b cells (Fig. 6E).

Screening of the cells by SNP array for the underlying molecular alterations revealed deletion of the same region on chromosome 15q in Ma-Mel-100a and Ma-Mel-100b cells, ranging from 15q12 to 15q22.2 (Fig. 6F). Thus, the shared loss of one *B2M* allele was the initial genetic alteration in the development of β2m deficiency. Additional alterations, acquired subsequently, were different in both cell lines. Sequence analysis of the coding region revealed a 12-bp deletion in Exon II of the *B2M* gene in Ma-Mel-100b cells. In contrast, only wild-type *B2M* mRNA was detected in Ma-Mel-100a cells, pointing to an unknown posttranscriptional defect in *B2M* expression (Fig. 6F; Supplementary Fig. S6B).

Discussion

During disease progression melanoma cells acquire genetic alterations that can affect their recognition by cytotoxic T cells. Although some mutations increase the T-cell sensitivity of melanoma cells (25–27), others decrease the tumor cell's immunogenicity and interfere with the effectiveness of immunotherapies. In terms of predicting responses to T-cell–based immunotherapy, it is important to understand the type and the sequence of genetic alterations that hamper the recognition of melanoma cells by cognate T cells.

Here, we studied the T-cell–stimulatory capacity of melanoma cells from three consecutive metastases of patient Ma-Mel-48 obtained at different times within one year of progressive stage IV disease. Although melanoma cells from skin metastasis Ma-Mel-48a strongly stimulated autologous $CD8^+$ T cells, tumor cells from the lymph node metastasis Ma-Mel-48b cells, excised half a year later, did not. The lower T-cell–stimulatory capacity of Ma-Mel-48b cells could not be explained by an altered expression of immunomodulatory B7 molecules or the adhesion molecule CD54. In addition, there was no expression of HLA-DR molecules, described as ligands of the regulatory receptor LAG3 (28–31). Furthermore, HLA allele alterations, known to protect melanoma cells from certain T-cell specificities, were not detected in Ma-Mel-48b cells (13, 15, 32). We therefore assume the lower immunogenicity of Ma-Mel-48b cells was more likely due to differences in the antigen expression pattern. Specific T-cell antigens could be downregulated in Ma-Mel-48b in comparison with Ma-Mel-48a cells, as observed for the MDA (33). Furthermore, alterations in proteasome subunits and peptidases involved in the processing and presentation of tumor antigens could have contributed to the differential immunogenicity of the tumor cells (34–36).

The gradual decrease of melanoma immunogenicity in patient Ma-Mel-48 culminated in the complete T-cell resistance of Ma-Mel-48c cells, as these cells acquired an irreversible HLA class I–negative phenotype. This was caused by a lack in β2m expression due to an inactivating mutation in one allele of the *B2M* gene and concomitant loss of the second allele. We previously reported that β2m-deficient melanoma cells are characterized by deletions on chromosome 15q including the *B2M* gene mapping to 15q21.1 (24). Notably, loss of chromosome 15q material in melanoma is not a very rare event (37, 38). Of 70 metastatic melanoma samples analyzed, 16% were positive for loss of heterozygosity in chromosome region 15q21-22 (39). In the near future, exome studies will provide more detailed information about the frequency of *B2M* gene mutations in melanoma metastases, which we expect to be lower than *B2M* allelic losses.

By SNP array analysis on DNA from Ma-Mel-48c cells, we could clearly narrow down the deletion on chromosome 15q including the *B2M* gene. Notably, exactly the same deletion on chromosome 15q was already present in Ma-Mel-48a and Ma-Mel-48b cells. A phylogenetic tree inferred from SNP array data demonstrated that in patient Ma-Mel-48 a lineage leading to metastasis Ma-Mel-48a cells evolved first, followed by the lineages leading to Ma-Mel-48b and Ma-Mel-48c. This branching order is consistent with the timing of the excision of the metastases. The localized deletion on chromosome 15q, including one *B2M* allele, detected in all metastatic cell lines is thus an early event on the lineage leading to all three studied melanoma metastases. To our knowledge, this is the first study demonstrating that allelic *B2M* loss is the initial genetic alteration in the development of β2m deficiency. The same chronology of genetic alterations was also observed in tumor cells of patient Ma-Mel-100. Here, two metastases shared a common deletion on chromosome 15q associated with loss of a *B2M* allele. Remarkably, both metastases were found to be β2m deficient due to different subsequent alterations. A specific *B2M* gene mutation was identified in Ma-Mel-100b cells, whereas Ma-Mel-100a cells expressed significant amounts of only wild-type *B2M* mRNA. We assume that an unknown posttranscriptional mechanism blocks *B2M* mRNA translation in Ma-Mel-100a cells that requires further investigations.

Although our data suggest deletion of a *B2M* allele can be an early event in the course of melanoma progression, most studies detected β2m-deficient melanoma cells in late-stage disease (12, 14). Interestingly, Del Campo and colleagues (40) recently found nests of β2m-negative tumor cells to be present in a very early lesion from a patient with melanoma, who later developed a completely HLA class I–negative metastasis. Although the first β2m-negative tumor cells were detectable before immunotherapy, the completely HLA class I–negative lesion occurred under dendritic cell vaccination, leading to the assumption that vaccine-induced T-cell activity enriched β2m-deficient tumor cells. This was proposed also by previous studies that detected β2m loss in several recurrent metastases from patients receiving immunotherapy, such as patient Ma-Mel-100 who was treated with IFNα (13, 41). Because patient Ma-Mel-48 did not receive immunotherapy, this suggests that spontaneous antitumor T-cell responses led to the outgrowth of the HLA class I–negative melanoma immunophenotype. Besides,

T-cell–independent mechanisms should also be taken into consideration. Recently, Garrido and colleagues demonstrated that HLA class I–negative melanoma cells showed enhanced proliferation, migration, and invasion in comparison with their HLA class I–positive counterparts, pointing to HLA class I molecules as tumor suppressors (42). Irrespective of the driving force, HLA class I alterations, in particular HLA class I loss, impede T-cell recognition of melanoma and will negatively influence all T-cell–based immunotherapies.

The infiltration of melanoma metastases by T cells is currently discussed as a biomarker that predicts responsiveness to immunotherapy, in which patients with T-cell–inflamed metastases more likely respond to treatment (43–45). Different mechanisms determine the migration of T cells into a metastatic lesion, such as the release of chemokines by tumor cells (45, 46). Furthermore, it has been described that the intensity of the T-cell infiltrate correlates with the level of HLA class I antigens expressed in the tumor (47). In accordance, $CD3^+$ T cells were not detected in the center of metastasis Ma-Mel-48c or Ma-Mel-100a. T cells were rather located at the periphery, an observation also noted in other HLA class I–deficient melanoma lesions (unpublished data). This suggests that within the group of patients with melanoma with non T-cell–inflamed tumors a subgroup might exhibit HLA abnormalities. Thus, we propose that biopsies from patients with melanoma should be screened for genetic HLA alterations, including losses on chromosome 15q21.1, as this could prove valuable for immunotherapeutic treatment decisions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Zhao, C. Heeke, S. Horn, R. Maltaner, P.A. Horn, B. Schilling, C.S. Falk, K. Griewank, A. Paschen, S. Ferrone, S. Massen

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Study supervision: A. Paschen

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Supplementary Material and Methods

HLA Genotyping

Genomic DNA was isolated from non-fixed peripheral blood cells and melanoma cell lines using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Molecular HLA class I genotyping was performed at the Institute for Immunology and Genetics Kaiserslautern (Kaiserslautern, Germany).

Immunohistochemistry

On serial cryostat tissue sections expression of specific marker proteins was detected with the indicated primary mAb in combination with a biotinylated goat anti-mouse antibody (Jackson ImmunoResearch). Antibody binding was visualized using the Vectastain ABC-AP kit (Vector) according to the manufacturer's instructions.

Western blot analysis

Proteins from tumor cell lysates were separated by SDS-PAGE, blotted on nitrocellulose membranes and probed with the specific primary antibodies. After washing, membranes were incubated with the appropriate secondary antibodies linked to horseradish peroxidase (HRP). Antibody binding was visualized with the ECL chemiluminescence system.

Flow cytometry

Cells were stained for surface marker expression with either directly labelled antibodies or non-labelled antibodies in combination with a secondary PE-labelled goat anti-mouse mAb (Beckmann Coulter) or Cy5-labelled goat anti-mouse mAb (Jackson ImmunoResearch). Stained cells were analysed either in a Calibur (Becton Dickinson) or Gallios (Beckmann Coulter) flow cytometer using CellQuest (Becton Dickinson) and Kaluza (Beckman Coulter) software, respectively, for data analysis. To determine the impact of interferons on the expression of specific surface molecules, cells were treated for 48 h with 1000 U/ml IFN-α or 500 U/ml IFN-γ.

Quantitative real-time RT-PCR

Total mRNA was isolated from tumor cells, melanocytes and CD8-depleted PBMC using the RNeasy Mini kit (Qiagen) and was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using specific TaqMan Gene Expression assays in combination with the

StepOnePlus™ Real-Time PCR system (Applied Biosystems). Relative RNA expression was calculated by the $2^{-\Delta\Delta CT}$ method after normalizing expression levels of candidate genes to *GAPDH* or *beta-actin* mRNA.

RT-PCR and cloning of mutated *B2M*

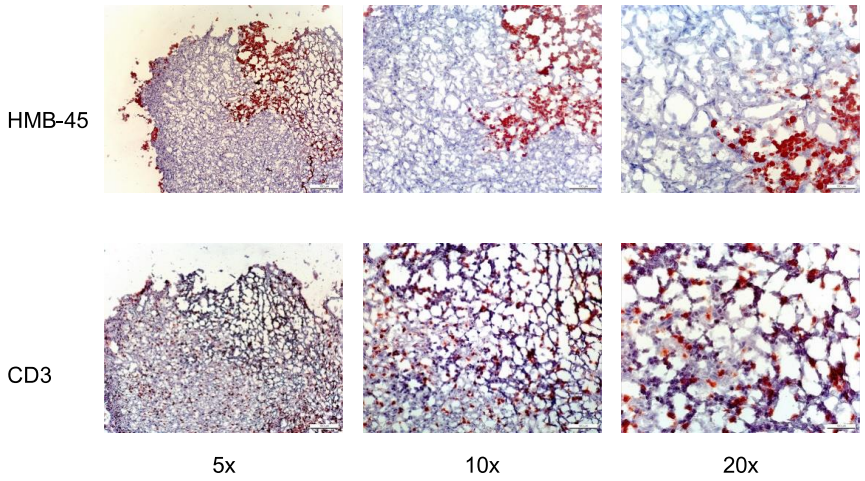
PolyA-RNA was reverse transcribed from total RNA using the first strand cDNA synthesis kit (Roche Diagnostics), according to the manufacturer's instruction. Specific amplification of *B2M* cDNA (sense primer 5'-cgagatgtctcgctccgtgg-3', antisense primer 5' ataacctctagaacctccatgatgctgcttaca-3') was carried out in a 30-cycle PCR using the proofreading polymerase Expand™ High Fidelity (Roche Diagnostics). PCR products were cloned into pCR2.1 (Invitrogen) and sequenced (MWG-Biotech).

Transfection

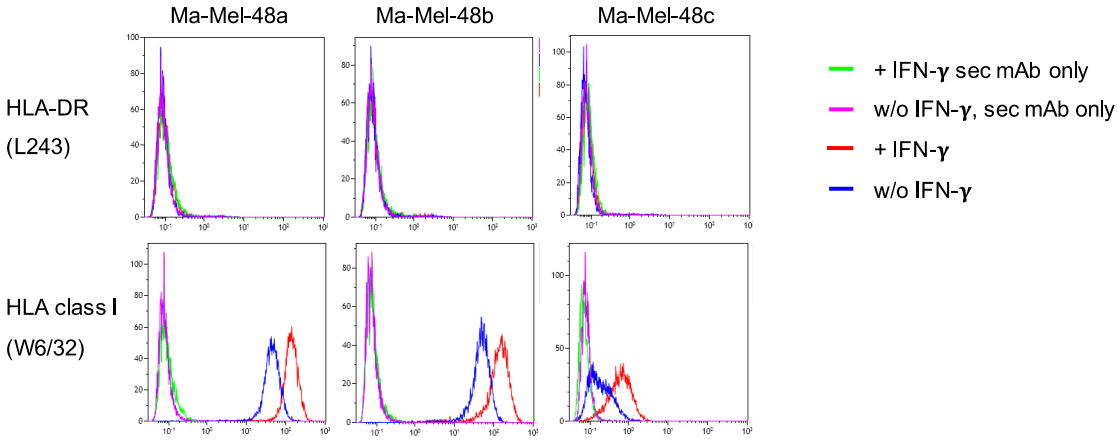
Lipofectamine (Life Technologies) was used for transient transfection of melanoma cells with a *B2M* expression plasmid. After 72 h, cells were analyzed for transient surface expression of HLA class I antigens by flow cytometry, as described above.

ELISPOT

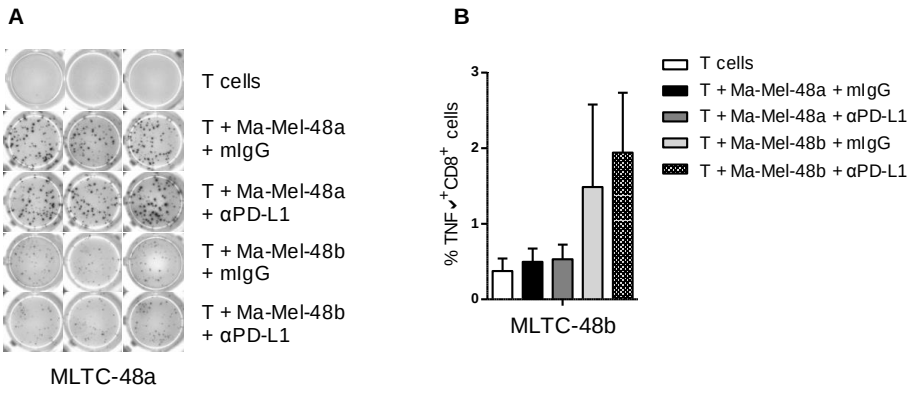
IFN-γ enzyme-linked immunospot (ELISPOT) assay was performed as follows: Multiscreen-HTS plates (Millipore) were coated with anti-hIFN-γ mAb 1-D1K (Mabtech). T cells were seeded into plates at $5x10^3$ cells/well in AIM-V complete medium and tumor cells ($1x10^4$ cells/well) were added. After incubation for 20 h, plates were washed and a biotinylated secondary anti-hIFN-γ antibody (clone 7-B6-1, Mabtech) was added. Captured cytokine was detected with ExtrAvidin alkaline phosphatase and BCIP®/NBT Liquid Substrate System (Sigma-Aldrich). Spots were imaged determined with the AID EliSpot reader (AID Diagnostika GmbH). All determinations were performed in triplicates. Where indicated, antibodies W6/32 (50 µg/ml) and anti-PD-L1 (10 µg/ml; Biolegend) were added to the co-culture in order to block the TCR/HLA class I and PD-1/PD-L1 interactions, respectively. Mouse IgG1 (R&D systems) was used as control in functional assays.



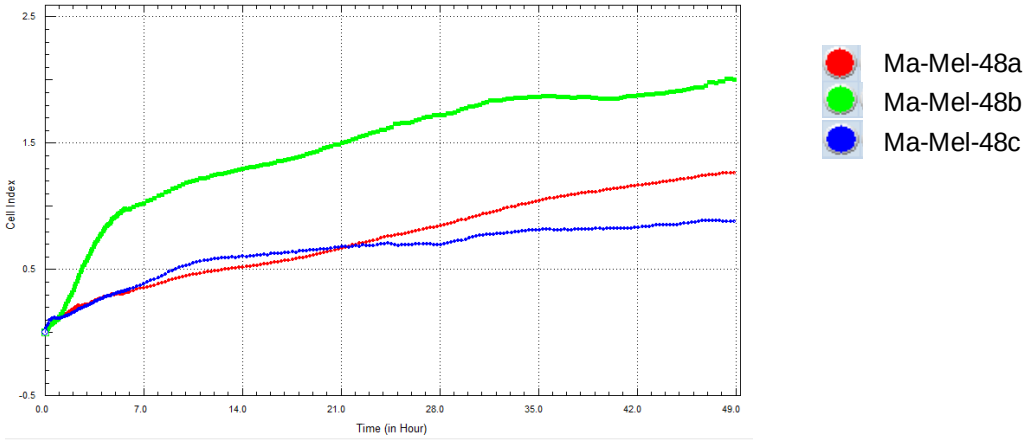
Supplementary Fig. S1: Analysis of CD3 (T cells) and HMB-45 (melanoma cells) expression in serial sections from cryopreserved metastasis Ma-Mel-48b. Red staining indicates positive cells.



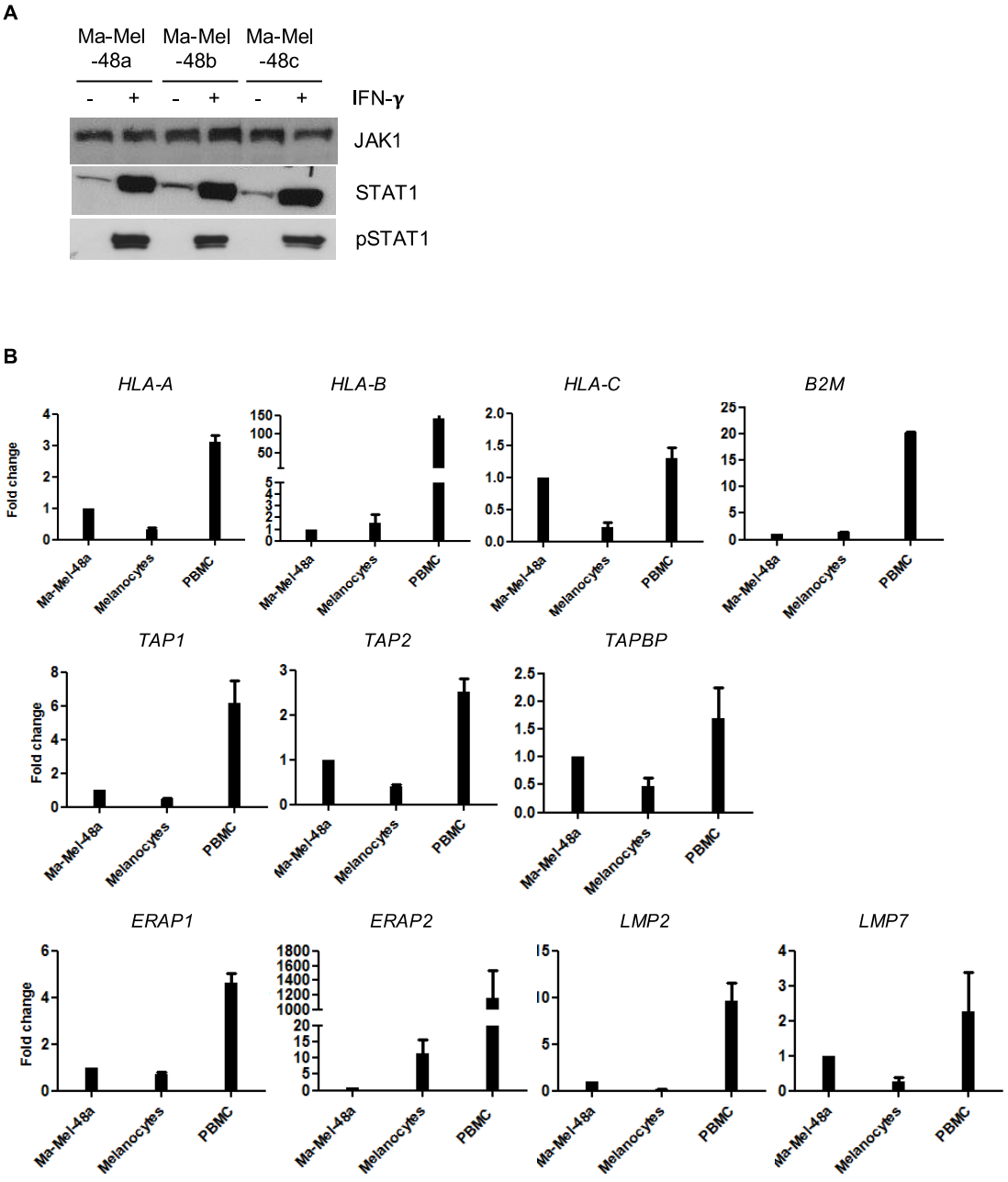
Supplementary Fig. S2: The different Ma-Mel-48 cell lines were treated with IFN-γ (500 U/ml) for 48 h, controls were left untreated. Cells were stained with mAb L243 and mAb W6/32 for detection of HLA-DR and HLA class I antigen expression, respectively. Representative histograms from one of three independent experiments are depicted.



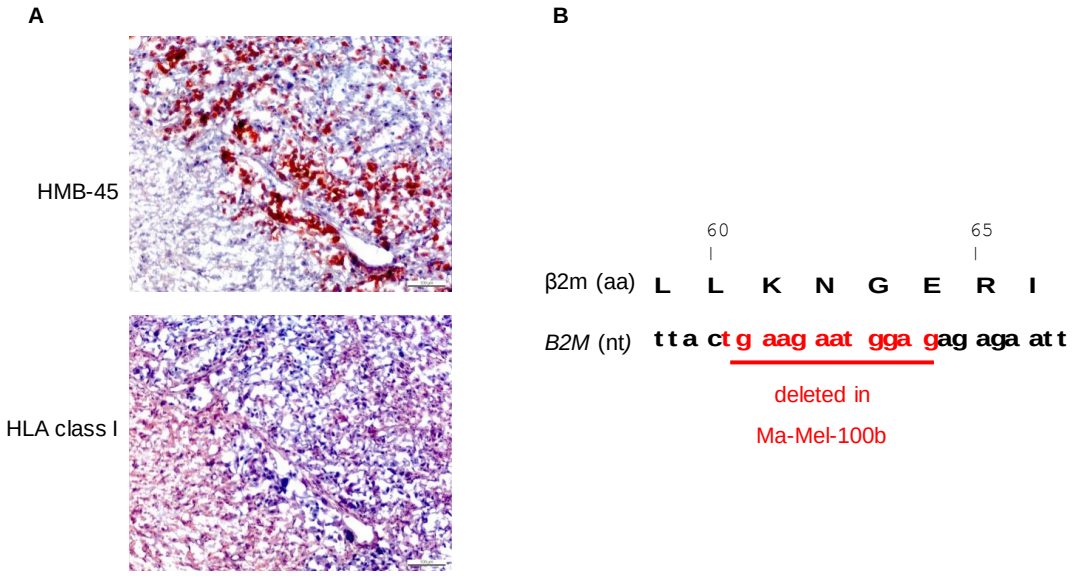
Supplementary Fig. S3: A, B, analysis of the T cell-stimulatory capacity of the different Ma-Mel-48 tumor cell lines. In autologous mixed lymphocyte-tumor cultures (MLTC) isolated $CD8^+$ T cells were stimulated twice with Ma-Mel-48a (MLTC-48a; A) or Ma-Mel-48b (MLTC-48b; B) cells and subsequently analysed for their response towards different target cells. A, T-cell activation was determined by IFN-! ELISPOT assay. One representative of 2 independent experiments is depicted. B, T-cell activation was measured by intracellular staining for TNF-α. The mean %TNF-α $CD8^+$ T cells within $CD8^+CD3^+$ T cells of three independent experiments is presented. A, B, where indicated αPD-L1 mAb was added to block inhibitory signaling to the T cells. Control cells were incubated with mouse IgG1 (mIgG).



Supplementary Fig. S4: Real-time proliferation of Ma-Mel-48 cell lines was determined in an xCelligence device. 1,5 x 10^4 cells were seeded per well and proliferation was determined over time. Representative data from one of three independent experiments are depicted.



Supplementary Fig. S5: A, The different Ma-Mel-48 cell lines were treated with IFN-γ 500 U/ml) for 48 h, controls were left untreated. Cell lysates were analyzed by Western blot for the protein levels of JAK1, STAT1 and pSTAT1. GAPDH served as loading control. One representative of three independent experiments is depicted. B, mRNA levels of the indicated APM components in Ma-Mel-48a cells, melanocytes from a normal donor and autologous CD8-depleted PBMC were quantified by qRT-PCR and normalized to endogenous beta-actin mRNA. Expression levels, given as means (+ SEM) of three independent experiments, are depicted relative to the expression in Ma-Mel-48a cells.



Supplementary Fig. S6: A, Analysis of HMB-45 (melanoma cells) and HLA class I antigen expression in serial sections from cryopreserved metastasis Ma-Mel-100b. Positive cells stain red. B, Localization of the 12 bp deletion in the *B2M* gene of Ma-Mel-100b cells. Capital letters (upper line) indicate the β2m amino acid sequence (aa), small letters (lower line) the nucleotide sequence of the corresponding *B2M* gene region (nt). Numbers indicate the aa position within the protein sequence.

7.2. Article II

Melanoma lesions independently acquire T-cell resistance during metastatic latency

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Running title: Lesion-specific immune escape of metastatic melanoma

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Precis

Individual melanoma lesions growing out after a latency period independently acquired genetically-defined resistance against autologous tumor-reactive $CD8^+$ T cells, limiting immunotherapeutic treatment options of recurrent disease and demanding for continuous genetic screening of evolving metastases.

Acknowledgments: Thanks to Dr. Klein-Hitpass for performing the SNP array analyses.

Abstract

Melanoma recurrence frequently occurs after a latency period of several years. *In vivo* studies demonstrated that tumor cells overcoming latency show a T cell-edited phenotype suggesting a relevant role for $CD8^+$ T cells in maintaining metastatic latency. Here, in a patient model of multiple recurrent lesions we illustrate the genetic evolution of poorly immunogenic melanoma phenotypes, evolving in the presence of autologous tumor antigen-specific $CD8^+$ T cells. Melanoma cells from two of three late recurrent metastases, developing within a 6-year latency period, lacked HLA class I expression. $CD8^+$ T cell-resistant, HLA class I-negative tumor cells became clinically apparent 1.5 and 6 years during stage IV disease. Genome profiling by SNP arrays revealed that HLA class I loss in both metastases originated from a shared chromosome 15q alteration and independently acquired focal *B2M* gene deletions. A HLA class I-haplotype deficient lesion developed in year 3 of stage IV disease that acquired resistance towards dominant $CD8^+$ T-cell clonotypes targeting stage III tumor cells. These early disease melanoma cells showed a dedifferentiated c-Jun^{high}/$MITF^{low}$ phenotype, recently suggested to be associated with immunosuppression, contrasting the c-Jun^{low}/$MITF^{high}$ phenotype of T cell-edited tumor cells from late metastases. In summary, our study demonstrates that tumor recurrences after long-term latency develop towards T-cell resistance by independent genetic events suggesting a mechanism of T cell-driven genetic evolution of melanoma as a means to evade immune recognition and tumor immunotherapy.

Introduction

$CD8^+$ T lymphocytes can efficiently kill autologous melanoma cells as indicated by the remarkable clinical responses to adoptive T-cell transfer and immune checkpoint-blocking antibody therapy (1, 2, 3, 4). Such T lymphocytes target peptide epitopes derived from different types of tumor antigens presented in the context of HLA class I surface molecules (5). Their capacity to mediate tumor rejection has also been demonstrated in different murine tumor models. However, in some mice selective T-cell pressure led to the outgrowth of poorly immunogenic antigen-loss tumor variants (6-8). This suggests tumor antigen-specific T cells are potent drivers of the so-called cancer immunoediting process, comprising an elimination, equilibrium and escape phase (9). Though different immune effectors and regulators are involved in this multi-mechanistic process, $CD8^+$ T cells seem to play a major role in all phases.

The capacity of $CD8^+$ T cells to kill tumor cells is well established. Additionally, evidence supports a role in the maintenance of tumor latency: In a genetically engineered murine model for spontaneous melanoma, depletion of $CD8^+$ T cells accelerated the outgrowth of visceral metastases from early spread tumor cells, indicating T cells delayed the metastatic progression of disseminated tumor cells (10). Similar observations were made in a carcinogen-induced tumor model where transformed cells established stable masses at the carcinogen injection site characterized by equilibrium of apoptosis and proliferation. Outgrowing tumors demonstrated an immunoedited, less immunogenic phenotype. Furthermore, depletion of $CD8^+$ T cells accelerated tumor formation (11). These and other studies demonstrate a role for $CD8^+$ T cells in maintaining tumor latency.

To date, reports examining the immunogenicity of recurrent patient metastases to gain insight into the immunoediting activity of $CD8^+$ T cells are rare. In 1997, Coulie and colleagues analyzed the T-cell responses towards tumor cell lines established from two stage IV melanoma metastases, obtained in 1988 and in 1993, after a 4-year disease free interval. Tumor cells from the second metastasis had acquired a poorly immunogenic phenotype expressing only one out of six HLA class I alleles (12). Similar observations were described by Yamshchikov et al. for cell lines established from two consecutive metastases excised approximately 5 and 11 years after primary melanoma diagnosis. Tumor cells from the first metastasis were poorly immunogenic due to HLA haplotype loss and those from the second lesion showed a mixed HLA class I-low/-negative phenotype (13). Both studies characterized phenotypic tumor cell alterations resulting in impaired T-cell recognition.

Although immunotherapies have evolved as the potentially most promising therapeutic approaches for a wide range of advanced malignant human neoplasia, tumor recurrences as well as non-responders still significantly impede therapeutic success. The immunoediting activity of $CD8^+$ T cells may well contribute to disease recurrence in responders to immune checkpoint-blocking antibody therapies, which mostly occurs after a prolonged period of therapy-induced regression (3). A detailed understanding of the mechanisms governing the immunogenicity of recurrent melanoma will be critical to optimize immunotherapy approaches both in terms of decreasing the rate of tumor recurrence and if this occurs, of choosing the best rescue therapy.

Here, we follow the genetic evolution of melanoma immunogenicity in a long-term survivor who developed multiple recurrent metastases over a period of six years in stage IV disease. We demonstrate distinct poorly immunogenic melanoma phenotypes evolving in a single patient after long-term latency and identify varying genetic mechanisms responsible for T-cell resistance in individual tumor metastases. Overall, our data support T cell-based immunoediting of disseminated tumor cells in melanoma patients.

Materials and Methods

Patient material. Tumor tissues and peripheral blood samples from melanoma patient Ma-Mel-86 were collected after approval by the institutional review board and patient informed written consent. Blood cells, collected in April 2004, were separated on a Ficoll gradient and peripheral blood mononuclear cells (PBMC) were cryopreserved. Tissues were mechanically dissected for generation of cell lines or cryopreservation. Melanoma cell lines were cultured in RPMI1640 medium supplemented with glutamine, 10% FCS and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere.

Targeted amplicon sequencing. To define known recurrent mutations in cutaneous melanoma a custom amplicon-based sequencing panel covering 29 genes (Supplementary Tab. S1) was designed, prepared, run on an Illumina MiSeq sequencer and analyzed by CLC Cancer Research Workbench from QIAGEN® as previously described (14).

SNP array analysis. SNP genotyping was performed with non-fixed PBMC and melanoma cell lines using CytoScan® HD arrays and analyzed with Chromosome Analysis Suite® (Affymetrix) as previously described (15). Genotypes with a p-value for confidence <0.005 were chosen using R (R Development Core Team; http://www.R-project.org), translated into single base letters and IUPAC ambiguity codes, then transposed to create an alignment. Of 8,4000 variable sites 1,456 were parsimony informative. We then used the maximum parsimony criterion, minimizing the total number of evolutionary steps required to explain the relationship of the tested samples. Using MEGA (16) we calculated a maximum parsimony tree with 500 bootstrap replicates, complete deletion of missing data and subtree-pruning-regrafting search method. A maximum likelihood tree was inferred using 100 bootstrap replicates, the kimura-2-parameter model and partial deletion of missing data. SNP array data are available via NCBI GEO (Accession number: GSE80736).

Transcriptome analyses. Total RNA was extracted from melanoma cell lines using the Qiagen RNeasy mini Kit according to the manufacturer's protocol (Qiagen). All preparations and analyses were done in triplicates. Sequencing (RNA-Seq-) libraries were generated using the TruSeq™ RNA sample preparation kit (Illumina) by GENterprise Genomics (Mainz, Germany). RNA-Seq libraries were subjected to high-throughput sequencing on the Illumina HiSeq2000 platform of the local NGS Core

facility (Biology Department, University of Mainz, Germany). On average, 69.4x10^6 100 bp paired-end reads were generated. Sequence reads were processed (quality filtering, adapter trimming) and mapped to the annotated human genome hg19 with CLC Genomics Workbench 8.5.1 (Qiagen). For analysis of differentially expressed genes and heatmap generation, the processed replicate reads of each sample were mapped together. Gene expression values were determined as RPKM (reads per kilobase of transcript per million mapped reads) normalized read counts. Transcriptome data are available via NCBI SRA as indicated in supplementary Table S2.

Mixed lymphocyte/tumor cell culture (MLTC) and T cell cloning. MLTC of selected autologous $CD8^+$ T cells and irradiated melanoma cells were set up as described previously (15). By limiting dilution in round-bottomed 96-well plates $CD8^+$ T cells from MLTC were cloned and stimulated with irradiated autologous tumor cells (3x10^3 cells/well) and allogeneic EBV-transformed B lymphoblastoid cells (5x10^4 cells/well) as feeders in AIMV medium supplemented with 250 IU/mL IL2 and 10% human AB serum. Restimulations were carried out in weekly intervals.

High-throughput T-cell receptor repertoire sequencing. DNA was extracted from autologous MLTC using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Total DNA extracts of MLTC-86a and MLTC-86c were sent to Adaptive Biotechnologies (Seattle, USA) for sequencing the T-cell receptor β chain (TCRβ) repertoire at "survey" level using their immunoSEQ assay. At Adaptive Biotechnologies, 400 ng of template DNA were used to perform multiplex PCR enrichment of the somatically rearranged TCRβ region followed by high-throughput sequencing on an Illumina MiSeq instrument (17). The generated TCRβ sequence data were analyzed using the immunoSEQ Analyzer. Analyses included amplification bias correction (18) as well as sequencing error correction (17). Following filtering for unique sequences and discarding sequences with mutations leading to frame shifts or stop codons, the total number of unique and productive TCRβ clonotypes was used for further analyses.

Results

Immunophenotype heterogeneity of consecutive melanoma metastases

Patient model Ma-Mel-86 was selected to dissect the genetic evolution of melanoma immunogenicity in the course of disease progression. This patient presented with primary melanoma in November 2001 but progression to stage III and stage IV was diagnosed already in January and September 2002, respectively. From then on the patient developed multiple lymph node lesions and visceral metastases in brain, breast and intestine (Fig. 1A). Accessible lymph node lesions and distant organ metastases were excised, while brain metastases regressed in response to irradiation. The patient received different types of immunotherapy including IFNα, peptide-based vaccination (Supplementary Tab. S3) and tumor lysate-loaded dendritic cell vaccines, that together with surgical interventions led to a 3-year clinically disease-free interval from 2005-2008. In December 2008 the patient developed recurrent lesions and died from metastatic disease in April 2009.

Tumor samples from patient Ma-Mel-86 were obtained at different time points in the course of disease including an early stage III lymph node metastasis (Ma-Mel-86a) excised 2 months after diagnosis and three late recurrent lymph node lesions excised in years 1.5 (Ma-Mel-86b), 3 (Ma-Mel-86c) and 6 (Ma-Mel-86f) of stage IV disease (Fig. 1A). From these metastases cell lines were established that, at first, were studied for the surface expression of HLA class I molecules as a precondition to T-cell recognition. As shown in Fig. 1B, tumor cells from early metastasis Ma-Mel-86a and the late recurrent lesion Ma-Mel-86c expressed HLA class I molecules, whereas those from lesions Ma-Mel-86b and Ma-Mel-86f were HLA class I-negative.

In line with the phenotype of the cell lines, staining of corresponding tissue sections with antibody W6/32, binding a structural epitope formed by complexes of the β2m light chain and the HLA class I heavy chains (HLA-A, -B, -C) revealed HLA class I expression by tumor cells from metastases Ma-Mel-86a (19) and Ma-Mel-86c (Fig. 1C), while lesions Ma-Mel-86b (19) and Ma-Mel-86f (Fig. 1C) contained HLA class I-negative melanoma cells. By staining for CD3, we found T cells to be present in both metastases, Ma-Mel-86c and Ma-Mel-86f (Fig. 1C), but screening of different tissue slices from metastasis Ma-Mel-86f suggested location of $CD3^+$ T cells primarily in the tumor periphery (Supplementary Fig. S1).

Divergent genetic evolution of early and late metastatic melanoma cells

To demonstrate their common origin, we screened genomic DNA from the distinct melanoma cell lines by targeted sequencing for known recurrent mutations in a total of 29 genes (Supplementary Tab. S1). As listed in Fig. 2A, all cell lines showed the same nucleotide alterations in *BRAF*, *CDK4, MAP2K1, PTEN* and *p53*. To further define their genetic relationship a genome-wide SNP array analysis on genomic DNA from the tumor cell lines and autologous PBMC as a constitutive normal control was performed. The phylogenetic tree based on maximum parsimony showed that tumor cells from the late recurrent lesions Ma-Mel-86b, Ma-Mel-86c and Ma-Mel-86f were closely related to each other. This grouping was present in 99% of the 500 bootstrap replicates of the analysis (Fig. 2B). Phylogenetic inference using maximum likelihood reached the same branching order and similar bootstrap support, indicating that the late tumor recurrences had separated from the Ma-Mel-86a lineage early on.

The divergent evolution was detectable also at the level of cellular differentiation. Only late tumor recurrences expressed the lineage-specific transcriptional activator MITF and its target gene Melan-A/MART-1. In contrast, expression of receptor tyrosine kinase AXL and transcription factor c-Jun was restricted to Ma-Mel-86a cells (Fig. 2C). Recently it was demonstrated that $AXL^{high}/MITF^{low}$ melanoma cells are characterized by elevated NF-κB activity (20), similar to $c\text{-}Jun^{high}/MITF^{low}$ cells inducing expression of pro-inflammatory genes (21). Consistently, analyses of transcriptome data revealed expression of *IL1B, IL6* and *CCL2* predominantly in Ma-Mel-86a cells (Fig. 2D) and by ELISA release of IL-6 was confirmed (Fig. 2E). Thus, despite the origin from a common ancestor early and late metastases formed genetically and phenotypically distinct groups.

Independent acquisition of *B2M* gene deletions by metastases Ma-Mel-86b and Ma-Mel-86f

Despite their close genetic relationship (Fig. 2B), the late recurrent melanoma cells displayed profound differences in their HLA class I phenotype. Ma-Mel-86b and Ma-Mel-86f cells, in contrast to Ma-Mel-86c, lost HLA class I surface expression due to a lack in β2m protein and corresponding mRNA expression (Fig. 3A, B). HLA class I surface expression on both cell lines could not be restored in response to type I or type II interferon treatment (data not shown) but was achieved upon transfection of Ma-Mel-86b and Ma-Mel-86f cells with a *B2M* expression plasmid (Supplementary Fig. S2). Previously, we demonstrated that loss of β2m expression in melanoma is associated with aberrations in chromosome 15q to which the *B2M* gene maps at 15q21.1 (15, 22,

23). Indeed, by SNP array the same large deletion on chromosome 15q encompassing the region 15q11.2 to 15q22.31 was detected in Ma-Mel-86b and Ma-Mel-86f cells, pointing to a shared chromosomal aberration associated with loss of one *B2M* gene (Fig. 3C, D). In addition, each of the cell lines displayed a specific small deletion, affecting the second *B2M* gene. In Ma-Mel-86b cells the *B2M* gene and flanking sequences were lost while in Ma-Mel-86f cells the deletion affected only the *B2M* gene (Fig. 3E). This suggested that β2m deficiency in Ma-Mel-86b and Ma-Mel-86f cells was due to a shared chromosome 15q aberration, acquired by a common precursor, and a subsequent cell line-specific *B2M* gene deletion. Thus, the late recurrent Ma-Mel-86b and Ma-Mel-86f cells independently acquired their HLA class I-negative phenotype gaining complete resistance against tumor-antigen specific $CD8^+$ T cells.

HLA class I-negative tumor cells could still be targeted by HLA class II-restricted $CD4^+$ T cells or innate $CD56^+$ NK/NKT cells. However, both HLA class I-negative cell lines lacked constitutive HLA class II expression except for a small subpopulation in Ma-Mel-86b and $CD4^+$ T cells could not be detected in metastasis Ma-Mel-86f (Supplementary Fig. S3). Furthermore, $CD56^+$ NK/NKT cells, if present, could not be distinguished from CD56-expressing melanoma cells. Generally, we detect very few or no $CD56^+$ lymphocytes in HLA class I-negative and -positive melanoma metastases (Supplementary Fig. S3; data not shown).

Stimulation of distinct T-cell repertoires by Ma-Mel-86a and Ma-Mel-86c cells

Of the four metastases, we detected HLA class I antigen expression only on Ma-Mel-86a and Ma-Mel-86c cells (Fig. 1C). The latency period of more than 3 years between the outgrowth of both metastases as well as their divergent genetic evolution led us to ask to which extent differences in their T cell-stimulatory capacity could be observed. To this end, autologous MLTC were set up, in which peripheral blood $CD8^+$ T cells of the patient, obtained in 2004, were stimulated twice with irradiated Ma-Mel-86a or Ma-Mel-86c cells for enrichment of tumor-reactive T cells. By screening for T-cell responses to the different tumor cell lines we observed that around 14% of $CD8^+$ T cells from MLTC with Ma-Mel-86a cells (MLTC-86a) released IFNγ in the presence of Ma-Mel-86a cells, but only 1.5% of these T cells reacted towards Ma-Mel-86c cells (Fig. 4A). On the other hand, approximately 13% of the $CD8^+$ T cells from MLTC-86c responded towards Ma-Mel-86c melanoma cells, but none of these T cells reacted towards Ma-Mel-86a cells (Fig. 4B). The same pattern of T-cell reactivity was observed in 3 independent MLTC experiments (Fig. 4C), indicating that each of the melanoma

cell lines stimulated the outgrowth of a specific T-cell repertoire with very limited to no cross-reactivity towards autologous tumor cells from distinct metastases.

Thus we studied the TCR repertoire usage of MLTC-86a and MLTC-86c. High-throughput TCRβ sequencing revealed 850,400 and 1,055,825 sequencing reads, representing an estimated number of 64,853 and 78,245 total haploid nuclear genome copies. After discarding 11.8% and 6.3% of the sequences due to errors resulting in premature stop codons and open reading frame shifts, as well as filtering for unique sequence haplotypes, a total number of 515 and 336 unique and productive TCRβ clonotypes were detected in MLTC-86a and MLTC-86c, respectively (data not shown). Both repertoires showed a high clonality score of 0.46 and 0.59. In MLTC-86a one clone was present at a frequency of 28.1%; this clone, however, was not detectable in MLTC-86c. In MLTC-86c two different clonotypes expanded to ~20% each, again neither of the clonotypes was present in MLTC-86a (Fig. 4D). In addition to those high frequency specific TCRβ clonotypes, a number of shared TCRβ clonotypes expanded to very low frequencies in both MLTC's (Fig. 4E), indicating that some subdominant T-cell epitopes remain presented on both Ma-Mel-86a or Ma-Mel-86c cells.

Amplification of differentiation antigen-specific CD8$^+$ T cells by Ma-Mel-86c cells

The amplification of cell line-specific T-cell repertoires led us to ask for the underlying mechanisms. We first studied melanoma cells for the surface expression of inhibitory PD-L1 dampening the proliferation and effector function of PD1$^+$ T cells (24). Comparable PD-L1 expression levels were detected on Ma-Mel-86a and Ma-Mel-86c cells, despite Ma-Mel-86a cells having elevated levels of the PD-L1 transcriptional activators c-Jun and pSTAT3 (25, 26) (Fig. 2C, supplementary Fig. S4). This suggests that differences in the strength of inhibitory signaling via the PD-L1/PD1 axis does not account for the amplification of different T-cell repertoires (27).

CD8$^+$ T-cell responses in melanoma patients are frequently directed towards melanoma differentiation antigens (MDA), including gp100, Melan-A/MART-1, TRP1, TRP2 and Tyrosinase. We detected MDA protein expression in Ma-Mel-86c but not Ma-Mel-86a cells (Fig. 5A), consistent with the results obtained by transcriptome analyses (Fig. 2D). This led us to assume that the pool of Ma-Mel-86c-reactive T cells contained MDA-specific CD8$^+$ T cells not responding to Ma-Mel-86a cells due to its stable dedifferentiation. To analyze this, we transiently transfected Ma-Mel-86a cells with MDA-encoding expression plasmids and used the transfectants as stimulators for bulk T cells from MLTC-86c. As shown in Fig. 5B, Ma-Mel-86a cells transiently

expressing Tyrosinase were recognized by a large proportion of the T cells from MLTC-86c in contrast to Ma-Mel-86a control cells. The same result was obtained also with a Tyrosinase-specific $CD8^+$ T cell clone established from MLTC-86c (Fig. 5C). Thus, the T-cell repertoire amplified by Ma-Mel-86c cells was dominated by MDA-specific $CD8^+$ T cells, not responding to dedifferentiated Ma-Mel-86a cells.

Tyrosinase-specific $CD8^+$ T cells had the capability of killing Ma-Mel-86c cells (Supplementary Fig. S5), but low *in vivo* antigen expression could have limited their anti-tumor activity (Fig. 5D). Interestingly, we observed a strong decrease of Tyrosinase expression in IFNγ-treated Ma-Mel-86c cells, abrogating antigen-specific T-cell recognition (Fig. 5E, F). This mechanism could also have applied to metastasis Ma-Mel-86c, as PD-L1-positive tumor cells were detected primarily in the vicinity of $CD8^+$ T cells, indicating IFNγ-mediated PD-L1 upregulation by activated T cells, as previously described (28) (Fig. 5G).

HLA haplotype loss in Ma-Mel-86c cells leads to resistance against $CD8^+$ T cells attacking early disease stage tumor cells

$CD8^+$ T cells from MLTC-86a killed autologous Ma-Mel-86a cells but ignored melanoma cells from metastasis Ma-Mel-86c (Fig. 4A and 4C, supplementary Fig. S6). Screening Ma-Mel-86c cells for mechanisms protecting from recognition by the MLTC-86a-specific T-cell repertoire, we asked for differences in the intensity and pattern of HLA class I expression. As shown in Fig. 6A, HLA class I expression levels on Ma-Mel-86c cells were lower compared to Ma-Mel-86a cells. Screening SNP array data for molecular alterations in the HLA class I region on chromosome 6p22.1-6p21.3, we detected a large deletion on one chromosome 6 in Ma-Mel-86c cells ranging from 6p25.3 to 6p21.1, expected to be associated with an HLA haplotype loss (Fig. 6B). Fittingly, HLA genotyping on DNA from both cell lines and autologous PBMC revealed loss of the HLA-A*24:02, HLA-B*15:01, HLA-Cw*03:03 haplotype in Ma-Mel-86c cells, whereas all *HLA* alleles were present in Ma-Mel-86a cells (Fig. 6C). Based on this result, we concluded that the haplotype loss protected Ma-Mel-86c cells from recognition by $CD8^+$ T cells of MLTC-86a. Indeed, T cells from MLTC-86a became activated in the presence of Ma-Mel-86c cells transiently re-expressing the HLA-A*24:02 and HLA-B*15:01 alleles (Fig. 6D). Notably, none of the tested MLTC T cells recognized peptides present in the vaccine applied in 2002 (Supplementary Tab. S3; data not shown).

Overall we demonstrated that lesions growing out after long-term latency in patient Ma-Mel-86 were protected from effector functions of tumor antigen-specific T cells by HLA haplotype loss or total HLA class I loss, the latter independently acquired by two different melanoma metastases.

Discussion

The mechanisms keeping tumor cells in latency are poorly understood but are most likely diverse, including a limited blood supply or an equilibrium of apoptosis and proliferation as observed in different mouse tumor models (29). $CD8^+$ T cells seem to contribute to equilibrium maintenance by directly killing tumor cells and releasing anti-proliferative cytokines (10, 11, 30). Thus, in order to switch from latency to proliferation, tumor cells either need to blunt T-cell activity by establishing an immunosuppressive microenvironment or have to acquire genetic/epigenetic alterations allowing escape from direct T-cell recognition (9).

Our study on melanoma recurrences suggests that tumor antigen-specific $CD8^+$ T cells modify tumor-cell immunogenicity by selective enrichment of genetically altered poorly immunogenic variants. We found melanoma cells forming overt metastases in patient Ma-Mel-86 after a latency period of approximately 1.5, 3 and 6 years in stage IV disease to be T cell-resistant albeit at varying degrees. Of the three late recurrent lesions Ma-Mel-86b and Ma-Mel-86f, excised in years 1.5 and 6, respectively, were completely T cell-resistant due to the loss of β2m expression. Based on genome profiling by SNP arrays we determined the chronology of genetic alterations leading to β2m deficiency as follows: a common ancestor of Ma-Mel-86b and Ma-Mel-86f cells acquired a large deletion on one chromosome 15q encompassing the *B2M* gene. From this ancestor the two cell lines diverged and independently acquired additional small deletions affecting the second *B2M* allele. Recently we detected the similar chronologies of genetic alterations in two additional patient models (15). Overall, these studies identify chromosome 15 alterations as early genetic events predisposing to total HLA class I loss in melanoma.

A remarkable fraction of melanoma cells shows constitutive HLA class II expression (31), sensitizing to $CD4^+$ T cells that can have direct perforin/granzyme-dependent cytotoxic activity against MHC class II-expressing tumor cells (32, 33). In addition, $CD4^+$ T cells have been demonstrated to halt tumor growth or eradicate established tumors by release of IFNγ and TNFα, acting on tumor and stroma cells (34, 35). HLA class I-negative Ma-Mel-86 cells lacked constitutive but still showed IFNγ-inducible HLA class II expression (19). NK cells targeting HLA class I-negative tumor cells could be a source of IFNγ, however rarely infiltrate melanoma lesions. In this regard, strategies mobilizing NK cells into the tumor, such as inhibitor treatment of BRAFV600E mutant melanoma (36, 37), local irradiation (38) or localized virotherapy (39) are of potential interest. Such treatments may also attract $CD4^+$ and $CD8^+$ T cells

that even in the absence of HLA class I/II expression on melanoma cells could eradicate tumors by recognition of cross-presented tumor antigen on stroma cells (40-42).

In contrast to metastases Ma-Mel-86b and Ma-Mel-86f, tumor cells derived from lesion Ma-Mel-86c, excised in year 3 of stage IV disease, still presented HLA class I antigens. Accordingly, Ma-Mel-86c cells were capable of stimulating autologous $CD8^+$ T cells, similar to HLA class I-positive Ma-Mel-86a cells obtained from an early stage III lesion. However, in short-term co-cultures with autologous $CD8^+$ T cells, each of the HLA class I-positive melanoma cell lines amplified a specific T-cell repertoire with distinct dominant T-cell clones. T lymphocytes stimulated twice with Ma-Mel-86c cells were dominated by MDA-specific T cells that did not cross-react to Ma-Mel-86a cells, which completely lack MDA expression. In contrast, T cells stimulated twice with Ma-Mel-86a cells were dominated by a T-cell repertoire recognizing their cognate antigens in the context of the HLA-A*24:02, HLA-B*15:01, HLA-C*03:03 haplotype. Due to an aberration in chromosome 6p, this haplotype was lost in Ma-Mel-86c cells protecting them from Ma-Mel-86a-reactive T cells. Despite the haplotype loss, Ma-Mel-86c cells were still recognized by autologous Tyrosinase-specific $CD8^+$ T cells. Low Tyrosinase expression in metastasis Ma-Mel-86c, potentially induced by T cell-derived IFNγ, might have limited T-cell efficacy *in vivo*. Furthermore, expression of this self-antigen in the thymus generates a tolerized specific T-cell repertoire that might not be capable of preventing metastasis progression (43). In contrast, T cells recognizing mutant neoantigens can effectively mediate tumor regression as demonstrated in different mouse tumor models, but can also select for an enrichment of immune escape variants (6-8, 44). The potential contribution of neoantigens to immunoediting in the Ma-Mel-86 melanoma model remains to be determined.

A broader relevance of chromosome 6p alterations in melanoma immune escape is indicated by different studies detecting HLA haplotype loss in late recurrent melanoma cells from a long-term survivor and in metastases from patients after immunotherapy (13, 45, 46). Recently, Rooney et al. found mutations in genes involved in antigen presentation (*B2M*, *HLA-A, HLA-B, HLA-C*) to be enriched in tumors with immune-cytolytic activity (47). Based on all these data we assume that continuous $CD8^+$ T-cell activity kept the precursors of Ma-Mel-86b, Ma-Mel-86c and Ma-Mel-86f cells in latency and that only upon acquisition of HLA alterations, overt metastases formation occurred, though an additional contribution of immune-suppressive mechanisms is still possible.

The establishment of an immune-suppressive microenvironment might have supported the outgrowth of the stage III lesion Ma-Mel-86a. Our data demonstrated that these early metastatic cells differed from late recurrent tumor cells in terms of genetic alterations as defined by SNP array and their $MITF^{low}cJUN^{high}$ phenotype. This phenotype has been associated with elevated c-Jun/NF-κB activity, which induces expression of pro-inflammatory genes recruiting myeloid cells and shares similarity to the $MITF^{low}AXL^{high}$ melanoma phenotype showing high invasive capacity (20, 21, 48). We could not study the immune cell composition of metastasis Ma-Mel-86a due to the lack of corresponding tissue, but detected expression of *IL1B* and *IL6* cytokines and chemokine *CCL2* an attractant of macrophages and myeloid derived suppressor cells generating an immune-suppressive microenvironment (49). Recently, the $MITF^{low}AXL^{high}$ phenotype was also reported to be associated with resistance to anti-PD1 therapy (3, 4, 50). Approximately 30% of patients objectively respond to therapy with anti-PD1 antibodies including a remarkable number of long-term responders. However, some patients show disease recurrence after a long period of therapy-induced regression (3). Though the underlying mechanisms are so far unknown, it is expected that some tumor cells might have acquired specific genetic alterations that directly interfere with T-cell recognition.

In summary, the comparative analysis of tumor cells derived from an early metastasis and multiple later recurrences has allowed us to identify genetic alterations associated with evolving T-cell resistance during metastatic latency. Our data suggest that under the selective pressure of tumor antigen-specific T cells individual immunoevasive subclones can emerge, generating a tumor heterogeneity counteracting complete tumor eradication by immunotherapy. New technologies directed towards identifying mechanisms of genetic and functional T-cell resistance in longitudinally collected tumor biopsies could potentially enable early appropriate adaptation of treatment regimens resulting in higher numbers of long-term therapy responders.

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Figures

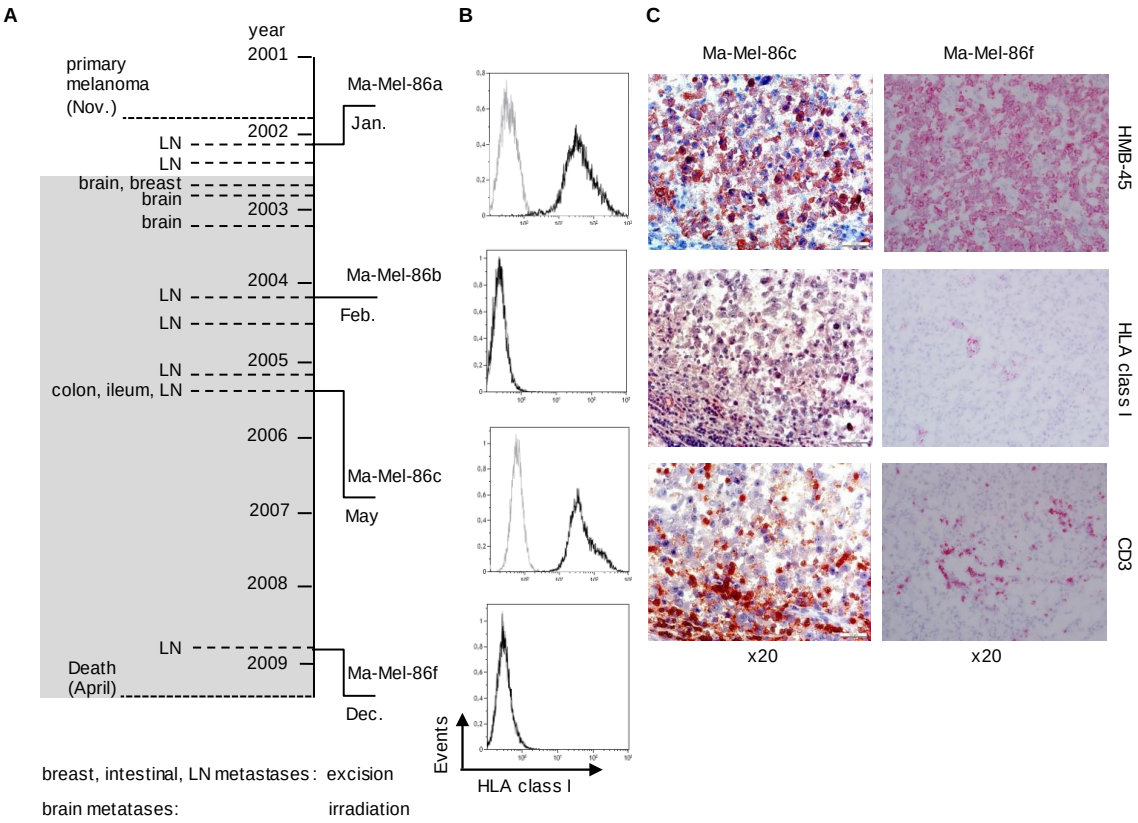


Figure 1.

Recurrent melanoma metastases of patient Ma-Mel-86 show heterogeneous HLA class I expression. A, clinical history of melanoma patient Ma-Mel-86. Lymph node (LN) metastases Ma-Mel-86a, Ma-Mel-86b, Ma-Mel-86c, Ma-Mel-86f were excised and cell lines established from corresponding tissue samples. Grey area indicates disease stage IV. B, expression of HLA class I antigen complexes on melanoma cell lines was analysed by flow cytometry. Representative histograms from one of three independent experiments. C, expression of melanoma marker HMB-45, HLA class I antigens and T-cell marker CD3 in serial cryostat tissue sections of melanoma metastases Ma-Mel-86c and Ma-Mel-86f, determined by immunohistochemistry. Red colour indicates marker-positive cells.

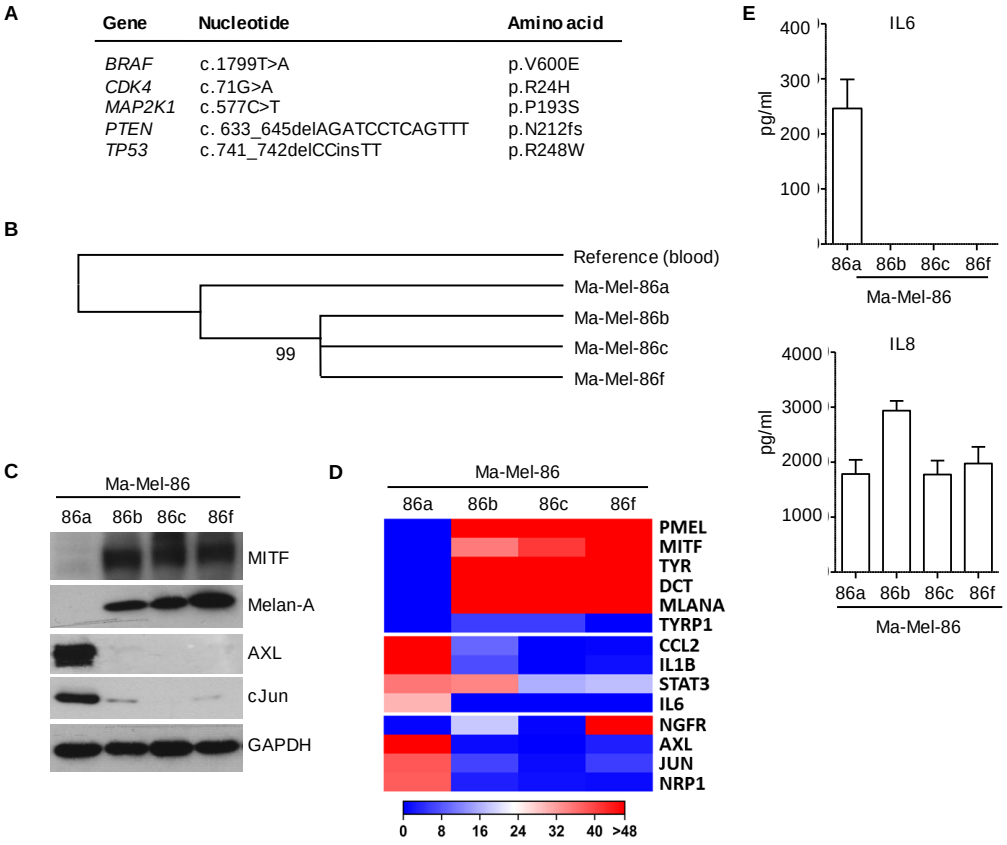


Figure 2.

Late recurrent metastases cluster in a phylogenetic subgroup distinct from the early lesion. A, mutations in melanoma cell lines defined by amplicon sequencing, known recurrent mutations among 29 genes tested. B, maximum parsimony tree showing the phylogenetic relationship of the melanoma cell lines and the autologous blood sample (Reference) used as outgroup. A melanoma lineage genetically divergent from the blood sample evolved in patient Ma-Mel-86, leading to the studied cell lines. The lineage giving rise to Ma-Mel-86a cells diverged from the melanoma ancestor and accumulated specific genotypic differences. Later the lineages of Ma-Mel-86b, Ma-Mel-86c and Ma-Mel-86f cells diverged. 99 percent of the bootstrap replicates showed this grouping. C, expression of indicated proteins in the different melanoma cell lines determined by Western blot. GAPDH served as loading control. Representative data from one of three independent experiments. D, expression profile of candidate genes compared for all cell lines and plotted as heatmap. Values represent RPKM (reads per kilobase of transcript per million mapped reads) normalized read counts. E, release of IL6 and IL8 by melanoma cell lines, measured by ELISA. Data represent means (+ SEM) of at least two independent experiments.

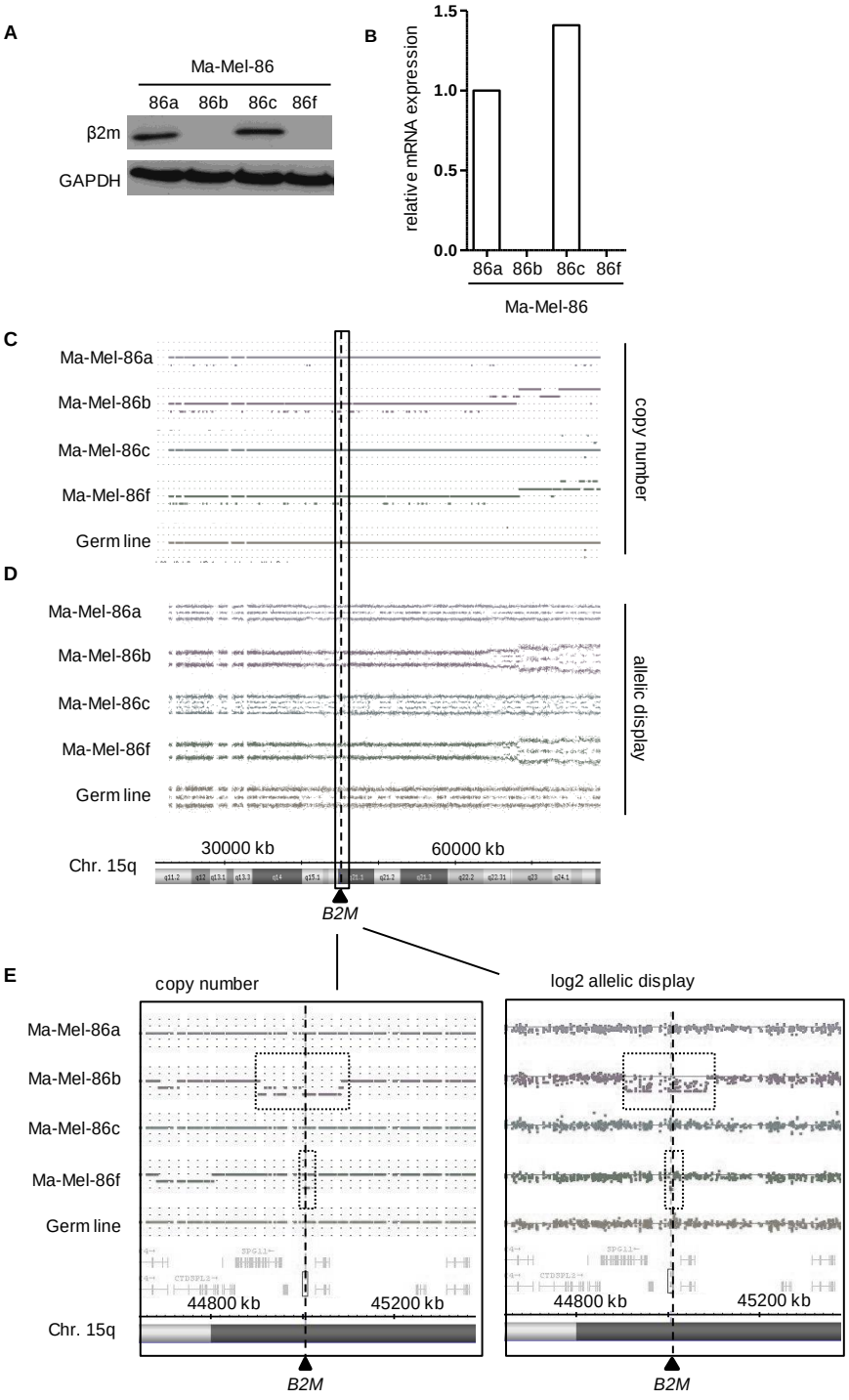

A
Ma-Mel-86
86a 86b 86c 86f
β2m
GAPDH
B
relative mRNA expression
1.5
1.0
0.5
0.0
86a 86b 86c 86f
Ma-Mel-86
C
Ma-Mel-86a
Ma-Mel-86b
Ma-Mel-86c
Ma-Mel-86f
Germ line
copy number
D
Ma-Mel-86a
Ma-Mel-86b
Ma-Mel-86c
Ma-Mel-86f
Germ line
allelic display
Chr. 15q
30000 kb
60000 kb
B2M
E
copy number
log2 allelic display
Ma-Mel-86a
Ma-Mel-86b
Ma-Mel-86c
Ma-Mel-86f
Germ line
Chr. 15q
44800 kb
45200 kb
B2M

Figure 3.

Ma-Mel-86b and Ma-Mel-86f cells independently acquired their HLA class I-negative phenotype. A, expression of β2m in melanoma cells was determined by Western blot. GAPDH was used as loading control. Representative data from one of three independent experiments depicted. B, mRNA levels of *B2M* in the different cell lines were quantified by qRT-PCR and normalized to endogenous *GAPDH* mRNA. Expression levels from one of two independent experiments are depicted relative to the expression in Ma-Mel-86a cells. C, D, SNP given as copy number (C) and allelic distribution (D) of chromosome 15q are shown for DNA obtained from the melanoma cell lines and autologous PBMC (germ line). Ma-Mel-86b and Ma-Mel-86f cells show a large deletion (42,982,458 bp) on chromosome 15q encompassing the region 15q11.2-15q22.31 (Chr.15: 22,752,398-65,734,856; hg19). The location of *B2M* at 15q21.1 (Chr.15: 45,003,675-45,011,075) is shown by the dashed line. E, higher magnification of the *B2M* gene region given as copy number (left) and log2 allelic display (right).

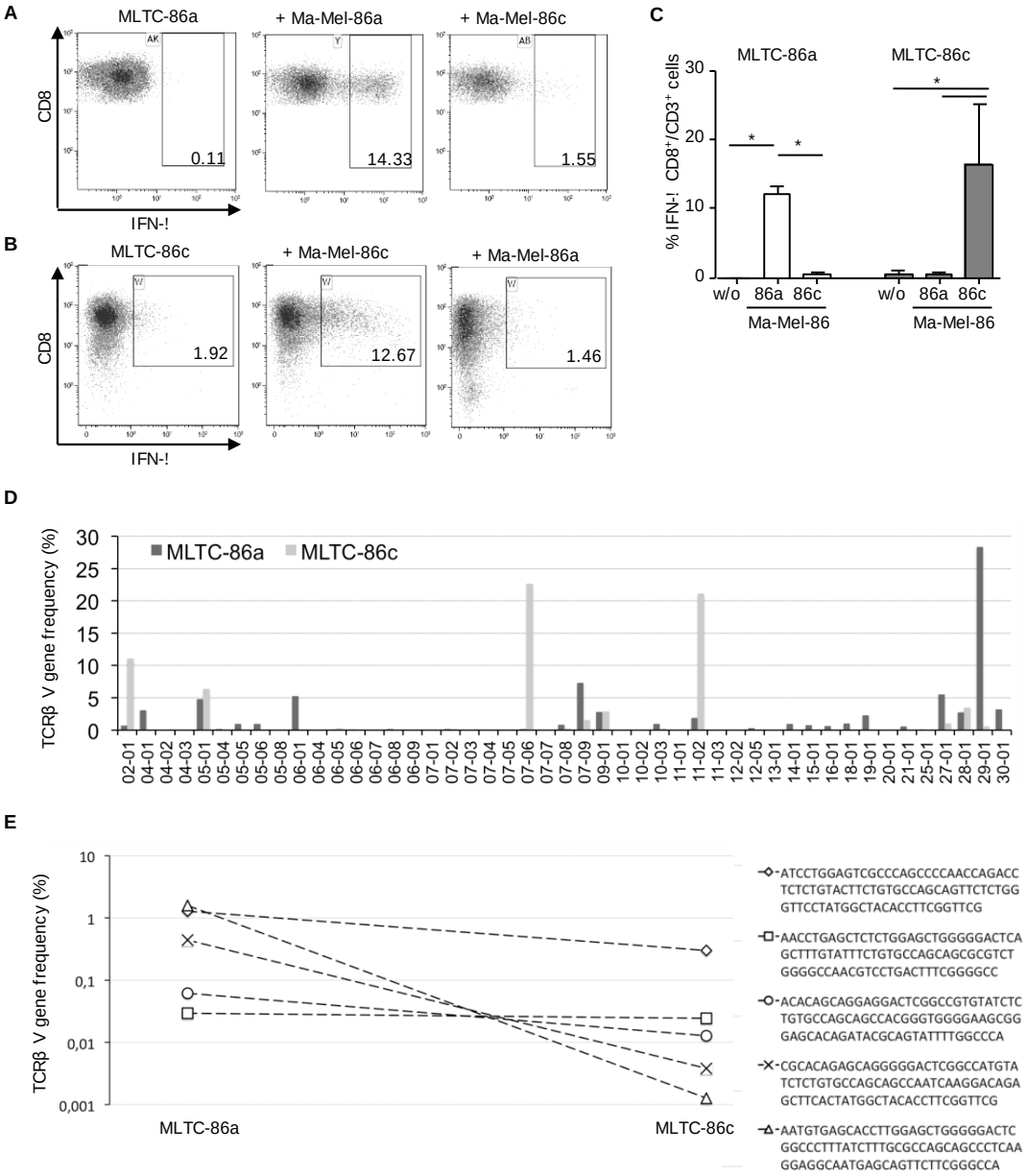

A
MLTC-86a
+ Ma-Mel-86a
+ Ma-Mel-86c
CD8
IFN-!
0.11
14.33
1.55
B
MLTC-86c
+ Ma-Mel-86c
+ Ma-Mel-86a
1.92
12.67
1.46
C
MLTC-86a
MLTC-86c
% IFN-! CD8+/CD3+ cells
w/o
86a
86c
Ma-Mel-86
D
MLTC-86a
MLTC-86c
TCRβ V gene frequency (%)
E
TCRβ V gene frequency (%)
MLTC-86a
MLTC-86c
ATCCTGGAGTCGCCCAGCCCCAACCAGACC TCTCTGTACTTCTGTGCCAGCAGTTCTCTGG GTTCCTATGGCTACACCTTCGGTTCG
AACCTGAGCTCTCTGGAGCTGGGGGACTCA GCTTTGTATTTCTGTGCCAGCAGCGCGTCT GGGGCCAACGTCCTGACTTTCGGGGCC
ACACAGCAGGAGGACTCGGCCGTGTATCTC TGTGCCAGCAGCCACGGGTGGGGAAGCGG GAGCACAGATACGCAGTATTTTGGCCCA
CGCACAGAGCAGGGGGACTCGGCCATGTA TCTCTGTGCCAGCAGCCAATCAAGGACAGA GCTTCACTATGGCTACACCTTCGGTTCG
AATGTGAGCACCTTGGAGCTGGGGGACTC GGCCCTTTATCTTTGCGCCAGCAGCCCTCAA GGAGGCAATGAGCAGTTCTTCGGGCCA

Figure 4.

Ma-Mel-86a and Ma-Mel-86c cells stimulate their specific T-cell repertoire. A-B, in mixed autologous lymphocyte-tumor cultures (MLTC) $CD8^+$ T cells were stimulated with Ma-Mel-86a (MLTC-86a) or Ma-Mel-86c (MLTC-86c) cells. After two stimulations T-cell reactivity towards the indicated target cells was determined by intracellular staining for IFNγ. A left, first dot plot: spontaneous IFNγ production by $CD8^+$ T cells from MLTC-86a; second to third dot plot: production of IFNγ In response to the different target cells indicated above. B left, first dot plot: spontaneous IFNγ production by $CD8^+$ T cells from MLTC-86c; second to third dot plot: production of IFNγ In response to the different target cells indicated above. Representative results from one of three independent experiments, numbers in dot plots indicate % of IFNγ^+ $CD8^+$ T cells. C, results from three independent experiments depicted as mean (+ SEM) of % IFNγ cells in $CD8^+CD3^+$ cells; *, $p<0.05$. D-E, results from high-throughput T-cell receptor β chain sequencing. D, clonal T-cell receptor β repertoire of MLTC-86a and MLTC-86c. E, selected intermediate to low frequency clones shared between both MLTC.

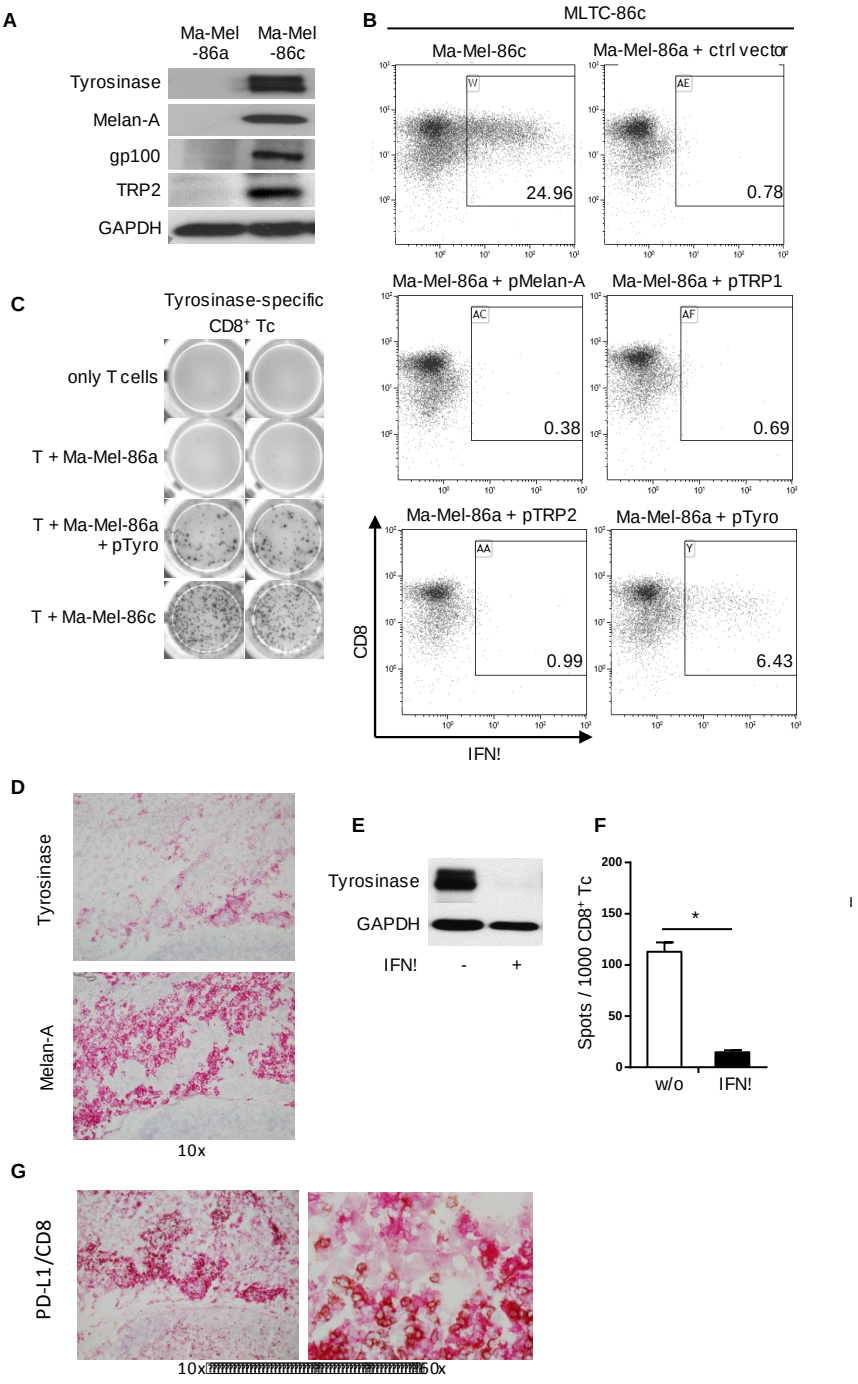

A
Ma-Mel -86a
Ma-Mel -86c
Tyrosinase
Melan-A
gp100
TRP2
GAPDH
B
MLTC-86c
Ma-Mel-86c
Ma-Mel-86a + ctrl vector
24.96
0.78
Ma-Mel-86a + pMelan-A
Ma-Mel-86a + pTRP1
0.38
0.69
Ma-Mel-86a + pTRP2
Ma-Mel-86a + pTyro
0.99
6.43
CD8
IFN!
C
Tyrosinase-specific CD8+ Tc
only T cells
T + Ma-Mel-86a
T + Ma-Mel-86a + pTyro
T + Ma-Mel-86c
D
Tyrosinase
Melan-A
10x
E
Tyrosinase
GAPDH
IFN!
-
+
F
Spots / 1000 CD8+ Tc
200
150
100
50
0
*
w/o
IFN!
G
PD-L1/CD8
10x
50x

Figure 5.

Ma-Mel-86c cells amplify MDA-reactive CD8$^+$ T cells. A, melanoma cells Ma-Mel-86a and Ma-Mel-86c were analysed for MDA expression (Melan-A/MART-1, gp100, TRP2, Tyrosinase) by Western Blot. GAPDH served as loading control. Representative data from one of three independent experiments. B, Ma-Mel-86a cells, transiently transfected with a control (ctrl) vector or expression plasmids encoding Melan-A, TRP1, TRP2 or Tyrosinase [Tyro] were co-cultured with T cells from MLTC-86c. After a 4 h coincubation, T cells were stained for intracellular IFNγ. Numbers in dot plots indicate % of IFNγ^+ CD8$^+$ T cells. Representative data from one of three independent experiments. C, activation of an autologous Tyrosinase-specific CD8$^+$ T cell clone (Tc) by different target cells was determined by IFNγ ELISpot. Each dot represents a cytokine-releasing T cell. Representative data from one of two independent experiments depicted. D, expression of Tyrosinase and Melan-A in serial cryostat tissue sections of metastasis Ma-Mel-86c determined by immunohistochemistry, Red colour indicates marker-positive cells. E-F, Ma-Mel-86c cells were treated with IFNγ (50 U/ml) in a 2-day interval for 7 d. E, Expression of Tyrosinase in IFNγ-treated and control Ma-Mel-86c cells, determined by Western blot. GAPDH served as loading control. Representative data from one of three independent experiments. F, activation of Tyrosinase-specific CD8$^+$ T-cell clones (Tc) in the presence of IFNγ-treated or control Ma-Mel-86c cells, determined by IFNγ ELISpot. Mean values (+SEM) of triplicates from one of four independent experiments is depicted; *, $p<0.05$. F, co-expression of CD8 (brown) and PD-L1 (red) in serial cryostat tissue section of metastasis Ma-Mel-86c determined by immunohistochemistry.

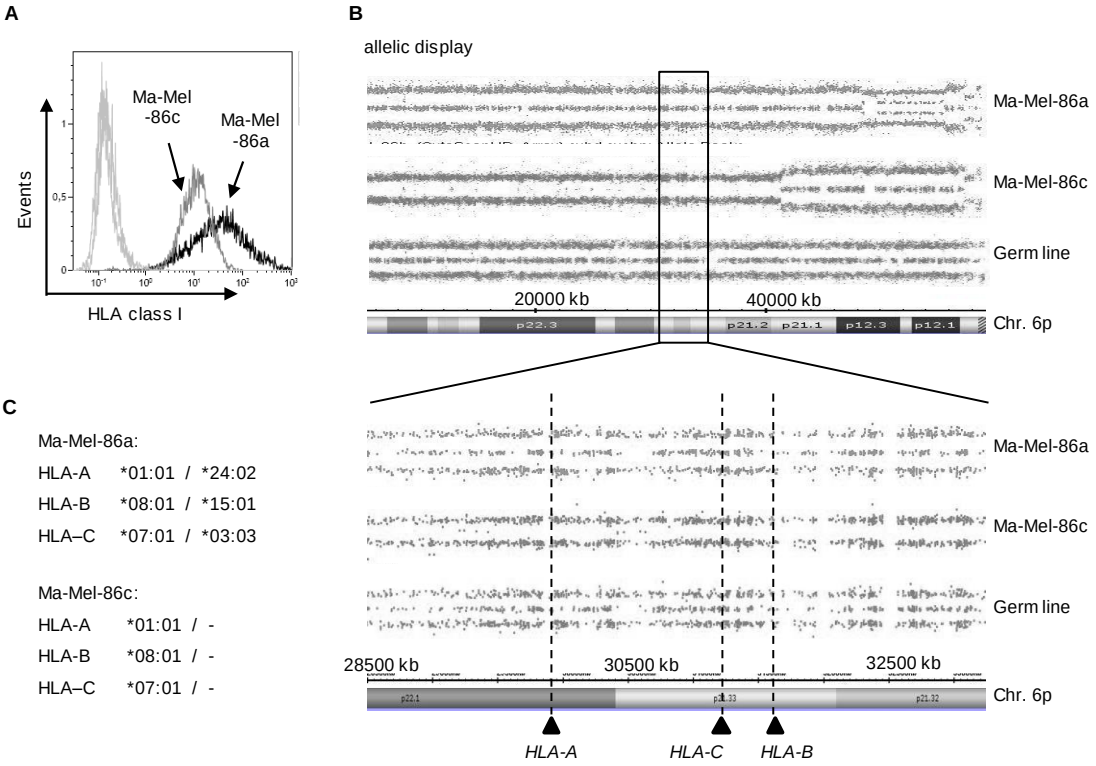

A
Ma-Mel
-86c
Ma-Mel
-86a
Events
HLA class I
B
allelic display
Ma-Mel-86a
Ma-Mel-86c
Germ line
20000 kb
40000 kb
Chr. 6p
28500 kb
30500 kb
32500 kb
HLA-A
HLA-C
HLA-B
C
Ma-Mel-86a:
HLA-A *01:01 / *24:02
HLA-B *08:01 / *15:01
HLA–C *07:01 / *03:03
Ma-Mel-86c:
HLA-A *01:01 / -
HLA-B *08:01 / -
HLA–C *07:01 / -
D
MLTC-86a
only T cells
T + Ma-Mel-86a
MLTC-86a
T + Ma-Mel-86c
+ pcDNA
+ pHLA-A*24
+ pHLA-B*15
+ pHLA-C*03

Figure 6.

HLA haplotype loss protects Ma-Mel-86c cells from Ma-Mel-86a-reactive T cells. A, HLA class I expression levels on Ma-Mel-86a and Ma-Mel-86c cells determined by flow cytometry. Representative data from one of two independent experiments. B, SNP results of chromosome 6p shown for DNA obtained from Ma-Mel-86a, Ma-Mel-86c and autologous PBMC (germ line): top, allelic display; bottom, magnification of the *HLA* gene region. Ma-Mel-86c cells show a large deletion (40,998,052 bp) on chromosome 6 encompassing the region 6p25.3 to 6p21.1 (Chr.6: 156,974-41,155,026; hg19), including the *HLA-A*, *HLA-B* and *HLA-C* genes. Location of *HLA* genes indicated by dashed lines. C, HLA class I genotype of Ma-Mel-86a and Ma-Mel-86c cells. D, reactivity of T cells from MLTC-86a towards Ma-Mel-86a cells as well as Ma-Mel-86c cells transfected with expression plasmids encoding *HLA-A*24:02* (pHLA-A*24), *HLA-B*15:01* (pHLA-B*15), *HLA-C*03:03* (pHLA-C*03) or an empty control vector (pcDNA) determined by IFNγ ELISpot assay. Representative data from one of two independent experiments.

Supplementary material and methods

Cell line authentication and HLA genotyping. The QIAamp DNA Mini Kit (Qiagen) was used for isolation of genomic DNA from melanoma cell lines and PBMC according to the manufacturer's instructions. For cell line authentication genetic profiling of genomic DNA was performed at the Institute for Forensic Medicine (University Hospital Essen) using the AmpFLSTR-Profiler Plus kit (Applied Biosystems). HLA class I genotyping on genomic DNA from cell lines and PBMC was carried out at the Institute for Immunology and Genetics Kaiserslautern (Kaiserslautern, Germany).

Immunohistochemistry. Serial cryostat tissue sections were stained for specific proteins with the indicated primary murine monoclonal antibodies (mAb) in combination with a biotinylated goat anti-mouse antibody (Jackson ImmunoResearch). Antibody binding was visualized using the Vectastain ABC-AP kit (Vector) according to the manufacturer's instructions. The following mAb were used: W6/32 for detection of HLA class I antigen complexes (Dianova); anti-HMB-45 (Dako) to stain melanoma cells, anti-CD3 for T cell detection (BD Pharmingen).

Quantitative real-time PCR. Total mRNA was isolated from tumor cells using RNeasy Plus Kit (Qiagen) and polyadenylate RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the instructions of the manufacturers. Real-time PCR was carried out with specific TaqMan Gene Expression assays (Applied Biosystems). Relative RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing expression levels of investigated mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Western blot. Tumor cells were lysed in 1 x Cell Lysis buffer (Cell Signaling) supplemented with 1 mM PMSF. Lysate was prepared by centrifugation at 13000 rpm for 15 min. Proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with primary non-labelled antibodies. After washing, membranes were incubated with the appropriate secondary antibodies linked to horseradish peroxidase. Antibody binding was visualized using the enhanced chemiluminescence system. The following primary mouse anti-human antibodies were used: anti-MelanA/MART-1 (Zytomed), anti-Tyrosinase, anti-gp100 and anti-MITF (Santa Cruz). Rabbit anti-TRP2/DCT was a generously provided by Dr. Vincent

Hearing (National Cancer Institute, National Institutes of Health, Bethesda). Rabbit anti-STAT3 and anti-pSTAT3 antibodies were purchased from Cell Signaling.

Flow cytometry. Cells were stained for HLA class I surface expression with the anti-HLA-A, -B, -C antibody W6/32, followed by staining with a PE-conjugated goat anti-mouse antibody (Beckmann Coulter). Background fluorescence was determined by staining with the secondary antibody only. Furthermore, cells were stained with for surface expression PD-L1 (Biolegend), HLA-DR (Beckmann Coulter) and CD56 (Beckmann Coulter) with fluorescence labelled antibodies, unstained cells served as controls. Cells were analysed by flow cytometry in a Gallios (Beckmann Coulter) flow cytometer using Kaluza (Beckman Coulter) or FlowJo software (TreeStar, Ashland, USA) for data analysis.

Transfection. FuGENE (Promega) was used for transient transfection of melanoma cells with plasmids. After a 72 h incubation, cells were collected and subjected to different assays.

IFNγ ELISPOT assay. Multiscreen-HA plates (Millipore, Bedford, MA) were coated with 5 µg/mL anti-hIFNγ mAb 1-D1K (Mabtech, Stockholm, Sweden). T cells were seeded at indicated numbers in AIM-V complete medium followed by addition of tumor cells ($1x10^4$ or $5x10^4$ cells/well). After 20 h incubation at 37°C in 5% CO_2, cells were removed and a biotinylated secondary anti-hIFNγ antibody (1 µg/mL, clone 7-B6-1, Mabtech) was added. Spots were developed by sequential addition of 1:1000 diluted ExtrAvidin alkaline phosphatase and BCIP®/NBT Liquid Substrate System (Sigma-Aldrich, St. Louis, MO). Spot numbers were determined with the AID ELISpot reader (AID Diagnostika GmbH, Strassberg, Germany).

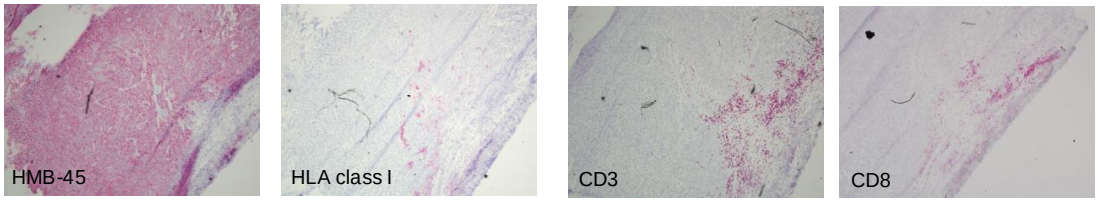
Cytotoxicity assay. To determine killing of melanoma cells, melanoma cells ($1x10^6$ cells/ml) were labeled with 20 nM CFSE (Invitrogen) at 37°C. After 10 min, labeling was stopped by the addition of fetal calf serum (FCS). Autologous tumor-specific CD8 T cells were then added to CFSE labeled melanoma cells at a 1:1 effector to target ratio. After 3 h of co-culture 7-AAD (BD Bioscience) was added to each sample at a final concentration of 1 µg/ml and measured directly on the flow cytometer.

Intracellular IFNγ staining. For detection of intracellular IFNγ, lymphocytes were stimulated for 4 h with the indicated tumor cells in AIM-V complete medium containing 10 µg/mL Brefeldin A (Sigma-Aldrich). Then cells were stained with anti-human CD3-PE/Cy7 and CD8-APC-Alexafluor700 antibodies (Beckman Coulter) followed by fixation and permeabilization using the Fixation/Permeabilization Concentrate and Diluent kit (eBioscience) and addition of an anti-IFNγ-FITC antibody (Beckman Coulter). Cells were analysed in a Gallios flow cytometer and the Kaluza software was used for data analysis (Beckman Coulter).

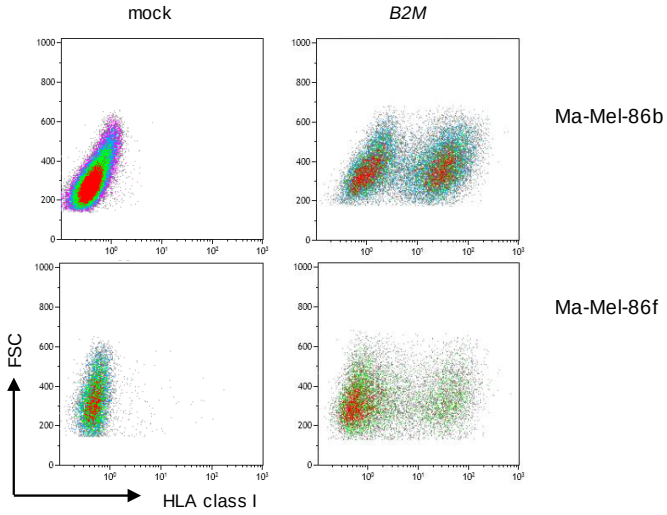
ELISA. Melanoma cells were seeded in 6-well plates at a concentration of 3x105 cells/well and cell culture supernatants were collected after 16 h. The concentration of IL6 (Mabtech) and IL8 (Biolegend) were determined by ELISA according to the manufacturer's instructions.

Statistical analysis. Quantitative data were plotted as mean + standard error (SEM). For comparison between experimental groups the two-tailed student's t-test was performed using the GraphPad Prism 5.03 software. Experimental groups were considered to be significantly different with $p < 0.05$.

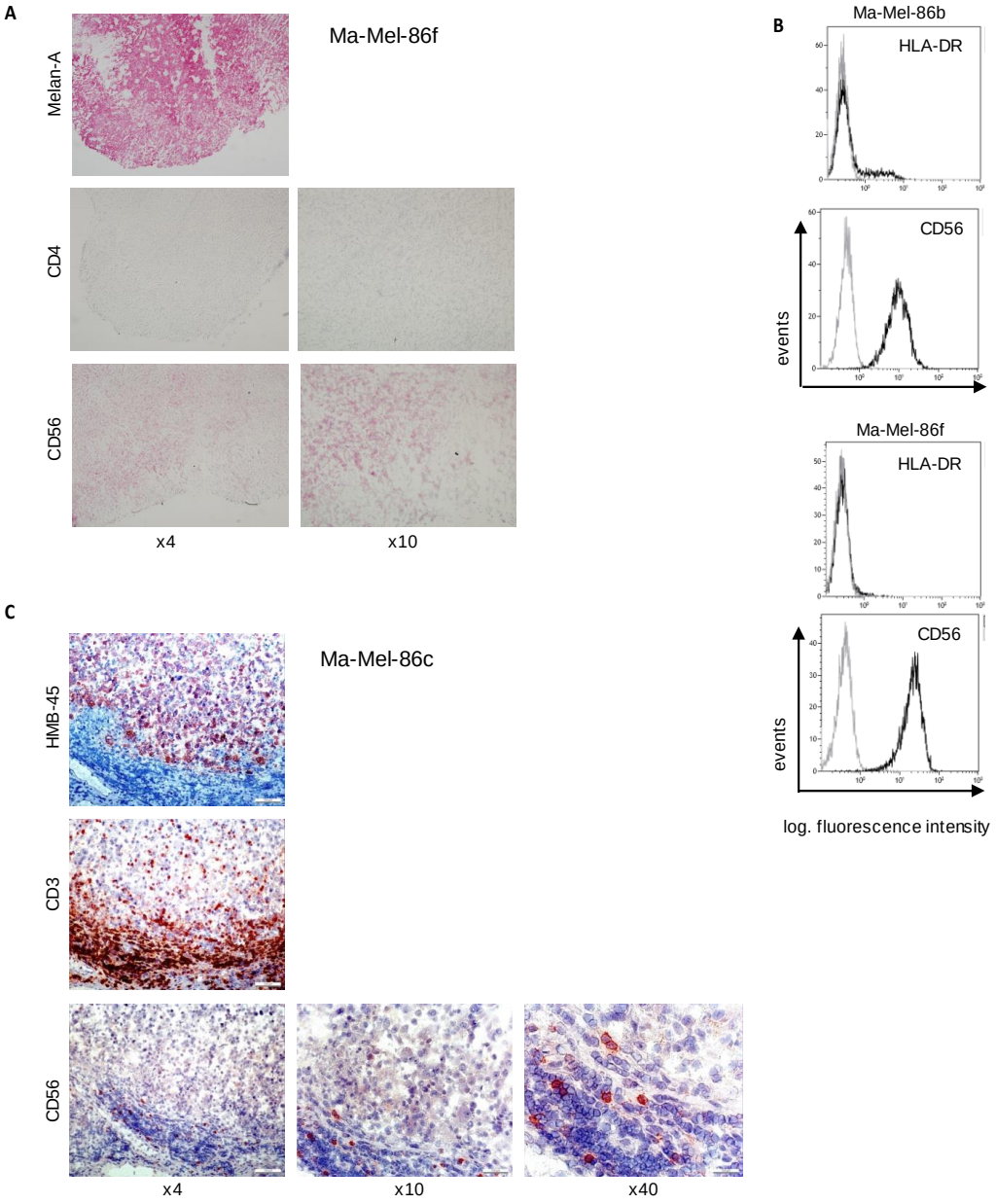
Supplementary figures



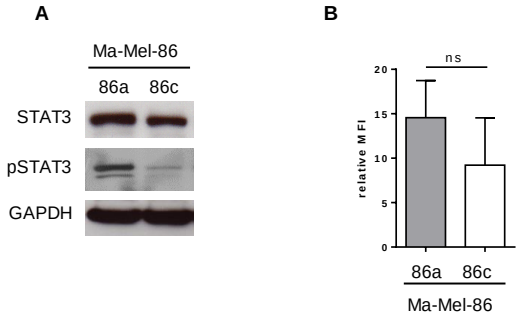
Supplementary Figure S1.
Expression of HMB-45, HLA class I, CD3, and CD8 in serial cryostat tissue sections of melanoma metastases Ma-Mel-86f, determined by immunohistochemistry. Red colour indicates marker-positive cells (magnification, x4).



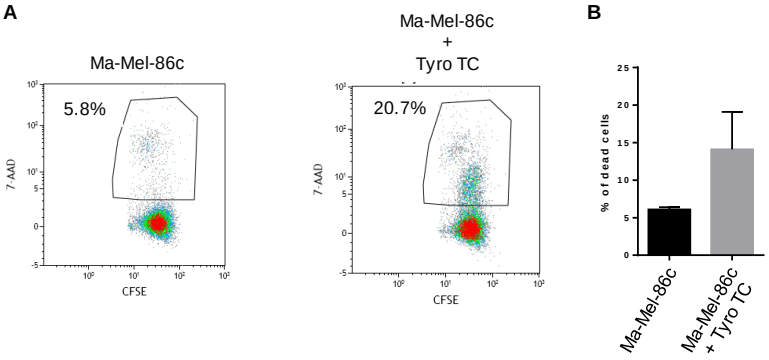
Supplementary Figure S2.
Re-expression of HLA class I on β2m-transfected melanoma cells. Ma-Mel-86b and Ma-Mel-86f cells were transiently transfected with a *B2M* expression plasmid. Expression of HLA class I antigens on transfectants was determined by flow cytometry after 72h. One representative of three independent experiments depicted.



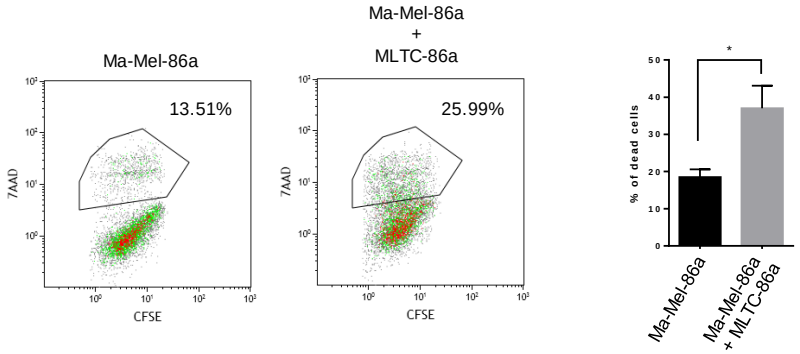
Supplementary Figure S3.
A, expression of Melan-A, CD4 and CD56 in serial cryostat tissue sections of melanoma metastases Ma-Mel-86f, determined by immunohistochemistry. Red colour indicates marker-positive cells. B, surface expression of CD56 and HLA-DR on Ma-Mel-86b and Ma-Mel-86f cells, determined by flow cytometry. One representative of two independent experiments depicted. C, expression of HMB-45, CD3 and CD56 in serial cryostat tissue sections of melanoma metastases Ma-Mel-86c, determined by immunohistochemistry. Red colour indicates marker-positive cells.



Supplementary Figure S4.
Similar PD-L1 expression on Ma-Mel-86a and Ma-Mel-86c cells. A, STAT3 and pSTAT3 levels determined by Western blot. GAPDH served as loading control. Representative data from one of three independent experiments. B, melanoma cells analyzed for PD-L1 surface expression by flow cytometry. PDL1 expression was determined as median fluorescence intensity (MFI) and normalized to the background MFI (unstained cells). Relative MFI (+SEM) of three independent experiments depicted.



Supplementary Figure S5.
Killing of CFSE-labeled Ma-Mel-86c cells by autologous Tyrosinase-specific CD8+ T cell clones (Tyro TC) analyzed by flow cytometry. A, dot plots indicate percentage of 7AAD$^+$ dead cells of one representative experiment. B, Mean percentage of dead cells (+SEM) from 2 independent experiments.



Supplementary Fig. S6.
Killing of CFSE-labeled Ma-Mel-86a cells by autologous bulk CD8 T cells (MLTC-86a) determined by flow cytometry. A, The percentages of 7AAD$^+$ dead cells as one representative data were indicated on the corresponding dot-plots. B, Mean percentage of dead cells (+SEM) from 4 independent experiments.

Supplementary Table S1. Genes covered in the applied sequencing panel

Nr.	Gene	Primary Melanoma type	Customary Mutation Type	Target Bases	Bases covered	Pri pa
1	*BRAF*	cutaneous	activating	2860	2456	
2	*NRAS*	cutaneous	activating	650	650	
3	*KIT*	cutaneous	activating	3354	3264	
4	*HRAS*	cutaneous	activating	780	667	
5	*KRAS*	cutaneous	activating	787	787	
6	*CDKN2A*	cutaneous	tumor suppressor	1184	713	
7	*PTEN*	cutaneous	tumor suppressor	1392	1248	
8	*CDK4*	cutaneous		1052	1052	
9	*TP53*	cutaneous	tumor suppressor	1503	1396	
10	*RAC1*	cutaneous		776	721	
11	*NF1*	cutaneous	tumor suppressor	9900	9167	
12	*PIK3CA*	cutaneous		3607	3313	
13	*MAP2K2*	cutaneous		1423	1240	
14	*PIK3R1*	cutaneous		2637	2627	
15	*MITF*	cutaneous		2066	2066	
16	*TERT*	cutaneous		3719	2371	
17	*ARID2*	cutaneous	tumor suppressor	5928	5830	
18	*ARID1A*	cutaneous	tumor suppressor	7258	6132	
19	*SMARCA4*	cutaneous	tumor suppressor	5761	5040	
20	*MAP2K1*	cutaneous		1436	1436	
21	*CTNNB1*	cutaneous		2626	2626	
22	*EZH2*	cutaneous		2680	2680	
23	*IDH1*	cutaneous		1405	1394	
24	*FBXW7*	cutaneous		2898	2808	
25	*WT1*	cutaneous		1784	1282	
26	*GNAQ*	uveal	activating	1220	1064	
27	*GNA11*	uveal	activating	1220	944	
28	*BAP1*	uveal	tumor suppressor	2599	2380	
29	*SF3B1*	uveal		4455	4412	

Supplementary Table S2: SRA accession numbers for transcriptomes

Cell line	**Accession Number**
Ma-Mel-86a transcriptome seq (rep. 1)	SRX1542616
Ma-Mel-86a transcriptome seq (rep. 2)	SRX1542617
Ma-Mel-86a transcriptome seq (rep. 3)	SRX1542618
Ma-Mel-86b transcriptome seq (rep. 1)	SRX1542619
Ma-Mel-86b transcriptome seq (rep. 2)	SRX1542620
Ma-Mel-86b transcriptome seq (rep. 3)	SRX1542621
Ma-Mel-86c transcriptome seq (rep. 1)	SRX1542622
Ma-Mel-86c transcriptome seq (rep. 2)	SRX1542623
Ma-Mel-86c transcriptome seq (rep. 3)	SRX1542624
Ma-Mel-86f transcriptome seq (rep. 1)	SRX1542625
Ma-Mel-86f transcriptome seq (rep. 2)	SRX1542626
Ma-Mel-86f transcriptome seq (rep. 3)	SRX1542627

Supplementary Table S3: List of vaccine peptides

Antigen	HLA allele	Sequence peptide epitope
Tyrosinase aa 243-251 analog	HLA-A*01	KSDICTDEY
MAGE-1	HLA-A*01	EADPTGHSY
MAGE3	HLA-A*01	EVDPIGHLY
Tyrosinase	HLA-A*A24	AFLPWHRLF
GP100	HLA-A*A24	VYFFLPDHL
MAGE-1	HLA-A*A24	NYKHCFPEI
MAGE-3	HLA-A*A24	IMPKAGLLI

7.3. Manuscript III

Development of stable HLA class II-deficient melanoma phenotypes in the course of disease progression leads to total T cell resistance in the context of HLA class I deficiency

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Running Title: Evolution of HLA class II-deficient melanoma phenotypes

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Key Words: HLA class II, melanoma, immune escape, $CD4^+$ T cell

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Translational Relevance

Clinical success of melanoma immunotherapy is mainly attributed to cytotoxic $CD8^+$ T cells, though *in vivo* evidences for tumor cell killing by $CD4^+$ T cells recognizing MHC class II antigens have been accumulated. Our study revealed strong intertumoral and intratumoral heterogeneity in constitutive HLA class II surface expression as well as heterogeneous expression among consecutive patient metastases. HLA class II-positive melanoma cells were sensitive to perforin-mediated killing by autologous $CD4^+$ T cells. However, development of stable HLA class II-negative phenotypes was demonstrated in the course of disease progression due to defective IFNγ signaling or epigenetic silencing of the HLA class II transcriptional activator CIITA, abrogating cytokine-mediated HLA upregulation. Since these escape mechanisms were active also in HLA class I-negative melanoma cells our data suggest that metastases should be screened for genetic and epigenetic alterations leading to complete T cell resistance in terms of predicting response to immunotherapy.

Abstract

Purpose: $CD8^+$ T cells are major effectors in anti-tumor immunity but recent *in vivo* studies provided evidence for direct anti-tumor responses by $CD4^+$ T cells. Constitutive expression of HLA class II antigens on melanoma cells already generated broad interest in $CD4^+$ T cell-based therapies. Here, we asked for the HLA class II expression of consecutive patient metastases and for phenotype alterations leading to $CD4^+$ T cell resistance.

Experimental Design: *In situ* and *in vitro* patterns of constitutive and IFNγ-induced HLA class II expression on melanoma cells from consecutive melanoma metastases were analyzed in different patient models. Dependency of HLA class II expression on its transcriptional activator CIITA and mechanisms of HLA class II deficiency protecting melanoma cells from killing by autologous $CD4^+$ T cells were determined.

Results: Heterogeneous, constitutive HLA class II expression was observed for melanoma cells from consecutive patient metastases. Negative tumor cells generally acquired HLA class II expression in response to IFNγ treatment enhancing their sensitivity towards autologous $CD4^+$ T cells showing perforin-dependent cytotoxicity. In the course of disease progression, development of stable HLA class II-negative phenotypes due to genetically acquired IFNγ signaling defects or epigenetic silencing of CIITA expression was detected, leading to absolute T-cell resistance in the context of a HLA class I-negative phenotype.

Conclusion: Melanoma cells can genetically and epigenetically evolve towards HLA class II deficiency suggesting $CD4^+$ T cells might not always be an option for targeting $CD8^+$ T cell-resistant melanoma.

Introduction

Cytotoxic CD8$^+$ T lymphocytes have long been considered the main actors against metastatic melanoma, as they are capable of specifically recognizing and killing neoplastic cells. Approaches like adoptive cell transfer of autologous CTLs or immune checkpoint blockade with αPD1 or αCTLA-4 monoclonal antibodies exploited this capacity leading to a proper cytolytic response against melanoma cells.[1, 2] Nevertheless, immunotherapies often fail to completely eliminate tumor cells. Thus, only a subset of cancer patients benefit from these immunotherapies, due to a a variety of interfering mechanisms including continuous immune suppression, selection of immune escape variants or the lack of T cell infiltration into the tumor microenvironment.[3, 4] Previously, we demonstrated the development of melanoma immune escape variants in the course of disease progression, showing melanoma cells lost expression of HLA class I molecules due to genetic alteration affecting the *B2M* gene.[5]

However, HLA class I deficient melanoma cells should still be capable of presenting tumor antigens to CD4$^+$ T cells in a MHC class II dependent manner. Of note, under normal conditions HLA class II expression is restricted to professional antigen presenting cells like dendritic cells or macrophages whereas on non-immune cells, HLA class II expression is generally inducible by IFNγ. IFNγ-dependent activation of JAK/STAT signaling leads to the expression of the transcriptional regulator CIITA and subsequently to the CIITA-dependent expression of HLA class II α- and β-chain. [6] Some solid tumors such as melanoma very frequently show constitutive HLA class II expression, becoming potentially accessible to tumor eradication by CD4$^+$ T cells.[7, 8] Several studies found peptides from differentiation antigens like TRP-1 and gp-100; cancer testis antigens like MAGE and NY-ESO or even mutated antigens to be presented on HLA class II molecules and to be recognized by CD4$^+$ T cells. [7, 9-12]

A role of CD4$^+$ T cells in antitumor immunity has long been described. Besides providing help for CD8$^+$ cytotoxic T lymphocytes to sustain and enhance their antitumoral activity, Th1 cytokines can also exhibit indirect antitumoral effects, e.g. via the induction of apoptosis or senescence or by acting on the tumor microenvironment.[13-15] Successful adoptive transfer of *in vitro* differentiated CD4$^+$ T cells into mice and human has opened the possibility of a significant role of these immune cells in anti-tumor immunity by leading to proper activation and preventing exhaustion of CD8$^+$ cytotoxic T lymphocytes.[10, 16, 17] Studies even suggested that CD4$^+$ T cells are able to outperform CTLs and are capable of clearing tumors that are

resistant to the eradication by $CD8^+$ T cells.[10] Additionally to their Th1 phenotype, naïve $CD4^+$ T cells apparently are able to gain cytotoxic features upon transfer into lymphopenic mice and eradicate established melanoma in a Granzyme/Perforin dependent manner. [18, 19] This cytotoxic phenotype of antigen-specific $CD4^+$ T cells could also be observed and even enhanced after treatment of four melanoma patients with anti-CTLA-4 antibody Ipilimumab.[20]

Concerning their pivotal role in immunotherapies, it is of importance to define mechanism either relevant for $CD4^+$ dependent tumor rejection or counteracting $CD4^+$ anti-tumor activity. So far nothing is known about the heterogeneity of HLA class II expression in consecutive melanoma metastases and whether tumors can acquire genetic/epigenetic alterations mediating HLA class II deficiency in the course of disease progression.

Therefore, we analyzed the expression pattern of HLA class II in human metastatic melanoma tissues as well as early melanoma cell lines. Additionally, we compared the HLA-DR and CIITA expression between cell lines established from consecutive metastases of melanoma patients and thereby identified mechanisms that counteracted recognition of tumor cells by autologous $CD4^+$ T cells.

Materials and Methods

Patient material

Samples from melanoma patients including tumor tissues and peripheral blood cells were collected after approval by the institutional review board and patient informed written consent. Blood cells were separated on a Ficoll gradient and peripheral blood mononuclear cells (PBMC) were cryopreserved. Melanoma cell lines were cultured in RPMI1640 medium supplemented with glutamine (PAA laboratories), 10% FCS (PAA laboratories), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO_2 atmosphere. Cell lines were confirmed to be mycoplasma-free in monthly intervals and authenticated by genetic profiling on genomic DNA at the Institute for Forensic Medicine (University Hospital Essen) using the AmpFLSTR-Profiler Plus kit (Applied Biosystems) every 6 months.

Immunohistochemistry

Serial cryostat tissue sections were (double-) stained for specific proteins with the indicated primary antibodies in combination with a Polymer Kit containing an AP-coupled secondary antibody (Dako REAL Detection System AP/RED Rabbit/Mouse, Dako) or a Polymer Kit containing a HRP-coupled secondary antibody (envision FLEX Mini Kit, Dako). The following mAbs were used: anti-pSTAT1 Y701 (1:25, Cell Signaling), anti-GBP1 (1:50, Santa Cruz), anti-CD3 (1:50, BD Pharmingen), anti-MART-1 (1:100, Dako), anti-HLA-DR,-DP,-DQ (1:100) antibody LGII-612.14, recognizing a linear epitope expressed on HLA-DR, -DQ, and -DP β chains. [21]

IFNγ treatment

20-24 hours prior to IFNγ treatment, tumor cells were seeded at a density of $1x10^6$ cells/ $75cm^2$ flasks. Cells were treated with IFNγ (500U/ml) or left untreated. After incubation for 48 hours at 37°C, cells were subjected to further analyses.

siRNA and plasmid transfection

20-24 hours prior to transfection, tumor cells were seeded at a density of $1\text{-}2x10^5$ cells/well in a 6-well culture plate. Cells were transfected with 20nM siCIITA (SMARTpool ON-TARGET plus, Dharmacon) or control siRNA using Viromer Blue transfection reagent (Lipocalyx). Transfection was performed according to manufacturer's instructions. Cells were subjected to further analyses after 48 hours incubation at 37°C. PMZ3F JAK1 SPA plasmid (1µg) was transfected using FuGENE

HD transfection reagent (Promega) following manufacturer's instructions. Stable transfectants were selected using Geneticin containing medium (1mg/ml).

Mixed lymphocyte tumor cell cultures (MLTC)

Tumor-reactive T cells were established following a previously described protocol.[5] Briefly, $CD4^+$ and $CD8^+$ T lymphocytes were isolated from patient PBMC using anti-CD4 or anti-CD8 MicroBeads (Miltenyi Biotech) (Supplemental Fig. S1). Isolated T cells ($1x10^6$) were co-cultured in 24-well culture plates with $1x10^5$ irradiated (120 Gy) autologous tumor cells per well in 2 ml of AIM-V (GIBCO/BRL) supplemented with 10% (vol/vol) human serum. $CD4^+$ T cell were additionally cultured with recombinant human IL-2 and IL-15 (20 IU/ml and 15ng/ml) while $CD8^+$ T cells were cultured solely with IL-2 (250U/ml). T cells were restimulated at weekly intervals with irradiated melanoma cells and medium was changed when necessary.

IFNγ ELISPOT assay

IFNγ enzyme-linked immunospot (ELISPOT) assay was performed as previously described.[22] Briefly, multicreen-HA plates (Millipore, Bedford, MA) were coated with 5 µg/mL anti-hIFN-γ mAb 1-D1K (Mabtech, Stockholm, Sweden). $1x10^4$T cells were seeded in RPMI complete medium. $5x10^3$ cells/well tumor cells or APC ($CD4^+$ /$CD8^+$-depleted PBMC) were added. After 20-24 hours incubation at 37°C in 5% CO_2, a biotinylated secondary anti-hIFNγ antibody (1 µg/mL, clone 7-B6-1, Mabtech) was used and spots were developed by the sequential addition of 1:1000 diluted ExtrAvidin alkaline phosphatase and BCIP®/NBT Liquid Substrate System (Sigma-Aldrich, St. Louis, MO). Spot numbers were determined with the AID EliSpot reader (AID Diagnostika GmbH, Strassberg, Germany).

Flow Cytometry

Cells were stained for surface marker expression with directly labelled antibodies (HLA-DR-PC7, Beckman Coulter; HLA-A, -B, -C -APC, ebioscience). Background fluorescence was determined by unstained cells. After fixation, cells were analyzed using the Gallios flow cytometer (Beckman Coulter) and the Kaluza software.

For CD107a Degranulation Assay and Intracellular Staining, Ma-Mel-61b cells were co-incubated with autologous tumor-specific $CD4^+$ T cells at an effector/target ratio of 1:1 for 1 hour in the presence of PE-conjugated CD107a mAb (Clone H4A3; BD Bioscience) or the corresponding isotype control. After co-culture for 1 hour, cells were

incubated in the presence of 2mM Monensin (Sigma-Aldrich) and 10µg/mL Brefeldin A (Sigma-Aldrich) for additional 3 hours to inhibit CD107a internalization and cytokine release. Thereafter cells were washed and stained with anti-CD4-PC7 (clone SFC/12T4D11, Beckman Coulter) or anti-CD3-PC7 (clone UCHT-1, Beckman Coulter) for 30 minutes followed by Intracellular Staining using the Fixation/Permeabilization Concentrate and Diluent kit (eBioscience) and addition of anti-IFNγ-APC antibody (clone 4S.B3, Biolegend) and anti-Perforin-BrilliantViolet (clone dG9, Biolegend) Cells were analyzed in a Gallios flow cytometer and the Kaluza software was used for data analysis (Beckman Coulter).

xCELLigence based Real-time cytotoxicity assay

To analyze $CD4^+$ T cell cytotoxicity, 50 µl RPMI medium was added to E-Plates 16 (Roche) for measurement of background values. Subsequently, melanoma cells were seeded in an additional 100 µl medium at a density of 0.5 x 10^4 cells per well. Cell attachment was monitored using the RTCA SP (Roche) instrument and the RTCA software Version 1.1 (Roche). After 24 hours incubation at 37°C, T cells were added at an effector to target ratios (E:T) of 1:1. Upon addition of effector cells, impedance measurements were performed every 15 min for up to 96 hours. All experiments were performed at least in duplicates. Changes in electrical impedance were expressed as a dimensionless cell index (CI) value, which derives from relative impedance changes corresponding to cellular coverage of the electrode sensors, normalized to baseline impedance values with medium only. To analyze the acquired data, CI values were exported and tumor lysis was calculated in relation to the control cells lacking any effector T cells.

CFSE Cytotoxicity Assay

Killing of melanoma cells was determined in a flow cytometry assay. Therefore, 1 x 10^6 melanoma cells were labeled with 20 nM CFSE (Invitrogen). After 10 minutes, labeling was stopped by the addition of complemented medium. Then cells were washed twice with complemented medium. $CD4^+$ cells were added to 5 × 10^4 CFSE-labeled melanoma cells at an effector to target ratio of 1:1. After 2 hours of co-culture, 7-AAD (BD Bioscience) was added to each sample at a final concentration of 1 µg/ml. Probes were measured directly by flow cytometry.

Quantitative real-time PCR

Total mRNA was isolated from tumor cells using RNeasy Plus Kit (Qiagen, Hilden, Germany) and RNA was reverse transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following the manufacturer's instructions. Real-time PCR was carried out using specific TaqMan Gene Expression assays. Relative RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing expression levels of investigated mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Western Blot

Tumor cells were lysed in 1x Cell Lysis buffer (Cell Signaling) supplemented with 1 mM PMSF. Lysate was then prepared by centrifugation at 13000 rpm for 15 min. Proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with the indicated primary non-labelled antibodies. After washing, membranes were incubated with the appropriate secondary antibodies linked to horseradish peroxidase (HRP). Antibody binding was visualized using the enhanced chemiluminescence system. The following primary anti-human antibodies were used: anti-STAT1, anti-IRF1 (Santa Cruz), anti-pSTAT1 Y701 and anti-GAPDH (Cell Signaling).

Combined bisulfite restriction analysis (COBRA)

To analyze two distinct regions of the CIITA promoter IV for the presence of cytosine methylation, COBRA Assay was used as previously described.[23] Briefly, CpG region GM2 was amplified by Touch Down PCR from bisulfite-converted gDNA (EZ DNA Methylation-Gold Kit, Zymo Research). The PCR products were controlled for appropriate size on 1.7% TBE gels and then purified using the Qiaquick PCR purification kit according to manufacturer's instructions. 17µl of the purified PCR products were subsequently used for enzymatic digest with BstUI restriction enzyme (Thermo Scientific), a restriction enzyme cleaving CpG sites retained because of methylation. The restricted DNA was subjected to 2% agarose gel electrophoresis and stained with Midori Green.

Results

IFN-independent *in situ* and *ex vivo* HLA class II expression by melanoma cells

Cultured melanoma cells frequently express HLA class II antigens, but it remained unclear whether expression occurs in corresponding metastatic lesions. Studying tissue sections from two consecutive metastases of patient UKE-Mel-34 we detected expression of HLA class II β chains (HLA-DR, -DP, -DQ) by immunohistochemistry in both lesions UKE-Mel-34a and UKE-Mel-34g (Fig. 1A). Short-term cultured cell lines established from the two metastases constitutively expressed HLA-DR surface molecules as demonstrated by flow cytometry (Fig. 1B). Consistently, we found melanoma marker Melan-A and HLA class II β chains to be co-expressed on tumor cells in corresponding tissues, but also detected single marker-positive cells (Fig. 1C). As shown in Fig. 1A, both tumors were infiltrated at low numbers by $CD3^+$ T cells that, by IFNγ release, could have induced HLA class II expression in tumor cells. To demonstrate that *in situ* HLA class II expression in melanoma is not necessarily IFNγ-driven, we again stained serial cryostat tissue sections from patient UKE-Mel-32a for CD3 and IFNγ-inducible GBP1 in addition to Melan-A and HLA class II β chains (Fig. 1D). While $CD3^+$ T cells were mainly located at the periphery of the lesions, corresponding to the GBP1-positive region, expression of Melan-A and HLA class II β chains was observed in the tumor area. Short-term cultured melanoma cell line UKE-Mel-32a established from this metastasis also showed HLA-DR expression on the cell surface (Fig. 1E).

Inter- and intratumoral heterogeneity of CIITA-dependent HLA-DR expression on short-term cultured melanoma cells

To estimate the intertumoral heterogeneity of HLA class II expression, we screened 28 short-term cultured melanoma cells (≤ passage 11) for *HLA-DRA* mRNA expression by qRT-PCR. Cell line Ma-Mel-61b was selected as reference since constitutive *HLA-DRA* mRNA expression in these cells was associated with strong HLA-DR surface expression. The screening approach revealed a very heterogeneous expression pattern: More than half of the total cell cultures expressed lower HLA-DRA mRNA levels as compared to the reference Ma-Mel-61b and 8 out of 28 cell cultures were completely negative (Fig. 2A).

From the early passage cells four cultures (UKE-Mel-20a, UKE-Mel-21a, UKE-Mel-23a, UKE-Mel-32a), showing different levels of *HLA-DRA* mRNA expression relative to Ma-Mel-61b cells, were selected for further analyses. In addition to the *HLA-DRA*

we determined expression levels of *CIITA*, the transcriptional regulator of HLA class II and accessory genes.[24, 25] As shown in Fig. 2B *HLA-DRA* and *CIITA* mRNA levels were clearly associated, indicating *CIITA*-controlled *HLA-DRA* transcription. Interestingly, within each of the short-term cell cultures we observed a strong heterogeneity in the intensity of HLA-DR surface expression, including small populations of HLA-DR-negative cells, indicating intratumoral heterogeneity of HLA class II expression (Fig. 2C)

Intrapatient heterogeneity of HLA class II expression in recurrent melanoma lesions

As shown in Fig. 1B, HLA-DR surface molecules were detected on cell lines UKE-Mel-34a and UKE-Mel-34g but UKE-Mel-34a showed higher and more homogenous expression compared to UKE-Mel-34g. Asking for heterogeneity of HLA class II expression in distinct patient lesions, we studied cell lines established from six different metastases of patient Ma-Mel-61 for HLA-DR surface expression. This patient presented with stage IV melanoma in November 2002. In the following three years, multiple lymph node lesions and distant metastases were excised and cell lines Ma-Mel-61a, Ma-Mel-61b, Ma-Mel-61c, Ma-Mel-61e, Ma-Mel-61f and Ma-Mel-61g were established from corresponding tissues (Fig. 3A). Staining for HLA-DR surface expression revealed a heterogeneous expression between the different cell lines. Ma-Mel-61b, Ma-Mel-61c and Ma-Mel-61f cells showed strong constitutive HLA-DR expression (HLA class II^{high}), whereas cell lines Ma-Mel-61a and Ma-Mel-61e were only slightly positive (HLA class II^{low}) and Ma-Mel-61g cells completely negative (HLA class II^{neg}) (Fig. 3A).

Comparison of *HLA-DRA* and *CIITA* mRNA expression levels demonstrated that both were tightly correlated again, suggesting CIITA-driven *HLA-DRA* transcription (Fig. 3B). Indeed, siRNA-mediated downregulation of *CIITA* mRNA in Ma-Mel-61b cells led to a decrease in HLA-DR mRNA levels (Fig. 3C). To determine the impact of type II IFN on HLA class II surface expression, the different cell lines were cultured in the presence of IFNγ. While HLA class II^{high} cells (Ma-Mel-61b, Ma-Mel-61e, Ma-Mel-61f) only slightly enhanced HLA-DR surface levels in response to IFNγ treatment, HLA class II^{low} cells (Ma-Mel-61a, Ma-Mel-61e) became strongly positive in contrast to Ma-Mel-61g cells remaining completely negative (Fig. 3D), indicating resistance to IFNγ as previously described (Sucker et al. submitted).

Recognition of HLA class II^{high} melanoma cells by autologous CD4^{+} T cells

Since HLA class II expression sensitizes to $CD4^+$ T cell recognition, we asked for the recognition of HLA class II^{high} melanoma cells from patient Ma-Mel-61 by autologous peripheral blood $CD4^+$ T cells. In order to enrich tumor-reactive $CD4^+$ T cells we set up mixed lymphocyte tumor cell cultures (MLTC) of sorted $CD4^+$ T cells and irradiated Ma-Mel-61b cells. After repeated restimulation, $CD4^+$ T cells were co-incubated with the different HLA class II^{high} Ma-Mel-61b, Ma-Mel-61c and Ma-Mel-61f cells and analyzed for activation by IFNγ ELISpot assay. $CD4^+$ T cells became activated in the presence of each of the three cell lines, strongest stimulation was achieved by Ma-Mel-61b cells and slightly lower activation by Ma-Mel-61c and Ma-Mel-61f cells and recognition of Ma-Mel-61b cells was impaired in the presence of an αHLA class II blocking antibody (Fig. 4A; supplementary Fig. S2).

In contrast to HLA class II^{high} cells $CD4^+$ T cells did not respond to HLA class II^{low} Ma-Mel-61a cells (Fig. 4B). When treated with IFNγ Ma-Mel-61a cells acquired a HLA class II^{high} phenotype, similar to Ma-Mel-61b cells (Fig. 4C). Interestingly, despite similar HLA-DR expression levels, $CD4^+$ T cells became barely activated in the presence of IFNγ-treated Ma-Mel-61a cells compared to Ma-Mel-61b cells, suggesting differences in antigen expression and/or processing between both cell lines (Fig. 4B). Specificity of $CD4^+$ T cells for tumor associated antigens was demonstrated by coincubation with Ma-Mel-61b as well as autologous APC ($CD4^+$/$CD8^+$-depleted PBMC) As shown in Fig. 4D, $CD4^+$ T cells became activated only in the presence of tumor cells.

Killing of HLA class II^{high} melanoma cells by autologous $CD4^+$ T cells

Recently evidences for cytolytic activity of $CD4^+$ T cells against melanoma cells have been accumulated in different mouse tumor models.[19, 20] Thus we asked whether autologous $CD4^+$ T cells expanded in MLTC with HLA class II^{high} Ma-Mel-61b cells would be capable of killing tumor cells. Using an xCELLigence device, the impact of $CD4^+$ T cells on Ma-Mel-61b cell survival was monitored real-time in parallel to the tumor cell killing by MLTC-expanded autologous $CD8^+$ T cells (Fig. 5A).[26] Activity of both T cell subsets led to strong and rapid decrease in Ma-Mel-61b cell numbers, though, $CD8^+$ T cell-mediated cytotoxicity was still more effective. Similar results were obtained when $CD4^+$ T cells were incubated with Ma-Mel-61f target cells (supplemental Fig. S3). Cytotoxic activity of $CD4^+$ T cells against Ma-Mel-61b cells was confirmed by 7-AAD/CFSE cytotoxicity assay (Fig. 5B; supplemental Fig. S4).

Interestingly, a subset of IFNγ-secreting $CD4^+$ T cells with perforin/granzyme-mediated cytolytic activity was recently defined in HIV as well as influenza infected individuals.[27,

[28] Thus, we stained MLTC-expanded CD4$^+$ T cells, in parallel to CD8$^+$ T cells, for degranulation marker CD107a, intracellular IFNγ and perforin (supplemental Fig. S5). As depicted in Fig. 5C, a subpopulation (35%) of CD4$^+$ T cells expressed perforin that was much higher (65%) for CD8$^+$ T cells. Coincubation with tumor cells increased frequency of CD107a$^+$/IFNγ$^+$ and CD107a$^+$/perforin$^+$, though not all CD107a$^+$ cells stained positive also for IFNγ or perforin. Overall, these data were in line with the suggested role of CD4$^+$ T cells as cytotoxic effectors in anti-tumor immunity.

The clinical significance of CD4$^+$ T cells in anti-tumor immune responses was demonstrated also by analyses of TCGA melanoma data revealing an association of high CD4 mRNA levels with improved overall patient survival (p=0.0012). Although this association was even stronger for CD8$^+$ T cells (p=0) (Fig. 5D), these data clearly indicate a positive role for CD4$^+$ T cells within the tumor microenvironment.

Stable HLA class II-negative phenotype acquisition due to defective IFN signaling in melanoma cells abrogates CD4$^+$ T-cell recognition

As shown in Fig. 3D, cell line Ma-Mel-61g retained its HLA-DR negative phenotype in the presence of IFNγ. Previously, we found *JAK1* mutated in Ma-Mel-61g cells, mediating resistance to IFNγ (Sucker et al., submitted). Thus, ectopic expression of *JAK1* in Ma-Mel-61g cells restored IFNγ signaling as demonstrated by the induction of HLA-DR expression on cytokine-treated transfectants (Fig. 6A). Consistently, autologous CD4$^+$ T cells that did not respond to Ma-Me-61g cells became activated in the presence of IFNγ-treated *JAK1*-transfected Ma-Mel-61g cells (Fig. 6B). These data demonstrated that IFNγ resistance of melanoma cells, allowing HLA-DR negative phenotype maintenance, could lead to CD4$^+$ T cell resistance.

HLA class I negative melanoma cells resist IFNγ-inducible HLA class II expression by CIITA promoter methylation

Expression of CIITA is driven by multiple promoters, including IFNγ-inducible promoter IV.[29] CpG islands of this promoter have been reported to be frequently methylated in cancer, thus leading to repression of CIITA transcription driven by this promoter.[23] Cell lines established from consecutively metastases of the patients Ma-Mel-48 (Ma-Mel-48a, Ma-Mel-48b, Ma-Mel-48c) and Ma-Mel-100 (Ma-Mel-100a, Ma-Mel-100b) were treated with IFNγ and analyzed for surface expression of HLA-DR and HLA class I. As shown in Fig.6C, none of the cell lines of patient Ma-Mel-48 showed upregulation of

HLA-DR after IFNγ treatment, although previous studies demonstrated an intact IFNγ signaling in these cells.[5] While Ma-Mel-100a cells increased HLA-DR expression in the presence of IFNγ, Ma-Mel-100b remained completely HLA-DR-negative (Fig.6D). Also in these cell lines, IFNγ signaling was found to be functional, indicated by phosphorylation of STAT1 and induction of IRF1 analyzed by Western Blot (Fig. 6E). Combined bisulfite restriction analysis (COBRA) was applied to analyze the role of DNA methylation in IFNγ resistance of HLA class II expression. Using primers for amplification of a previously defined CpG island (designated GM2) in promoter IV of the CIITA gene,[23] we demonstrated that Ma-Mel-48a, Ma-Mel-48b and Ma-Mel-48c cells show complete methylation in GM2, in line with the non-reactivity of HLA class II to IFNγ treatment. Interestingly, while Ma-Mel-100a showed only partial CpG methylation of the amplified DNA region, extensive methylation was observed for Ma-Mel-100b cells, in line with maintenance of its HLA class II-negative phenotype in the presence of IFNγ In summary, these data underline DNA methylation as an important mechanism of tumor cells to become resistant to IFNγ mediated upregulation of HLA class II molecules, thus leading to immune escape towards antitumoral $CD4^+$ T cells.

Discussion

Many barriers of $CD8^+$ T cell mediated immunotherapy of cancers have been described over the last years. It is widely accepted that selective pressure of the immune system can lead to the development of immune escape variants resistant to the activity of cytotoxic $CD8^+$ T cells. Thus, it is of importance to define recognition and sensitivity of melanoma cells for other potential anti-tumor effectors to be targeted by immunotherapy.

In recent years $CD4^+$ T cells came into focus, mainly because of their ability in boosting CTL cytotoxicity and preventing their exhaustion.[17, 30] Basis for this anti-tumor immunity is the differentiation capacity of $CD4^+$ T cells into multiple different subsets. The Th1 lineage of $CD4^+$ T cells is producing large amounts of IFNγ, TNFα and IL-2, by which they activate $CD8^+$ T cell responses or other immune cells.[31, 32]

Our results clearly show that melanoma cells expressing MHC class II molecules can be recognized and killed by autologous $CD4^+$ T cells. However, we also observed a heterogeneous pattern of constitutive HLA class II expression on melanoma cells *in situ* and *in vitro.* At baseline, variations in the expression level of HLA class II molecules led to differences in $CD4^+$ T cell recognition, as shown in patient system Ma-Mel-61. Most of the cell lines studied showed an upregulation of HLA class II expression in response to IFNγ treatment. In the case of IFNγ-treated Ma-Mel-61a cells, expression level of HLA class II were comparable to those of Ma-Mel-61b cells, but still, the recognition by autologous $CD4^+$ T cells was very weak. Possible explanation is a lower immunogenicity due to different antigen expression pattern, e.g. downregulation of specific immune-dominant antigens that are still present in Ma-Me-61b, as described e.g. for differentiation antigens.[33] Another explanation could be the expression of immune-inhibitory molecules like PD-L1, leading to suppression of T cells.

Upon activation $CD4^+$ T cells can secrete large amounts of IFNγ described to exhibit anti-proliferative and pro-apoptotic features acting on tumor cells, leading to so-called bystander-killing of tumor cells. Interestingly, the metastasis Ma-Mel-61g, excised late in metastatic disease, showed a defect in the IFNγ pathway leading to loss of sensitivity towards IFNγ mediated anti-proliferative activity (Sucker et al., submitted), but also to a non-inducibility of HLA class II molecules on the cell surface and subsequently to a resistance towards $CD4^+$ T cell recognition. Additionally, epigenetic alterations like DNA methylation also might drive melanoma cells to escape of immune surveillance either of direct or indirect antitumoral $CD4^+$ T cells or to a complete immune escape towards both $CD4^+$ and $CD8^+$ T cells. Of note, in case of the HLA class I negative Ma-

Mel-48c and Ma-Mel-100b cells, this resistance is even more of significance, because it indicates a complete resistance conventional T cell based therapies.[5] Besides MHC dependent T cells, CAR T cells could be an alternative for targeting tumor cells with described immune escape mechanisms, because they are able to recognize cell surface molecules independent of MHC expression.[34]

Besides release of IFNγ, $CD4^+$ T cells also showed production of the degranulation marker CD107a and perforin in response to co-culture with Ma-Mel-61b cells, indicating cytotoxic T cell activity, as already described for $CD4^+$ T cells from the peripheral blood of melanoma patients. [20] In the mentioned study, PBMC from melanoma patients were expanded in vitro and then analyzed for a cytotoxic phenotype. Expression of EOMES, perforin and Granzyme B was detectable in NY-ESO specific $CD4^+$ T cells and was even higher after Ipilimumab (αCTLA-4) treatment. In our setting, addition of IL-2 and IL-15 to MLTC cultures could have boosted the amplification of such tumor-specific cytotoxic $CD4^+$ T cells. IL-15 is known to enhance IFNγ production of $CD8^+$ T cells together with IL-2 in an in vitro breast cancer model or in $CD4^+$ T cells in multiple sclerosis.[35, 36]

Other publications identified engagement of the costimulatory molecule OX-40 on $CD4^+$ T cells to be responsible for expansion, memory and cytokine secretion and even for differentiation of $CD4^+$ T cells into a cytolytic phenotype with Th1 and Th2 properties that is able to kill melanoma cells *in vitro*.[37-39] These OX-40 induced $CD4^+$ CTLs were expressing Granzyme B and its transcription factor EOMES.[40] However, differences in cytolytic activity between autologous $CD4^+$ and $CD8^+$ T cells were visible in xCELLigence based assay: the decrease in proliferation of the tumors cells occurred faster and more profound when $CD8^+$ T cells were added.

Interestingly, expression of HLA class II molecules have to be considered also as a mechanism potentially related to immune suppression. The group of Svane et al. showed that an increase in HLA class II expression in human melanoma tumors was associated with enhanced $CD4^+$ T cell recognition by TILs.[41] They additionally observed TNFα production to be an inherent feature of tumor specific $CD4^+$ T cell response, but this TNFα in turn dampened $CD8^+$ T cell response. Thus, upregulation of HLA class II molecules by melanoma cells might reflect an immune escape mechanism towards the cytolytic function of $CD8^+$ T cells. It indicates that the immune-negative features of TNFα possibly outpace the potential anti-tumor activity of tumor specific $CD4^+$ T cells. We did not analyze TNFα production in our $CD4^+$ T cells, thus it could be possible that TNFα production might have an impact on $CD8^+$ T cells in a

more physiological setting. Furthermore, HLA class II engagement by LAG-3 on T cells has been reported to contribute to resistance to Fas-mediated or drug-induced apoptosis of melanoma cells.[42] Expressed on $CD4^+$ and $CD8^+$ T cells, LAG-3 shows structural similarities to CD4 and thereby binds to HLA class II molecules. Following binding, it guides inhibitory signals into the T cells and subsequently impairs proper T cell function.[43]

Additionally, only recently a publication showed that melanoma specific MHC class II expression can be used as a surrogate marker for tumor cells with an inflammatory signature marking cells that are more responsive to PD-1 targeted therapies. [44]

In summary, we confirmed the expression of HLA class II molecules on human melanoma tissue and early melanoma cell lines. This expression seems to be very heterogeneous, even within consecutive patient metastases, pointing towards possible barriers in the usage of tumor-specific autologous $CD4^+$ T cells in immunotherapy. Moreover, genetic and epigenetic changes affecting CIITA transcription or induction have to be taken into consideration when predicting the effectiveness of T cell based therapies. In addition, we confirmed the existence of $CD4^+$ T cells in the peripheral blood of melanoma patients that are able to differentiate into tumor-specific cytotoxic effectors expressing Perforin and CD107a and are able to lyse target cells.

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Figures

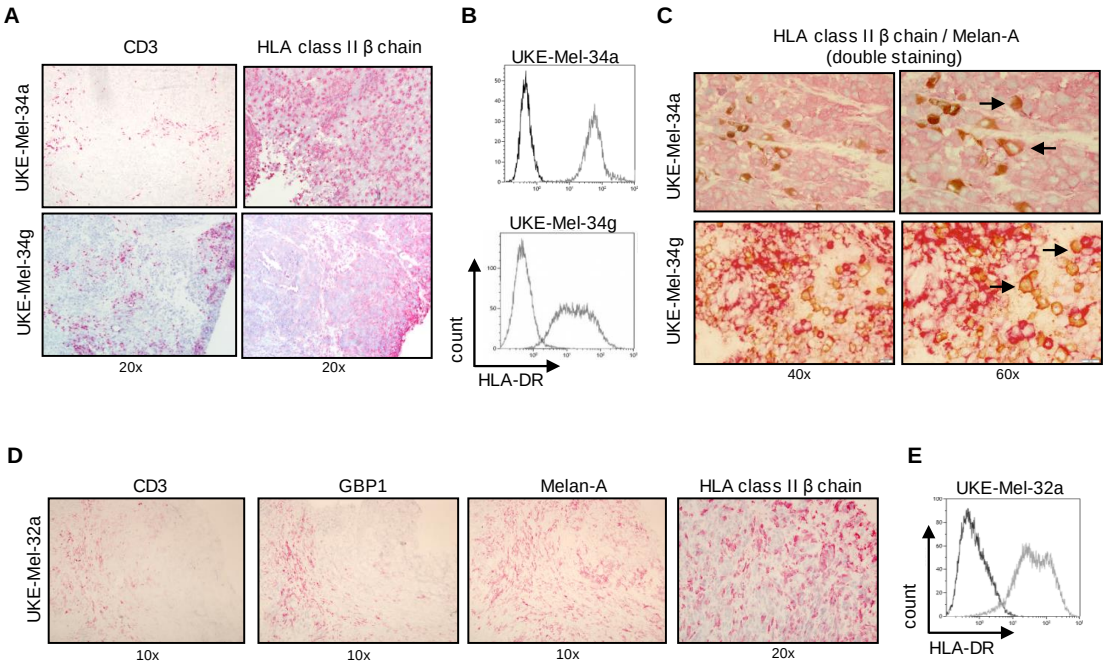


Figure 1.
Constitutive IFNγ-independent HLA class II expression on melanoma cells *in situ* and *in vitro*. A, Expression of CD3 and HLA class II β chain in serial cryostat tissue sections of melanoma metastases UKE-Mel-34a and UKE-Mel-34g, determined by immunohistochemistry. B, HLA-DR expression on corresponding melanoma cell lines. C, Co-staining of tissue sections for HLA class II β chain (red color) and melanoma marker Melan-A (brown color). Black arrows indicate HLA class II^{positive} melanoma cells. D, Expression of CD3, IFNγ-inducible GBP1, Melan-A and HLA class II β chain in serial cryostat tissue sections of melanoma metastasis UKE-Mel-32a, determined by immunohistochemistry. E, HLA-DR expression on corresponding melanoma cell line UKE-Mel-32a.

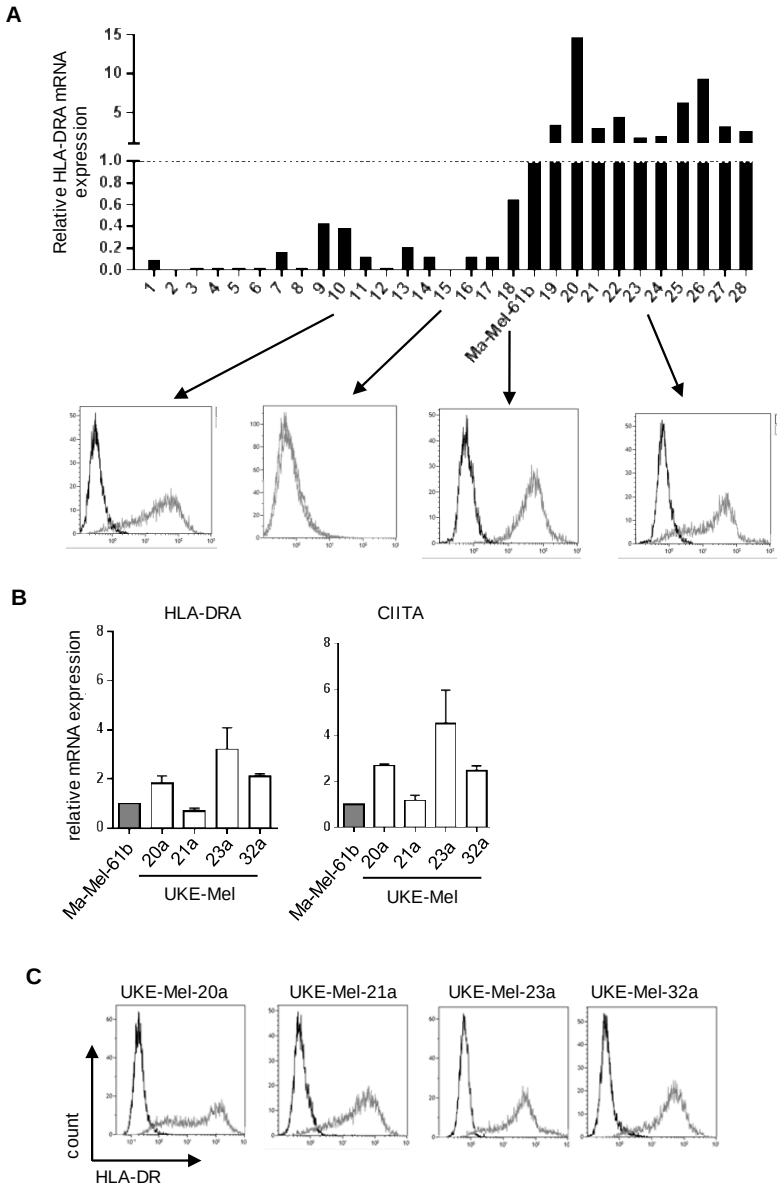


Figure 2.
Heterogeneous CIITA-dependent HLA-DR expression on short-term cultured melanoma cells. A, Early passage melanoma cell lines (≤ passage 11) screened for HLA-DRA mRNA expression by qRT-PCR. Specific mRNA levels were normalized to endogenous GAPDH mRNA levels and depicted relative to HLA-DRA mRNA amounts in Ma-Mel-61b cells. B, Cells analyzed for mRNA expression of HLA-DRA and CIITA by qRT-PCR. Specific mRNA levels were normalized to endogenous GAPDH mRNA levels. Mean expression levels (+SEM) of two independent experiments depicted relative to the expression in Ma-Mel-61b cells. C, Cell surface expression of HLA-DR on early melanoma cell lines measured by flow cytometry. Representative data from one of two independent experiments.

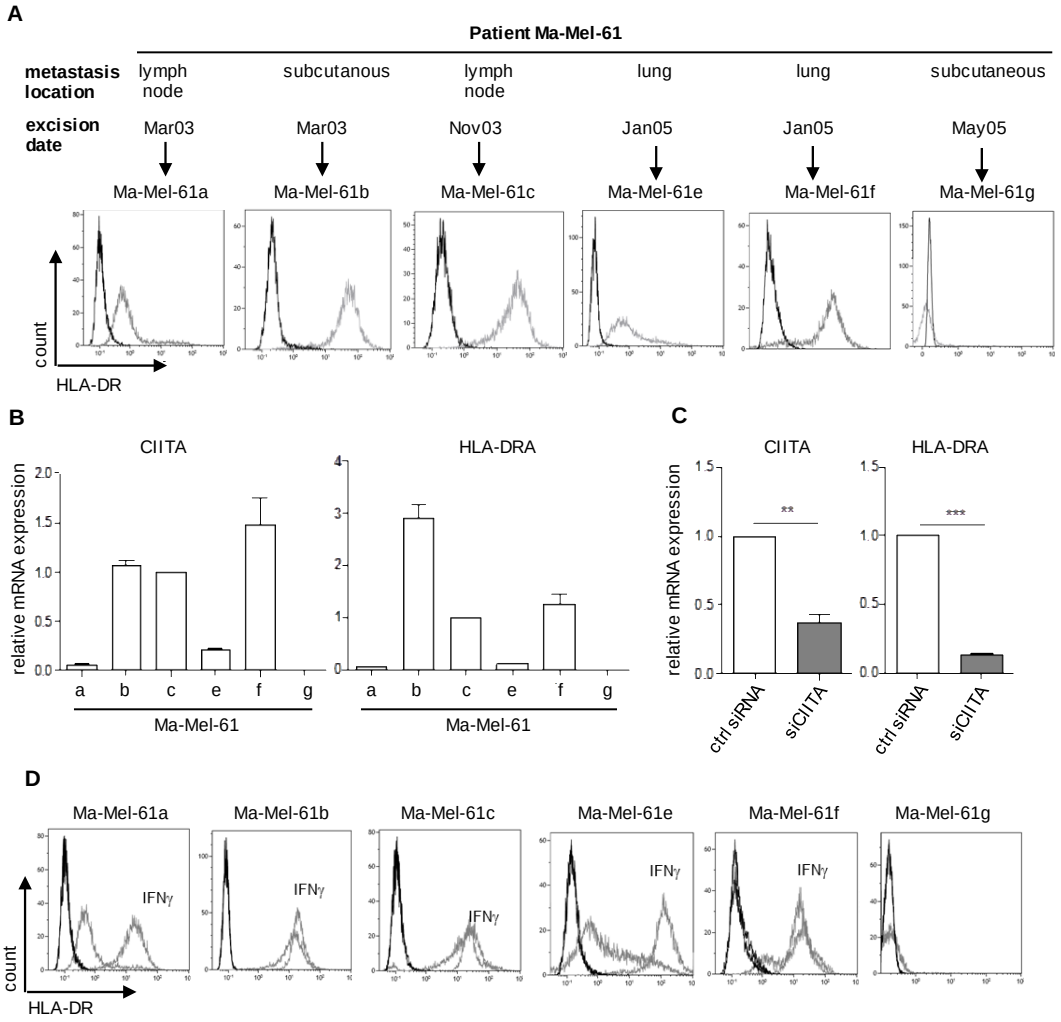


Figure 3.
Heterogeneous HLA class II expression on melanoma cells from recurrent lesions of patient Me-Mel-61. A, Metastases excised from melanoma patient Ma-Mel-61 in the course of disease progression. Cell lines Ma-Mel-61a, Ma-Mel-61b, Ma-Mel-61c, Ma-Mel-61e, Ma-Mel-61f and Ma-Mel-61g established from corresponding tissue samples. HLA-DR cell surface expression measured by flow cytometry. Representative data from one of at least two independent experiments. B, Cell lines analyzed for CIITA and HLA-DRA mRNA expression by qRT-PCR. Specific mRNA levels were normalized to endogenous GAPDH mRNA levels. Mean expression levels (+SEM) of two independent experiments depicted relative to the expression in Ma-Mel-61c cells. C, Ma-Mel-61b cells transfected with a pool of CIITA-specific siRNA (20nM) for 48 h. CIITA and HLA-DRA mRNA expression measured by qRT-PCR. Specific mRNA levels normalized to endogenous GAPDH mRNA levels. Mean expression levels (+SEM) of three independent experiments presented relative to control siRNA transfected cells. Experimental groups considered significantly different at $p < 0.01$ (**), $p < 0.005$ (***). D, Melanoma cell lines treated with IFNγ (500 U/ml) for 48 h. Surface expression of HLA-DR measured by flow cytometry. Representative data from one of at least two independent experiments.

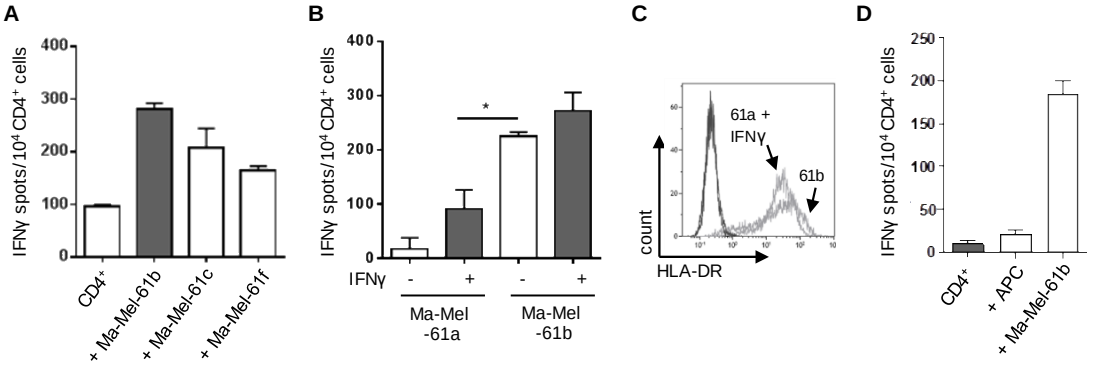


Figure 4.
Differential CD4⁺ T cell sensitivity of melanoma cells from consecutive patient lesions. A, Activation of CD4⁺ T cells in the presence of different autologous melanoma cell lines measured by IFNγ ELISPOT assay. Numbers indicate mean IFNγ spots/10^4 CD4⁺ T cells of duplicate determinations. Representative data from one of three independent experiments. B, Activation of CD4⁺ T cells by IFNγ-treated melanoma cells and control cells measured by IFNγ ELISPOT assay. Data represent means of three independent experiments (+SEM) shown as number of IFNγ spots/10^4 CD4⁺ T cells. Experimental groups considered significantly different at $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.005$ (***). C, Melanoma cell lines Ma-Mel-61a and Ma-Mel-61b treated with IFNγ (500 U/ml, 48 h) and analyzed for surface expression of HLA-DR by flow cytometry. Representative data from one of three independent experiments. D, Activation of CD4⁺ T cells in the presence of autologous APC or Ma-Mel-61b cells measured by IFNγ ELISPOT assay. Numbers indicate mean IFNγ spots/10^4 CD4⁺ T cells of duplicate determinations. Representative data from one of two independent experiments.

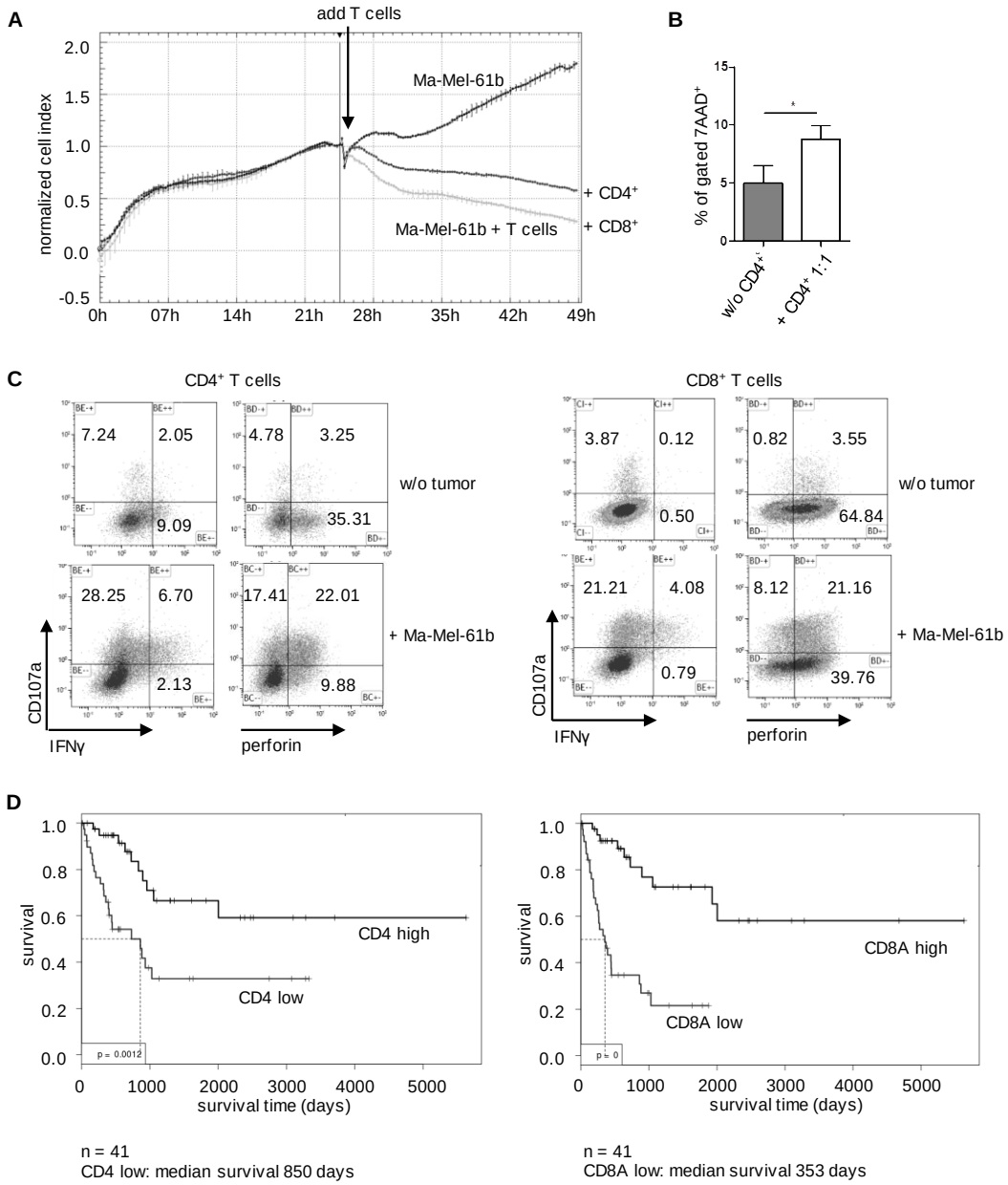


Figure 5.
Cytolytic activity of CD4+ T cells against autologous melanoma cells. A, Ma-Mel-61b cells seeded into xCELLigence E-plates for measurement of proliferation/survival over time, after 20 h addition of autologous CD4+ and CD8+ T cells (ratio 1:1). Representative data from one of three independent experiments. B, Co-culture of CSFE-labeled Ma-Mel-61b cells with CD4+ T cells for 2 h followed by staining with 7-AAD. Measurement of dead (CFSE+/7AAD+) melanoma cells by flow cytometry. Data represent means of four independent experiments (+SEM). Experimental groups considered significantly different at $p < 0.05$ (*). C, CD4+ and CD8+ T cells stained for CD107a, IFNγ and perforin expression after co-incubation with Ma-Mel-61b cells for 4 h. D, Patient survival curves for *CD4* or *CD8A* mRNA expressing melanomas from TCGA samples.

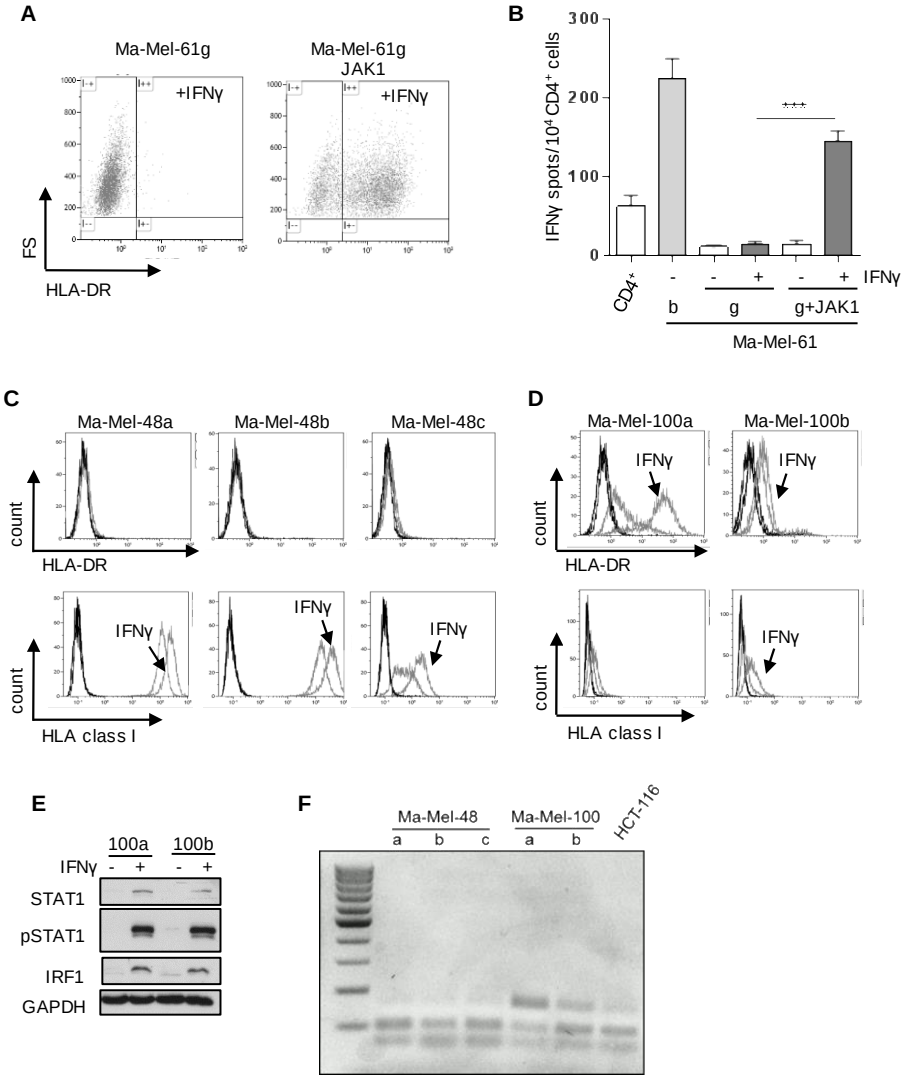


Figure 6.
Genetic and epigenetic alterations associated with CD4⁺ T cell resistance. A, Ectopic JAK1 expression in Ma-Mel-61g cells, treatment of transfectants and control cells with IFNγ (48 h) and measurement of HLA-DR surface expression by flow cytometry. Representative data from one of three independent experiments. B, Activation of CD4⁺ T cells in the presence of IFNγ-treated and non-treated melanoma cells measured by IFNγ ELISPOT assay. Data represent means from two of three independent experiments (+SEM) shown as number of IFNγ spots/10^4 CD4⁺ T cells. Experimental groups considered significantly different at $p < 0.005$ (***). C, D, Cell lines established from consecutive metastases of patient Ma-Mel-48 (Ma-Mel-48a, Ma-Mel-48b, Ma-Mel-48c) and Ma-Mel-100 (Ma-Mel-100a, Ma-Mel-100b) treated with IFNγ (500 U/ml, 48 h) and subjected to analysis of HLA-DR and HLA class I surface expression measured by flow cytometry. Representative data from one of at least two independent experiments. E, Expression of indicated proteins analyzed by Western Blot. GAPDH served as loading control. Representative data from one of at least two independent experiments are shown. F, Methylation of CpG island GM2 in promoter IV of the *CIITA* gene of HLA class I-positive and -negative melanoma cell lines. COBRA assay performed on genomic DNA of indicated cell lines, HCT116 cells served as positive control.

Supplementary material and methods

Mixed lymphocyte tumor cell cultures (MLTC)

Tumor-reactive T cells were established following a previously described protocol.(Sucker et al., 2014) Briefly, $CD4^+$ and $CD8^+$ T lymphocytes were isolated from patient PBMC using anti-CD4 or anti-CD8 MicroBeads (Miltenyi Biotech) (Supplemental Fig. S1). Isolated T cells ($1x10^6$) were co-cultured in 24-well culture plates with $1x10^5$ irradiated (120 Gy) autologous tumor cells per well in 2 ml of AIM-V (GIBCO/BRL) supplemented with 10% (vol/vol) human serum. $CD4^+$ T cell were additionally cultured with recombinant human IL-2 and IL-15 (20 IU/ml and 15 ng/ml) while $CD8^+$ T cells were cultured solely with IL-2 (250 U/ml). T cells were restimulated at weekly intervals with irradiated melanoma cells and medium was changed when necessary.

IFNγ ELISPOT assay

IFNγ enzyme-linked immunospot (ELISPOT) assay was performed as previously described.(Paschen et al., 2005) Briefly, multicreen-HA plates (Millipore, Bedford, MA) were coated with 5 µg/mL anti-hIFNγ mAb 1-D1K (Mabtech, Stockholm, Sweden). $1x10^4$T cells were seeded in RPMI complete medium. After preincubation with a specific HLA class II blocking antibody or its isotype, $5x10^3$ cells/well tumor cells were added. After 20-24 hours incubation at 37°C in 5% CO_2, a biotinylated secondary anti-hIFNγ antibody (1 µg/mL, clone 7-B6-1, Mabtech) was used and spots were developed by the sequential addition of 1:1000 diluted ExtrAvidin alkaline phosphatase and BCIP®/NBT Liquid Substrate System (Sigma-Aldrich, St. Louis, MO). Spot numbers were determined with the AID EliSpot reader (AID Diagnostika GmbH, Strassberg, Germany).

Flow Cytometry

Cells were stained for surface marker expression with directly labelled antibody (HLA-DR-PC7, Beckman Coulter). Background fluorescence was determined by unstained cells. After fixation, cells were analyzed using the Gallios flow cytometer (Beckman Coulter) and the Kaluza software.

To determine purity of enriched $CD4^+$ and $CD8^+$ T cells, cells were stained with directly coupled CD3-PC7, CD4-FITC and CD8-APC antibody or isotype control (Beckman Coulter) and fluorescence was measured by flow cytometry (Supplemental Fig. S1).

For CD107a Degranulation Assay and Intracellular Staining, Ma-Mel-61b cells were co-incubated with autologous tumor-specific $CD4^+$ T cells at an effector/target ratio of 1:1 for 1 hour in the presence of PE-conjugated CD107a mAb (Clone H4A3; BD Bioscience) or the corresponding isotype control. After co-culture for 1 hour, cells were incubated in the presence of 2mM Monensin (Sigma-Aldrich) and 10µg/mL Brefeldin A (Sigma-Aldrich) for additional 3 hours to inhibit CD107a internalization and cytokine release. Thereafter cells were washed and stained with anti-CD4-PC7 (clone SFC/12T4D11, Beckman Coulter) or anti-CD3-PC7 (clone UCHT-1, Beckman Coulter) for 30 minutes followed by Intracellular Staining using the Fixation/Permeabilization Concentrate and Diluent kit (eBioscience) and addition of anti-IFNγ-APC antibody (clone 4S.B3, Biolegend) and anti-Perforin-BrilliantViolet (clone dG9, Biolegend) Cells were analyzed in a Gallios flow cytometer and the Kaluza software was used for data analysis (Beckman Coulter).

xCELLigence based Real-time cytotoxicity assay

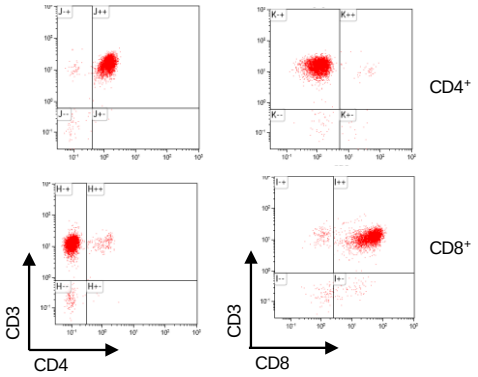
To analyze $CD4^+$ T cell cytotoxicity, 50 µl RPMI medium was added to E-Plates 16 (Roche) for measurement of background values. Subsequently, melanoma cells were seeded in an additional 100 µl medium at a density of 0.5 x 10^4 cells per well. Cell attachment was monitored using the RTCA SP (Roche) instrument and the RTCA software Version 1.1 (Roche). After 24 hours incubation at 37°C, T cells were added at an effector to target ratios (E:T) of 1:2.5 and 1:5. Upon addition of effector cells, impedance measurements were performed every 15 min for up to 96 hours. All experiments were performed at least in duplicates. Changes in electrical impedance were expressed as a dimensionless cell index (CI) value, which derives from relative impedance changes corresponding to cellular coverage of the electrode sensors, normalized to baseline impedance values with medium only. To analyze the acquired data, CI values were exported and tumor lysis was calculated in relation to the control cells lacking any effector T cells.

CFSE Cytotoxicity Assay

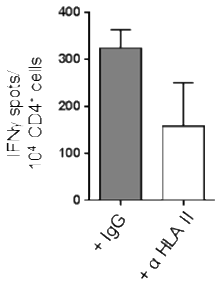
Killing of melanoma cells was determined in a flow cytometry assay. Therefore, 1 x 10^6 melanoma cells were labeled with 20 nM CFSE (Invitrogen). After 10 minutes, labeling was stopped by the addition of complemented medium. Then cells were washed twice with complemented medium. $CD4^+$ cells were added to 5 × 10^4 CFSE-labeled melanoma cells at an effector to target ratio of 1:1. After 2 hours of co-culture, 7-AAD

(BD Bioscience) was added to each sample at a final concentration of 1 µg/ml. Probes were measured directly by flow cytometry.

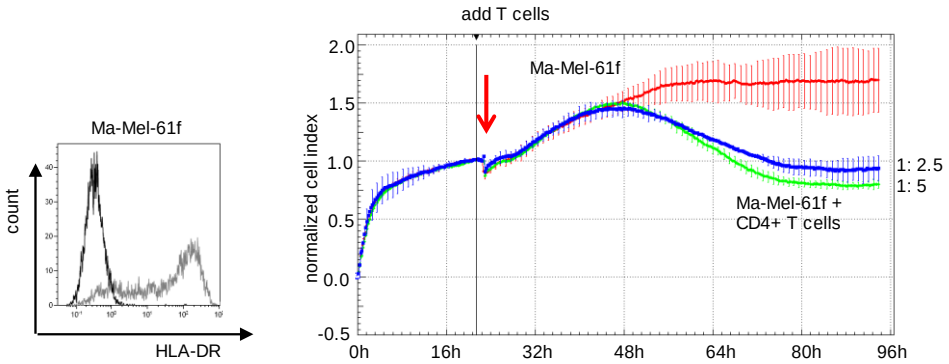
Supplemtentary Figures



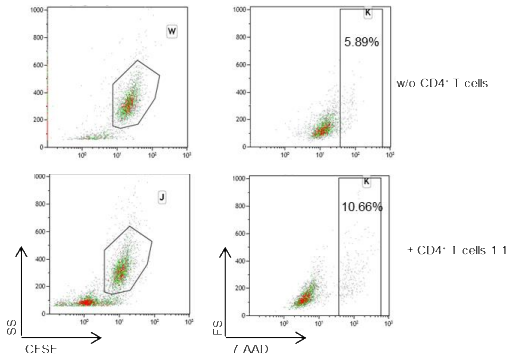
Supplementary figure S1.
Purity of enriched CD3⁺/CD4⁺ or CD3⁺/CD8⁺ T cells determined by flow cytometry. Autologous PBMC of patient Ma-Mel-61 were enriched with magnetic beads for CD4⁺ and CD8⁺ T cells.



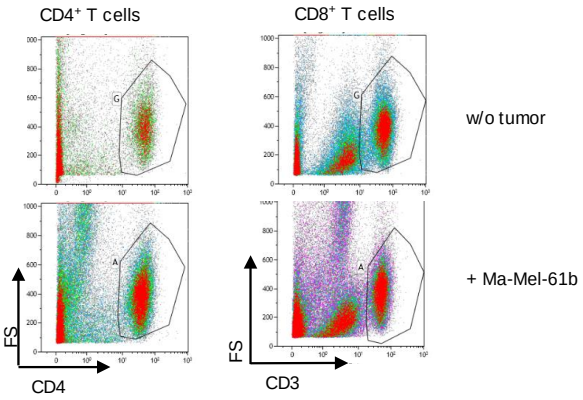
Supplementary figure S2.
Dependency of tumor recognition by CD4⁺ T cells on MHC class II determined by blocking with an anti-HLA class II antibody. Activation of CD4⁺ T cells in the presence of Ma-Mel-61b preincubated with blocking antibody or isotype control, measured by IFNγ ELISPOT assay. Numbers indicate mean IFNγ spots/10^4 CD4⁺ T cells of two independent exeriments.



Supplementary figure S3.
Cytolytic activity of CD4⁺ T cells against autologous melanoma cells. Ma-Mel-61f cells seeded into xCELLigence E-plates for measurement of proliferation/survival over time, after 20 h addition of autologous CD4⁺ (ratio 1:2.5 and 1:5). Representative data from one of three independent experiments.



Supplementary figure S4.
Killing of CFSE-labeled Ma-Mel-61b by autologous CD4+ T cells, determined by flow cytometry. Co-culture of CSFE-labeled Ma-Mel-61b cells with CD4⁺ T cells for 2 h followed by staining with 7-AAD. Measurement of dead (CFSE⁺/7AAD⁺) melanoma cells by flow cytometry. Representative data from four independent experiments is shown.



Supplementary figure S5.
Cytolytic activity of CD4+ T cells against autologous melanoma cells. CD4+ and CD8+ T cells stained for CD4 or CD3 expression after co-incubation with Ma-Mel-61b cells for 4 h, determined by flow cytometry.

8. Discussion

8.1. The variety of T cell resistance mechanisms

Generally, different steps in the development of immune resistance are conceivable, and it is important to define them to consider further steps in T cell based immunotherapy.

As discussed in the manuscripts, in different melanoma patient systems consisting of consecutive metastases and autologous $CD4^+$ and $CD8^+$ T cells, evolution of melanoma cells towards an edited, less immunogenic phenotype was clearly shown. Immune escape mechanisms range from downregulation of antigens or presentation molecules to epigenetic alterations or genetic defects in signaling pathways. Also, heterogeneity within one tumor affecting expression of MHC molecules has been demonstrated in manuscript III.

Besides described mechanisms, other evasion mechanisms have been characterized in melanoma and other immunogenic tumor entities. Kelderman *et al*. classified resistance mechanisms by human tumors into intrinsic, naturally acquired and therapy induced resistance to define patient groups by immunogenic characteristics and their likelihood to benefit from therapies (Kelderman et al., 2014).

As naturally acquired resistance they define mechanisms of immune evasion that occur due to a first inherent immune reaction against the tumor that subsequently leads to resistance against immune mediated destruction. Patients showing this phenotype of resistance show immune infiltration and ongoing tumor immunity but will eventually not further benefit from immunotherapy. Underlying mechanisms are for instance the attraction and infiltration of immune suppressive cells like MDSCs or Treg cells as well as the expression of immune suppressive molecules such as PD-L1 and TIM-3 by tumor cells and PD-1 and LAG-3 by T cells (Dong et al., 2002; Sakaguchi et al., 2008; Sledzinska et al., 2015; Weide et al., 2014). IFNγ release by infiltrating, activated $CD4^+$ or $CD8^+$ T cells has been shown to upregulate PD-L1 on the tumor cell surface as a negative feedback loop, consequently leading to immune suppression (Taube et al., 2012). Simultaneously, studies claim PD-L1 expression to be a prognostic factor being positively correlated with response to immunotherapy, because it can be seen as a marker for an active inflamed tumor microenvironment and thus favoring a positive clinical outcome in treatment with cancer vaccines or Ipilimumab (Gajewski et al., 2010).

For patients that exhibit T cell infiltration and PD-(L)1 expression within the tumor but that at the same time do not benefit from immune checkpoint blockade, other resistance mechanisms have to be considered for further therapy options.

Another acquired type of immune resistance is the therapy-induced resistance that is triggered by constant but artificial immune pressure induced by immunotherapies and defines a subgroup of patients that experience only temporary disease regression but relapse under therapy. Mechanisms of tumors with this kind of resistance might be similar to naturally occurring resistance mechanisms but the source of immune pressure is different (Kelderman et al., 2014). Acquired immune resistance during disease progression was also observed for patient system Ma-Mel-48 (Article I) and Ma-Mel-86 (Article II), whereby the resistance could also potentially be acquired under the pressure of a therapy-induced immune response.

Besides naturally acquired or therapy-induced resistance mechanisms, intrinsic resistance is important to be considered when applying immunotherapy. In this regard, difficulties of lymphocytes in homing into the tumor, due to the lack of a tumor inflammatory microenvironment, are important mechanisms (Gajewski et al., 2010; Kelderman et al., 2014). Do lymphocytes infiltrate the tumor, their activation and anti-tumor activity can still be impaired due to immune suppressive cytokines or inhibitory molecules. Intrinsic expression of PD-L1 by tumor cells can be activated due to oncogenic mutations as shown for the induction of PD-L1 after loss of PTEN in glioblastoma cells (Parsa et al., 2007). In contrast to that, melanoma $BRAF^{V600E}$ as well as $BRAF^{wt}$ patient samples with a PTEN loss were shown to be less infiltrated by lymphocytes, but didn't show conclusive upregulation of PD-L1 due to PTEN loss (Peng et al., 2016). These patients are more likely not to respond to antibody treatment against PD-1 or CTLA-4 due to the lack of TILs.

8.2. MHC class II expression on melanoma cells and its role in anti-tumor immunity and tumor survival

One major reason for $CD8^+$ T cells as the main actors in cancer immunotherapy is their potency in inducing a cytotoxic response against cancer cells. However, defective presentation of antigens on MHC class I molecules can impair this immune response. Cancer cells express if only little MHC class II molecules on the cell surface. Melanoma, colorectal cancer cells as well as breast cancer cells, however, display an exception of this paradigm as they frequently show constitutive expression of MHC

class II presenting molecules (Goodwin et al., 2001; Shi et al., 2006; Warabi et al., 2000).

We showed in the last manuscript that melanoma cells with constitutive or IFNγ inducible expression of MHC class II are basically recognizable by autologous $CD4^+$ T cells in a MHC class II dependent manner (Heeke et al., to be submitted). However, we also detected limitations of this recognition, namely non-inducibility due to defects in the IFNγ signaling pathway or methylation of the CIITA promoter. Although direct recognition or killing of MHC class $II^{negative}$ melanoma cells would not be possible, bystander killing of MHC class $II^{negative}$ tumor cells is still conceivable. For this way of tumor eradication, the release of tumor antigen and subsequent uptake by APCs is required. They in turn activate tumor specific $CD4^+$ T cells leading to further activation of macrophages and NK cells by the secretion of IFNγ (Haabeth et al., 2014; Perez-Diez et al., 2007). Th1/M1 macrophages have been reported to indiscriminately kill surrounding tumor cells independently of their MHC class II expression in a myeloma model (Corthay et al., 2009). Bystander killing has been reported to act indiscriminately regardless whether the tumor cells express one specific antigen or lost its expression due to immune evasion mechanisms. A similar mechanism is of course also conceivable for MHC class $I^{negative}$ tumor cells with regard to the recognition by $CD8^+$ T cells.

Moreover, IFNγ exhibits pro-apoptotic and anti-proliferative effects on tumor cells and can inhibit cancer-angiogenesis (Beatty and Paterson, 2001; Takeda et al., 2002; Zaidi and Merlino, 2011). By boosting apoptosis of tumor cells, antigen release would be enhanced, leading to increase in tumor antigen uptake and their presentation on MHC class I and II and thereby to enhanced activation of priming and activation of naïve tumor specific $CD4^+$ and $CD8^+$ T cells. This initial antigen release by tumor cell death is e.g. manageable by combining small molecule inhibitors or radiotherapy with adoptive transfer of tumor specific T cells.

In contrast to that, IFNγ can also possibly upregulate inhibitory ligands such as PD-L1 and the immune-suppressive enzyme IDO in tumor associated DCs or tumor cells themselves leading to inhibition of immune response (Kondo et al., 2010; Wu et al., 2009).

However, MHC class II expression on tumor cells could possibly not only stimulate anti-tumor $CD4^+$ T cells but also antigen-specific $CD4^+$ Tregs. Immune suppressive $CD4^+$ Tregs can be divided into two subgroups of Tregs, namely naturally occurring Tregs that develop in the thymus, as well as adaptive Tregs, that are induced in the

periphery. While natural Tregs are able to recognize self-antigens via their T cell receptor and suppress $CD8^+$ or $CD4^+$ T cells in a contact dependent manner, adaptive Tregs recognize tissue specific or foreign antigens and suppress T cells by release of suppressive cytokines (Bluestone and Abbas, 2003; Seddon and Mason, 1999). Of note, adaptive Tregs can be induced *ex vivo* from mature T cell subsets by culturing with antigens and suppressive cytokines such as IL-10 (Barrat et al., 2002).

It has been observed that Tregs are also involved in restricting cancer immunity, because they are able to recognize antigens presented by tumor cells such as NY-ESO-1, MAGE-A3, gp100 and TRP-1 (Francois et al., 2009; Jandus et al., 2009; Vence et al., 2007). Tumor antigen-specific Tregs therefore limit the efficacy of immunotherapy approaches. As shown in manuscript III, melanoma cells frequently express MHC class II molecules presenting antigens to $CD4^+$ T cells. These $CD4^+$ T cells also possibly differentiate into adaptive regulatory T cells and thus limiting the activity of other T cells in the tumor microenvironment. In one study, it has been conclusively shown that effector $CD4^+$ T cells and Tregs from one melanoma patient can recognize the same epitope of NY-ESO-1 presented by the same HLA allele (Ebert et al., 2012). These antigen-specific Tregs were not only circulating in the blood of the melanoma patients, but were also detectable in high numbers in the tumor microenvironment. Additionally, it has been shown that numbers of circulating Tregs are higher in melanoma patients with advanced disease compared to patients with resected tumors (Correll et al., 2010; Nicholaou et al., 2009). Thus, it is conceivable that presentation of antigens in an HLA class II restricted manner could not only induce effector populations of $CD4^+$ T cells but also Tregs that limit immunotherapy approaches.

Of note, tumor-infiltrating $CTLA^{high}$ Tregs have been shown to be one of the main targets of αCTLA-4 therapy, as administration of the blocking antibody leads to an elevated ratio of effector T cells to regulatory T cells, mainly by the depletion of Tregs via an FcγR mediated mechanism involving tumor-associated macrophages (Quezada et al., 2006; Romano et al., 2015). Thus, the observation of $CD4^+$ T cell subsets, their differentiation state and antigen specificity is of importance in immunotherapy, also with regard to combination of $CD4^+$ T cells mediated anti-tumor immunity and immune checkpoint blockade.

The most widely recognized function of MHC class II molecules is the presentation of antigens to T lymphocytes but these molecules are also important signaling receptors. Signals mediated by the engagement of the MHC class II molecules have been shown

to influence antigen-presenting function, adhesion, apoptosis, growth, differentiation and cytokine production of the antigen presenting cells (Barbieri et al., 2011). It has been shown that ligation of MHC class II with the specific mAb L243 inhibits Fas-mediated apoptosis of melanoma cells by the activation of the MAPK-Erk pathway (Aoudjit et al., 2004). Furthermore, activated $CD4^+$ and $CD8^+$ T cells are able to express LAG-3, a CD4 homolog type I membrane protein that binds to MHC class II but with higher affinity and leads to impaired lymphocyte proliferation and function (Huard et al., 1994; Workman and Vignali, 2003). Simultaneously, binding of LAG-3 to MHC class II on melanoma cells renders them resistant to Fas-mediated and drug-induced apoptosis by upregulation of the MAPK/Erk pathway (Hemon et al., 2011). Thus, MHC class II expression on melanoma cells can exhibit, beside antigen presentation, negative effects that counteract apoptosis-inducing properties of immunotherapy and may represent an additional immune escape mechanism.

8.3. Defining tumor antigens

For immunotherapeutic approaches that are based on T cells as cytotoxic mediators, the understanding of what T cells recognize on tumor cells is essential. It has become clear over the last decades that tumor vaccination approaches or ACT based on tumor-associated self-antigens such as differentiation antigens or cancer-testis antigens potentially fail because tolerance against some of these antigens has already been induced in early T cell development in the thymus (Gilboa, 1999; Klein et al., 2014). Thus, if T cells against self-antigens survive, their TCR normally shows a lower affinity against these epitopes compared to epitopes from mutated origin (Kvistborg et al., 2013). Therefore, it is of importance to define epitopes for specific T cells that are not sorted out in T cell development and show a high binding affinity to the TCR.

Non-synonymous mutations within a tumor may generate neo-nonself epitopes that can lead to T cell recognition. Simultaneously, exome sequencing has revealed that most of the epitopes recognized by TILs of melanoma patients that showed objective tumor regression, appeared to be mutant epitopes. Similar conclusions were made when clinical benefit positively correlated with the mutational landscape of melanoma patients receiving αCTLA-4 mAb and in patients with non-small cell lung cancer (NSCLC) receiving αPD-1 mAb (McGranahan et al., 2016; Rizvi et al., 2015; Robbins et al., 2013; Snyder et al., 2014; Van Allen et al., 2015). In a study with 515 cancer patients, RNA sequencing analysis revealed that higher mutational load correlated with

increased patient's survival, higher infiltration by CTLs and upregulation of PD-1 and CTLA-4 genes (Brown et al., 2014; Ward et al., 2016). Despite melanoma and NSCLC, neoantigen specific T cells were also detected in gastrointestinal cancer (Tran et al., 2015; Tran et al., 2014). In general, mutant epitopes are considered to be ideal targets for vaccination and ACT approaches, since they are unique for a tumor cell and not expressed in normal tissue.

Melanoma has shown to be the cancer with the highest load of somatic mutations among various types of solid and non-solid malignancies. Approximately 10 somatic mutations per megabase (mb) of coding DNA can be found, pointing to a UV related signature (Alexandrov and Stratton, 2014). Most of these mutations seem to be passenger mutations but some of them have also been proven to be oncogenic (Wolfel et al., 1995). The high mutational load in melanoma is thus increasing the probability of inducing mutation-specific T cell responses. Several studies indicate that at least half of the tumor specific T cells are recognizing mutated antigens, although neoantigens specific T cells are not detectable in every patient (Lennerz et al., 2005; Linnemann et al., 2015).

It already has been shown that mutation-specific T cells can infiltrate human melanomas and thus can be helpful for immunotherapies, as shown in multiple approaches of ACT or vaccination (Robbins et al., 2013; van Rooij et al., 2013). Additionally, immune checkpoint blockade seems to act on the reactivity of neoantigen specific TILs as administration of CTLA-4 mAb alters their frequency and magnitude of response (Tumeh et al., 2014; van Rooij et al., 2013). Thus, mutated antigens are of special interest in cancer immunotherapies.

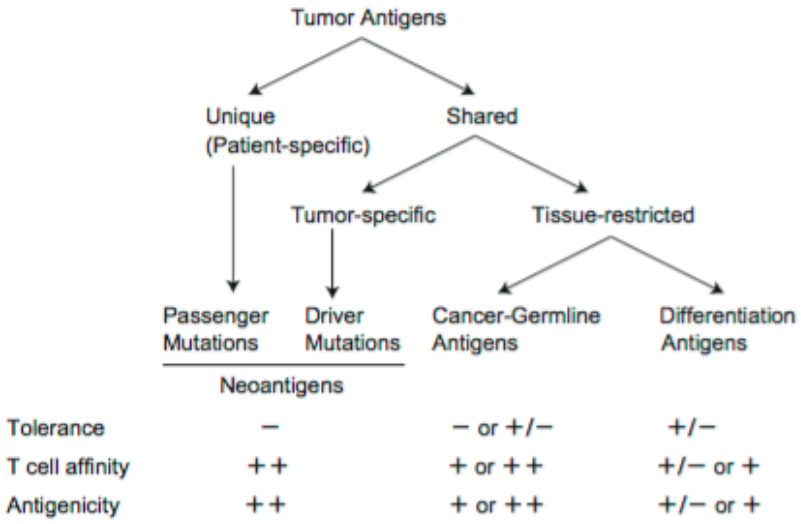


Figure 3: Human tumor antigens and their antigenicity (Kakimi et al., 2016).

Over the last years, effort has been made to define immunogenic non-synonymous mutations in various cancer models for $CD8^+$ T cells, regarding the challenge of the diversity of antigens possibly recognized by T cells. Deep sequencing based analysis of a tumor's 'mutanome', thus somatic mutations in individual tumors, can be applied to identify potential targets for T cell based therapies (Castle et al., 2012; Matsushita et al., 2012). Also in clinical settings, Next Generation Sequencing (NGS) based approaches are useable to predict reactivity of TILs for melanoma patients but also in other tumor entities (Robbins et al., 2013; Tran et al., 2014; van Rooij et al., 2013). Of notice, most of these mutations are not shared between patients, consequently meaning that identification of tumor mutations has to be based on an individual patient's tumor compared to healthy tissue (Schumacher and Schreiber, 2015). With new advanced methods as for example exome-capture it is now feasible to detect and list somatic mutations of one cancer patient within a couple of days (Cabanski et al., 2014). Various prediction algorithms can then valuate the probability of a specific epitope to be presented on MHC class I molecules *in silico* based on its binding affinity to the MHC class I molecule, its abundance and its processing (Gubin et al., 2015).
As immune-editing by T cells takes place it is conceivable that also neoantigen expressing melanoma cells are affected and consequently, tumor variants evolve that either lost expression of the antigen or are not able to express antigens due to defects in antigen processing or presentation. In the case of MHC class I deficiency or loss of antigens presented on MHC class I, endogenous $CD8^+$ T cells would not be able to detect malignant cells anymore (Matsushita et al., 2012).

Surprisingly, it has been shown that neoantigens are also presented on MHC class II molecules and therefore induce specific responses of $CD4^+$ T cells in melanoma patients upon ACT (Linnemann et al., 2015). Even though only around 0.5% of the total mutanome is leading to spontaneous $CD4^+$ T cell reactivity, it seems to be a frequent event in melanoma patients. In one study, most of the immunogenic mutated epitopes appeared to be presented on MHC class II (Kreiter et al., 2015). Thus, MHC class II restricted neoantigens might have a potential clinical relevance in spontaneous or induced $CD4^+$ T cell reactivity against cancers. Spontaneous $CD4^+$ T cell reactivity against neo-epitopes can also be triggered by vaccination, as shown in mice (Kreiter et al., 2015). In this study 16/17 of mutated epitopes led to cytokine secretion by $CD4^+$ T cells after vaccination of B16F10 mice with synthetic peptides. In the same study they showed in correlation analysis that immunogenic mutations have a better binding score for MHC class II molecules than non-immunogenic ones. One explanation they quote, is that length and sequence requirements for binding to MHC class II molecules are less strict compared to MHC class I molecules, thus facilitating presentation of neo-epitopes to $CD4^+$ T cells (Arnold et al., 2002; Kreiter et al., 2015). On the other hand, these less strict requirements for peptide binding seem to problematize prediction of MHC class II epitopes, as the peptide binding groove is opened compared to MHC class I molecules, thus leading to higher variation in epitopes that are able to bind to MHC class II (Gubin et al., 2015).

In another study with a cholangiocarcinoma patient, they found that TILs from the patient's tumor contained $CD4^+$ T cells specific for the mutated form of ERBB2IP. Adoptive transfer of the expanded TIL population containing 95% neoantigen specific Th1 $CD4^+$ T cells led to an objective response that was durable for 20 months (Tran et al., 2014). Importantly, no adverse immune effects were detectable after transfer of neoantigen specific T cells, stressing the specificity of the T cells and the absence of off-target effects.

8.4. Advances in T cell based immunotherapy

As mentioned before, ACT protocols using autologous TILs eventually shows clinical response in 40-50% of melanoma patients but success is restricted by the limitations of TILs that can be obtained and enriched from the tumor as well as potential side effects, mainly attack of healthy tissue, when TILs recognize cognate tumor associated selfantigens. With respect to other cancer types such as childhood leukemia, breast or colon cancer, tumors are often not infiltrated by lymphocytes, presumably due to low

mutational load, or the specificity and function of TILs is unknown (Ogino et al., 2011; Ruffell et al., 2012). To circumvent the problem with the availability of TILs for ACT as well as to obtain T cells with highest avidity and specificity for desired epitopes, T cells with a transgenic TCR can be engineered (Abad et al., 2008; Johnson et al., 2009; Kerkar et al., 2011). For example, $CD8^+$ T cells specific for the immunogenic TAAs gp100 or Melan-A have been developed by transduction of patient's lymphocytes with retroviral vectors encoding for the TAA-specific α/βTCR genes (Duval et al., 2006; Johnson et al., 2009). Clinical response has been seen in 30% of melanoma patients that received T cells expressing the human TCR. As a consequence of the expression of gp100 also in normal tissue, T cells attacked healthy tissue leading to severe off-target effects. Also, unwanted assembly of the transgenic and the endogenous T cell receptor can lead to unknown specificity and attack of healthy tissue (Bonini and Mondino, 2015; Johnson et al., 2009; Restifo et al., 2012).

To circumvent these side effects, T cells with a chimeric antigen receptor (CAR) have been developed, which are synthetic receptors consisting of an antibody with desired specificity fused to the signaling domain of a TCR (Sadelain et al., 2003).

Engineering of CAR T cells has broadened adoptive immunotherapy also to less immunogenic malignancies that lack TILs for isolation and expansion (Duong et al., 2015). The CAR consists of a monoclonal antibody that binds tumor antigens or intact cell surface molecules. Since CARs are fused to intracellular signaling components, T cells get activated upon epitope binding (Jena et al., 2010; Willemsen et al., 2005; Zhang et al., 2003). Additionally, they are able to recognize cell surface molecules in a MHC non-restricted manner, thereby circumventing immune escape mechanisms such as MHC downregulation or defects in antigen processing or presentation as we saw in melanoma patient systems (Duong et al., 2015; Sucker et al., 2014; Zhao et al., 2016). Beside cell surface proteins CAR T cells can recognize carbohydrates and lipid antigens (Westwood et al., 2005). Several other manipulations of CAR T cells are possible, like the insertion of ICOS signaling domains for the differentiation of $CD4^+$ T cells into Th17 cells (Paulos et al., 2010), the over-expression of survival genes as well as the down regulation of pro-apoptotic molecules (Dotti et al., 2005; Rufer et al., 2001). Initiating differentiation into specific subsets of $CD8^+$ or $CD4^+$ T cells, e.g. cytotoxic $CD4^+$ T cells as we saw in our model, is conceivable by the inserted expression of lineage-specific transcription factors (Restifo et al., 2012). Because of their high avidity CAR-engineered T cells bypass the problem of central tolerance and side-effects due to unknown specificity. Nevertheless, side-effects such as cytokine

release syndrome of CAR-therapy have been observed in the treatment of B cell malignancies (Brentjens et al., 2013). Obviously, success of engineering of CARs is restricted to the availability of appropriate targetable structures on the tumor cells. However, targeting of intracellular molecules such as the tumor antigen NY-ESO-1, as shown in a preclinical myeloma xenograft mouse model, led to delayed tumor growth (Schuberth et al., 2013).

As observed for ACT, also vaccine-based immunotherapy approaches failed when targeting shared tumor antigens due to low binding affinity of TAA specific T cells, induced tolerance or off-target effects (Rosenberg et al., 2004). In one recent study, exome sequencing of metastases of three stage III melanoma patients was applied in order to identify neoantigens expressed by the tumor (Carreno et al., 2015). The identified neoantigens then served for the development of a DC based vaccine and led to a broad and specific $CD8^+$ T cell response. Two of the three analyzed patients had a stable disease and one patient no sign of recurrence. Importantly also here, no visible signs of adverse immune-related effects were observed.

Occurrence of suppressive mechanisms in immune therapy settings is leading to the rationale to combine ACT or cancer vaccines with immune checkpoint inhibitors. As combinatorial therapy with Ipilimumab (αCTLA-4) and Nivolumab (αPD-L1) induced response in over 50% of the patients with advanced melanoma compared to 20-30% with the single-agent therapy, it has become apparent that combining therapy approaches is the most feasible way to mount the best-possible anti-tumor response in order to attack different immune evasion checkpoints in tumor development (Wolchok et al., 2013).

8.5. Outlook on personalized medicine in immunotherapy of melanoma

Identification of biological processes within tumor cells and the patient's immune system has paved the way to a more direct approach to cancer therapies and has improved therapy tremendously. As mentioned before and as shown in the included manuscripts resistance against targeted therapy as $BRAF^{V600E}$ inhibitor treatment and immune resistance as well as escape mechanisms are a huge obstacle in successful melanoma therapy.

A complex interplay of tumor genetics and its microenvironment, including stroma cells and infiltrating immune cells, leads to immune responsive and unresponsive tumors.

Identifying underlying mechanism as well as the analysis of biomarkers as the basis for treatment decisions is a crucial objective in future oncological care.

As shown in manuscript I, resistance to tumor specific $CD8^+$ T cells arose within the metastasis process due to gradual acquisition of mutations leading to tumor escape. In manuscript II melanoma metastases of the patient Ma-Mel-86 were found to be poorly immunogenic, but to different degrees. Manuscript III therefore describes $CD4^+$ T cells as important mediators of cytotoxic activity against melanoma cells but also describes immune escape mechanisms protecting against $CD4^+$ T cells. Above all, there is the observation that heterogeneity plays an important role in the limitations of current therapy approaches. The gradual development of immune-resistant metastases as well as the outgrowth of such plus the heterogeneity within a patient and even within a tumor as shown for the HLA class II expression, renders immunotherapy nearly incapable to catch each tumor cell. Developments such as Genome Sequencing give the possibility of genetically characterizing tumors even before therapy to tailor therapy to the patient's mutations such as BRAF or NRAS (Stratton, 2011).

In relation to immunotherapy, identification of encoded (neo) antigens by whole exome sequencing is useful in order to address T cell epitopes. Prediction algorithms with computational tools are on the way to allow a comprehensive impression of a tumor's mutational burden as well as to predict the likelihood of the presentation on MHC molecules (Beroukhim et al., 2010; Coulie et al., 2014; Duan et al., 2014; Yadav et al., 2014). Mutational load of a tumor can then be used to predict response to αCTLA-4 therapy, as it has been shown to correlate with a cytolytic gene signature in melanoma patients (Van Allen et al., 2015). Of note, targeting neoantigens with specific vaccines or ACT approaches can still lead to immune evasion by cancer cells, resulting in the outgrowth of less immunogenic metastases, as it has been shown in mice (Matsushita et al., 2012).

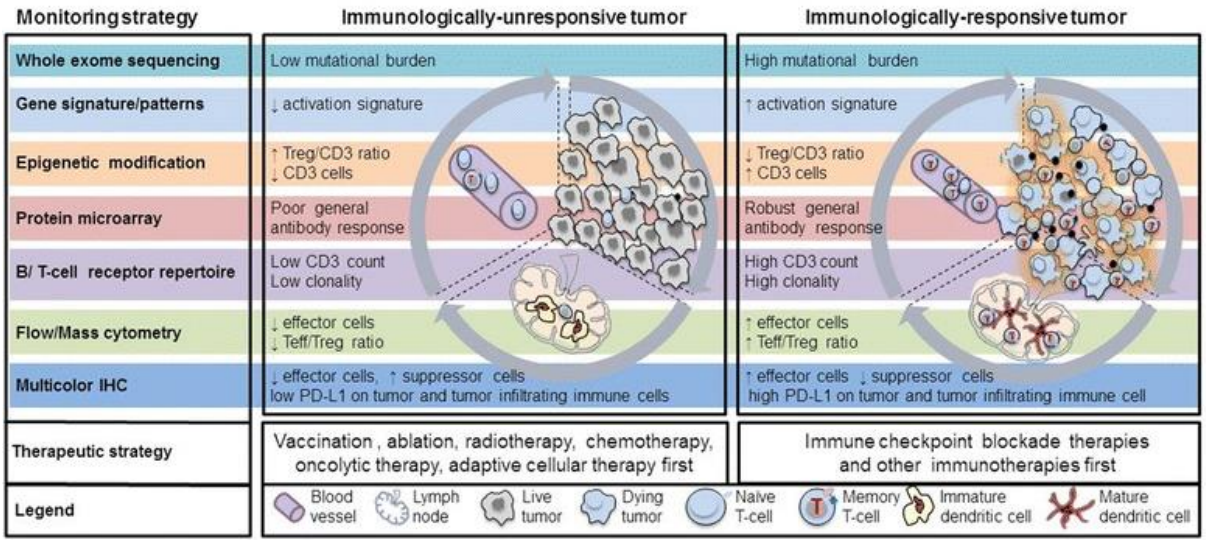


Figure 4: Biomarker prediction and personalized immunotherapy. Immunological responsive and unresponsive tumors are classified by the presence and the composition of immune cells in the tumor microenvironment. High-throughput technologies can further characterize the tumor by analyzing mutations, genetic and proteomic signatures and the phenotype and function of associated immune cells. Based on these assays, assessment of personal cancer immunotherapy choices could be possible. Arrows indicate decrease (↓) or increase (↑) (Yuan et al., 2016).

Analysis of gene signatures and expression patterns from fresh tumor tissue and isolated PBMCs from the patient's peripheral blood are an important tool to define the immune status of a patient, before considering certain immune therapies as well as for immune monitoring alongside with therapy. Up- or down-regulated gene signatures of the tumor microenvironment have been validated for several tumors in different studies (Erdag et al., 2012; Galon et al., 2012; Griewank et al., 2013). Infiltration by specific immune cells and their activation state can be evaluated from deep analysis of those signatures. Via flow/mass cytometry, the ratio of different lymphocyte subsets within tumors as well as antigen-specificities in terms of neoantigens can be determined in the peripheral blood or in tissue of cancer patients. Also baseline numbers of specific subsets of immune cells as MDSCs or $CD4^+$ T cells can be predicted by flow cytometry. Other techniques that can be used alongside with above discussed, are summarized in figure 5 and reviewed by Yuan *et al.*, emphasizing the development of high standardized sample collection procedures and the development of high-throughput assays in order to define biomarkers for personalized immunotherapy (Yuan et al., 2016).

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10. Abbreviations

aa	amino acid
ACT	adoptive cell transfer
AMP	adenosine monophosphate
AP-1	activator protein 1
APC	antigen-presenting cell
ATF	activating transcription factor
β2m	beta-2-microglobulin
CD	cluster of differentiation
$CD8^+$	CD8 positive
$CD4^+$	CD4 positive
CDK4	cyclin dependent kinase 4
CDKN2A	cyclin dependent kinase inhibitor 2A
CIITA	Class II transactivator
CREB	cAMP responsive element binding protein
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte associated protein 4
DNA	deoxyribonucleid acid
DC	dendritic cell
EOMES	Eomesodermin
ER	endoplasmatic reticulum
ERAP	endoplasmatic reticulum amino peptidase
ERK	extracellular signal-regulated kinases
FDA	Food and Drug Administration
FOXP3	forkhead box P3
gp-100	glycoprotein 100
HLA	human leukocyte antigen
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
LMP	low molecular weight protein
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
mb	megabase
MCA	methylcholanthrene

MCSF	macrophage colony stimulating growth factor
MDA	melanoma differentiation antigen
MDSC	myeloid derived suppressor cell
MEK	extracellular signal-regulated kinases
MICA/B	MHC class I chain related proteins A and B
MHC	major histocompatibility complex
MITF	microphtalmia-associated transcription factor
NF-Y	nuclear transcription factor Y
NGS	Next Generation Sequencing
NK cell	natural killer cell
NKT cell	natural killer T cell
NSCLC	non-small cell lung cancer
p53	glycoprotein 53
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
PFS	progression-free survival
PI3K	phosphatidyl-inositol-3-kinase
PLC	peptide loading complex
PTEN	phosphatase and tensin homolog
RAF	rapidly accelarated fibrosarcoma
RAG	recombination-activating genes
RAS	rat sarcoma protein
RFX	regulatory factor X
RGP	radial growth phase
STAT	signal transducer and activator of transcription
TAP	transporter associated with antigen processing
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper
TIL	tumor-infiltrating lymphocyte
TNF	tumor necrosis factor
Treg	regulatory T cell
UPS	Ubiquitin-Proteasome system
UV	ultraviolet

VEGF	vascular endothelial growth factor
VGP	vertical growth phase

11. List of figures

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13. Acknowledgments

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14. Author Contributions

Article I

Genetic evolution of T-cell resistance in the course of melanoma progression.

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Authors: Antje Sucker, Fang Zhao, Birgit Real, **Christina Heeke**, Nicola Bielefeld, Maβen S, Susanne Horn, Iris Moll, Raffaela Maltaner, Peter A. Horn, Bastian Schilling, Sabbatino F, Volker Lennerz, Matthias Kloor, Soldano Ferrone, Dirk Schadendorf, Christiane S. Falk, Klaus Griewank, Annette Paschen

I hereby certify that Christina Heeke contributed to the concept and experimental design of this article. She performed experiments depicted in figure 3B (PDL-1 and PDL-2 flow cytometry staining), figure 3C (CD54 flow cytometry staining) as well as Supplemental figures S2 and S5 A and was responsible for planning, execution, analysis and illustration of depicted results and corresponding figures.

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Place, Date Antje Sucker

_____ _____

Place, Date Annette Paschen

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Place, Date Christina Heeke

Article II

Melanoma lesions independently acquire T-cell resistance during metastatic latency.

Journal: Cancer Res. Accepted in May 2016

Authors: Fang Zhao, Antje Sucker, Susanne Horn, **Christina Heeke**, Nicola Bielefeld, Barbara Schrörs, Anne Bicker, Monika Lindemann, Alexander Roesch, Gustav Gaudernack, Mathias Stiller, Jürgen C. Becker, Volker Lennerz, Thomas Wölfel, Dirk Schadendorf, Klaus Griewank, Annette Paschen

I hereby certify that Christina Heeke contributed to the concept and experimental design of this article. She performed experiments depicted in figure 2 E as well as Supplemental figure S4 and S3 B. and was responsible for planning, execution, analysis and illustration of depicted results and corresponding figures. Additionally, she contributed to the experimental design and interpretation of Figure S3 A.

_____	_____
Place, Date	Fang Zhao
_____	_____
Place, Date	Annette Paschen
_____	_____
Place, Date	Christina Heeke

Manuscript III

Development of stable HLA class II-deficient melanoma phenotypes in the course of disease progression leads to total T cell resistance in the context of HLA class I deficiency

prepared for submission

Authors: **Christina Heeke**, Fang Zhao, Antje Sucker, Nicola Bielefeld, Raffaela Maltaner, Ulf Dittmer, Soldano Ferrone, Klaus Griewank, Dirk Schadendorf, Matthias Kloor, Annette Paschen

I hereby certify that Christina Heeke was the main contributor to the concept and experimental design of this manuscript. She was the principal scientist in the planning and execution of the experiments and in the writing of the manuscript. She performed all experiments shown, except for producing slices from cryopreseved tumors for immunohistochemistry and of generating stable JAK1 transfectants.

_____	_____
Place, Date	Annette Paschen
_____	_____
Place, Date	Christina Heeke

15. Curriculum Vitae

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

Der Lebenslauf ist in der Online-Version aus Gründen des Datenschutzes nicht enthalten.

16. Eidesstattliche Erklärung

Hiermit erkläre ich, gem. § 7 Abs. 2, d + f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _____ _____

Unterschrift der Doktorandin

Hiermit erkläre ich, gem. § 7 Abs. 2, e + g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Essen, den _____ _____

Unterschrift der Doktorandin

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Molekulare Grundlagen für T Zellresistenz im malignen Melanom“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Christina Heeke befürworte.

Essen, den _____ _____

Unterschrift d. wisschenschaftl. Betreuers/
Mitglieds der Universität Duisburg-Essen