Impact of IFN-γ resistance & MAPK inhibition on the immune surveillance of malignant melanoma - relevance for immune-based therapies

Auswirkungen von IFN-γ Resistenz & MAPK-Inhibition auf die Immunüberwachung des malignen Melanoms - Relevanz für immunbasierte Therapieansätze

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Kumulative Dissertation
zur Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für Biologie
an der
Universität Duisburg-Essen

vorgelegt von
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Juni 2018

Die der vorliegenden Arbeit zugrundeliegenden Experimente wurden in der Hautklinik im Labor „Molekulare Tumorimmunologie“ des Universitätsklinikums Essen durchgeführt.

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Tag der mündlichen Prüfung: 07.11.2018

DOI: 10.17185/duepublico/47582
URN: urn:nbn:de:hbz:464-20191127-122410-2

1. List of content

2. Preface

The herein presented thesis consists of a selection of three published original articles.

The work presented in this thesis was carried out between October 2014 and March 2018 and was supervised by principal investigator Prof. Dr. Annette Paschen in the Dermatology Department of the University Hospital Essen, Group ‘Molecular Tumor Immunology’.

3. Summary

The $BRAF^{V600E}$ mutation, expressed in approximately 50% of melanomas, mediates constitutive activation of the BRAF-MEK-ERK (MAPK) signaling pathway and therefore tumor proliferation. Rapid and high rate of clinical responses can be achieved by applying MAPK inhibitors (MAPKi), such as BRAFi and MEKi as single agents or in combination. Nevertheless, disease progresses in the majority of treated patients due to resistance. Combining targeted therapy with immune checkpoint blockade is proposed to improve the long-term survival of patients. However, to which extent BRAFi may affect melanoma immunogenicity over time remains largely unknown. Moreover, a considerable number of patients does not respond to immune checkpoint blockade pointing to immune escape mechanisms. Since IFN-γ is known to play an essential role in tumor control mediated by $CD8^+$ T cells, which are the anti-tumor effectors in immunomodulating antibody therapy, the first aim of the thesis was to explore mechanisms contributing to insensitivity of melanoma cells towards IFN-γ. The second objective was to investigate the impact of MAPK inhibition on the recognition of human melanoma cells by autologous $CD8^+$ T cells and NK cells, in order to provide mechanistic insights and valuable suggestions concerning treatment strategies for malignant melanoma. To accomplish this, several melanoma patient models consisting of tumor tissue, corresponding cell lines, autologous $CD8^+$ T cells and NK cells were used in the studies.

The first article included in this work demonstrates that IFN-γ-resistant melanoma clones can evolve in the course of disease under the selective pressure of an effective anti-tumor immune response in patients receiving different types of immunotherapy. Here, loss of chromosomal material and subsequent inactivating mutations in genes of the type II IFN signaling pathway (e.g. *JAK1*, *JAK2*) were found to be causative for the insensitivity of melanoma cells towards IFN-γ-mediated antitumorigenic effects. Moreover, it was demonstrated that IFN-γ resistant cells can further evolve into HLA class I negative populations due to silencing of genes involved in antigen presentation, the re-expression of which could no longer be induced by IFN-γ. This mechanism can lead to melanoma cell escape from $CD8^+$ T cell recognition rendering immunotherapeutic approaches ineffective. These results indicate that screening of tumor lesions for genetic defects in the IFN-γ signaling pathway should be considered in selecting patients for immunotherapy.

The two following publications deal with the effects of MAPKi on the recognition of different patient-derived melanoma cell lines by NK cells and T cells. Article II presented in this thesis demonstrates that already after 2-3 days of BRAFi treatment melanoma cell variants evolve, which escape NK cell-mediated recognition by strongly decreasing the surface expression of ligands for NK cell activating receptors. However, ligand downregulation and thus impaired NK cell recognition of BRAFi-treated melanoma cells could be counteracted by simultaneous application of the HDAC inhibitor, sodium butyrate, favoring combination therapy that in addition might combine the direct pro-apoptotic effects of both drugs towards melanoma cells. The third article demonstrates the evolution of melanoma cross-resistance to the pre-existing tumor-specific T cell repertoire during prolonged BRAFi treatment. While efficiently recognizing short-term (3, 7 days) BRAFi-treated melanoma cells, antigen-specific $CD8^+$ T cells were less responsive towards long-term (14, 21 days) exposed tumor cells due to a time-dependent downregulation of target antigens. Thus, MAPKi strongly alters the tumor antigen expression profile over time favoring outgrowth of melanoma variants cross-resistant to both T cells and targeted therapy, suggesting that MAPKi treatment preceding T cell-based immunotherapy might be disadvantageous for melanoma treatment.

Therefore, to induce long-lasting benefit and to avoid cross-resistance it seems reasonable to complement targeted therapy by an early sequential or simultaneous immunotherapy, but only if no genetic defects in IFN-γ signaling pathway and/or antigen presentation are present within the tumor.

4. Zusammenfassung

Die $BRAF^{V600E}$-Mutation, die in etwa 50 % der kutanen Melanome auftritt, bewirkt eine konstitutive Aktivierung des BRAF-MEK-ERK (MAPK)-Signalwegs und damit die Tumorproliferation. Ein schnelles und hohes klinisches Ansprechen kann durch die Anwendung von MAPK–Inhibitoren (MAPKi) wie BRAFi und MEKi, als Einzelwirkstoffe oder in Kombination, erreicht werden. Dennoch schreitet die Erkrankung bei der Mehrzahl der behandelten Patienten aufgrund von Resistenzen voran. Um das Langzeitüberleben von Patienten zu verbessern wird eine Kombination aus gezielter Therapie und Immun-Checkpoint-Blockade vorgeschlagen. Inwieweit BRAFi die Immunogenität des Melanoms über die Zeit beeinflussen kann, ist jedoch weitgehend unbekannt. Darüber hinaus zeigt ein beträchtlicher Teil der Patienten kein Ansprechen auf die Immun-Checkpoint-Blockade, was auf "immune escape"-Mechanismen hindeutet. Da IFN-γ bekanntermaßen eine essentielle Rolle bei der Tumorkontrolle durch $CD8^+$ T-Zellen spielt, welche die Anti-Tumor-Effektoren in der immunmodulierenden Antikörpertherapie darstellen, war es das erste Ziel dieser Arbeit, Mechanismen zu untersuchen, die zur Insensitivität von Melanomzellen gegenüber IFN-γ beitragen. Das zweite Ziel bestand darin, die Auswirkungen der MAPK-Hemmung auf die Erkennung von humanen Melanomzellen durch autologe $CD8^+$ T-Zellen und NK-Zellen zu untersuchen, um Erkenntnisse hinsichtlich der zugrundeliegenden Mechanismen zu gewinnen und Vorschläge zu möglichen Behandlungsstrategien für das maligne Melanom zu entwickeln. Dazu wurden mehrere Patientenmodelle bestehend aus Gewebe, korrespondierenden Zelllinien, autologen $CD8^+$ T-Zellen und NK-Zellen verwendet.

Der erste Artikel in dieser Arbeit zeigt, dass IFN-γ-resistente Melanomzell-Populationen im Krankheitsverlauf unter dem selektiven Druck einer wirksamen Anti-Tumor-Immunantwort bei Patienten, die verschiedene Arten von Immuntherapie erhalten, entstehen können. Dabei wurden der Verlust von chromosomalem Material und nachfolgende inaktivierende Mutationen in Genen des Typ II IFN-Signalwegs (z.B. *JAK1*, *JAK2*) als ursächlich für die Unempfindlichkeit von Melanomzellen gegenüber den antitumorigenen Eigenschaften von IFN-γ identifiziert. Darüber hinaus konnte demonstriert werden, dass sich IFN-γ-resistente Zellen zu HLA Klasse I-negativen Populationen entwickeln können, in dem Gene, die an der Antigenpräsentation beteiligt sind, expressionell stillgelegt werden und nicht länger

durch IFN-γ induziert werden können. Dieser Mechanismus kann dazu führen, dass Melanomzellen vollständig der Erkennung durch $CD8^+$ T-Zellen entkommen, was immuntherapeutische Ansätze unwirksam machen würde. Diese Ergebnisse weisen darauf hin, dass das Screening von Tumorläsionen auf genetische Defekte im IFN-γ-Signalweg bei der Auswahl von Patienten für eine Immuntherapie in Betracht gezogen werden sollte.

Die zwei folgenden Veröffentlichungen befassen sich mit den Auswirkungen von MAPKi auf die Erkennung verschiedener Patienten-abgeleiteter Melanomzelllinien durch NK-Zellen und $CD8^+$ T-Zellen. Der in der Arbeit vorgestellte Artikel II zeigt, dass bereits nach 2-3 Tagen unter BRAFi-Behandlung Melanomzellvarianten entstehen, die der Erkennung durch NK-Zellen entgehen, in dem sie die Oberflächenexpression von NK-Zell-aktivierenden Liganden stark reduzieren. Der Herabregulierung von Liganden und der dadurch verschlechterten Erkennung von BRAFi-behandelten Melanomzellen durch NK-Zellen konnte durch die gleichzeitige Anwendung des HDAC-Inhibitors, Natriumbutyrat, entgegengewirkt werden. Darüber hinaus können die pro-apoptotischen Eigenschaften von BRAFi und HDACi gegenüber Melanomzellen durch die immunologisch favorisierte Doppelbehandlung kombiniert werden. Der dritte Artikel demonstriert die Entwicklung einer Kreuzresistenz des Melanoms gegenüber dem bestehenden tumorspezifischen T-Zell-Repertoire im Verlauf einer BRAFi-Behandlung. Während die antigenspezifischen $CD8^+$ T-Zellen kurzzeitig (3, 7 Tage) BRAFi-behandelte Melanomzellen effizient erkannten, zeigten sie eine eingeschränkte Reaktionsfähigkeit gegenüber langzeitexponierten (14, 21 Tage) Tumorzellen. Der Grund dafür war, die behandlungszeitabhängige Herunterregulation von Zielantigenen. Den Ergebnissen zu Folge verändert MAPKi das Expressionsprofil von Tumorantigenen über die Zeit der Behandlung stark und begünstigt das Auswachsen von Melanomvarianten, die sowohl gegen T-Zellen als auch gegen zielgerichtete Therapien resistent sind. Dies weist darauf hin, dass eine MAPKi-Behandlung, die der T-Zellen-basierten Immuntherapie vorangeht, für die Melanombehandlung nachteilig sein könnte.

Um einen langfristigen Nutzen zu erzielen und eine Kreuzresistenz zu vermeiden, erscheint es daher sinnvoll, die gezielte Therapie durch eine frühe sequenzielle oder simultane Immuntherapie zu ergänzen, jedoch nur dann, wenn keine genetischen Defekte im IFN-γ-Signalweg und/oder in der Antigenpräsentation im Tumor vorliegen.

5. Introduction

5.1. Malignant melanoma

Of all skin cancers, malignant melanoma is the most aggressive and deadly variant with an incidence of 15-25/100.000 cases per year (rising steadily) causing 75 % of skin cancer related deaths (Miller and Mihm, 2006; Schadendorf and Hauschild, 2014). Due to very early dissemination of tumor cells during progression and subsequent formation of lymph node and distant organ metastases patients diagnosed with stage IV melanoma have poor prognosis with a median survival reaching from 6-12 months (Schadendorf, Fisher, *et al.*, 2015). However in 80 % of melanoma cases the tumor can be surgically removed when detected very early (Gray-Schopfer, Wellbrock and Marais, 2007), demonstrating the great importance of an effective screening of early lesions as prevention of malignant melanoma.

5.1.1. Origin of malignant melanoma

Melanoma arises upon malignant transformation of melanocytes, which are pigment-producing cells of neuroectodermal origin that can be found in the skin, iris and mucosa (Schadendorf, Fisher, *et al.*, 2015). Consequently, melanoma occurs not only in the skin (cutaneous melanoma) but in rare cases also in eyes and in mucosal surfaces (Laver, McLaughlin and Duker, 2010; Seetharamu, Ott and Pavlick, 2010). Under normal circumstances the dark brown pigment melanin protects the skin melanocytes and keratinocytes from UV-induced DNA damage by reducing absorption and scattering of the radiation as well as absorbing reactive oxygen species (Gilchrest *et al.*, 1999). The amount of melanin correlates therefore with the sensitivity to sunlight making light-skinned and fair-haired individuals who often expose to solar radiation a high-risk group for development of melanoma (Gilchrest *et al.*, 1999). Other risk factors, next to UV exposure, include high number of melanocytic and atypical nevi, genetic background e.g. germline mutations in *CDKN2A* associated with familial melanoma, chronic immunosuppression and an age of over 50 years (Psaty *et al.*, 2010). Based on histological alterations the development of malignant melanoma has been described over 30 years ago by Clark and colleagues as a gradual process (Clark *et al.*, 1984). In the first step of this process a benign melanocytic nevus is arising as a consequence of altered melanocyte proliferation, which in the second step forms a dysplastic nevus

(melanoma *in situ*). In the following stage called radial-growth phase (RGP melanoma) the premalignant lesion grows and spreads through the epidermis, but exhibits only a very limited migratory potential. These changes in the critical transition step from the radial-growth phase to vertical-growth phase (VGP melanoma) in which melanoma cells break through the basement membrane and gain the ability to invade the surrounding tissue (dermis). In the final step of melanoma progression – the metastatic melanoma, tumor cells can enter the bloodstream and distribute through the body reaching distant organs and forming metastasis.

However, the development of melanoma does not necessarily have to progress through all the stages described by Clark et al. or reach the metastatic stage in any cases (Clark *et al.*, 1984; Miller and Mihm, 2006). Indeed, studies have shown that the majority of cutaneous melanoma appear to arise *de novo* and less frequent from preexisting nevi (Duffy and Grossman, 2012). Furthermore, the rate of malignant transformation of a nevus has been estimated to be very low (0.00005 to 0.003 % of nevi per year) (Tsao *et al.*, 2003).

5.1.2. Molecular alterations in malignant melanoma

At the molecular level cancer cells display a multitude of alterations that protect them from apoptosis, allow unlimited growth independently of growth factor availability, facilitate angiogenesis and ultimately lead to metastasis (Hanahan and Weinberg, 2011). In melanoma, one very important genetic alteration leading to unimpeded proliferation of the malignant cells, found in 50-60 % of the patients, concerns a mutation in the BRAF serine/threonine protein kinase causing an amino acid substitution from valine to glutamate at the position 600 (*BRAF*^{V600E}) (Brose *et al.*, 2002). Another, however, less frequently occurring mutation is BRAF^{V600K}, which accounts for approx. 10-30 % of patients bearing a BRAF^{V600} mutation (Rubinstein *et al.*, 2010; Li, Umbach and Li, 2017). As part of the RAS-RAF-MEK-ERK (mitogen-activated protein (MAP) kinase) signaling cascade the BRAF$^{V600E/K}$ mutant leads to constitutive activation of the pathway and subsequent expression of genes involved in growth and survival without the need for additional extrinsic growth signals (Davies *et al.*, 2002; Pratilas *et al.*, 2009). Another 15-20 % of melanoma cases exhibit activating mutations in the NRAS GTPase which likewise constantly activates the MAP kinase pathway (Omholt *et al.*, 2003; Giehl, 2005). Since mutated *BRAF* activates the MAPK pathway independently of RAS, *BRAF* and *NRAS* mutations co-

occur only very rarely in the same tumor (Goel *et al.*, 2006; Flaherty, Hodi and Fisher, 2012). Next to the MAP kinase pathway the PI3K-AKT pathway is also often hyper-activated in melanoma. Aberrant regulation of this signaling pathway can be caused by mutated RAS or by the loss of expression and/or function of the tumor-suppressor PTEN, which is a negative regulator of PI3K (Kwong and Davies, 2013). Interestingly, the combination of *PTEN* loss with *BRAF* mutation is found in up to 40 % of human melanomas while the association with NRAS is by far less prominent and described in about 4 % of cases (Schadendorf, Fisher, *et al.*, 2015). Another molecular mechanism has been implicated in the pathogenesis of familial melanoma and affects the Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) locus encoding for two distinct tumor-suppressor proteins ($p16^{INK4A}$ and $p14^{ARF}$). Mutations, deletions or transcriptional silencing of *CDKN2A* lead to loss of the cell cycle regulator $p16^{INK4A}$ and therefore promote cell cycle progression from G1- to S-phase releasing tumor cells from possible cell cycle arrest. Mutations in *CDKN2A* are described in 40 % of melanoma patients with a familial melanoma background (FitzGerald *et al.*, 1996; Goldstein *et al.*, 2006; Schadendorf, Fisher, *et al.*, 2015). In contrast, amplifications of Cyclin D1 and/or mutations in the Cyclin depended kinase CDK4 occur at much lower frequency in melanoma (Curtin *et al.*, 2005). Furthermore, amplifications of microphthalmia-associated transcription factor (MITF) - a master regulator of melanocyte development and differentiation, are found in 5-20 % of human melanomas, and shown to have oncogenic properties (Garraway *et al.*, 2005; Kawakami and Fisher, 2017). However, it should be noted that a single genetic aberration is not enough to transform normal melanocytes into metastatic melanoma. Rather, it is a combination of several alterations that occur gradually in the course of the transformation (Flaherty, Hodi and Fisher, 2012).

5.2. Immune effector cells & their role in immune surveillance of melanoma

Malignant melanoma is considered one of the most immunogenic tumors and is often used as a model to study tumor immunogenicity (Maio, 2012). Two main observations from the clinics contributed to this reputation – the occurrence of spontaneous, partial or even complete, remissions of primary melanoma lesions and the presence of tumor infiltrating lymphocytes (TILs) in these lesions. Importantly, these two facts have been shown to be directly associated with each other,

supporting the idea of immune surveillance in regressing melanomas already over 20 years ago (Ferradini *et al.*, 1993; Mackensen *et al.*, 1994). Recently, it was demonstrated that malignant melanoma displays the highest prevalence of somatic mutations among numerous cancer entities (Alexandrov *et al.*, 2013) and that high mutational load predicts clinical benefit of immune-based therapies e.g. adoptive T cell therapy, anti-PD-1 therapy or anti-CTLA-4 blockade (Van Allen *et al.*, 2015; McGranahan *et al.*, 2016; Lauss *et al.*, 2017). The multitude of immune therapeutic approaches used in the clinics, demonstrate the great importance of host immunity in melanoma defense. The main immune effector cells deeply involved in the tumor immune surveillance are $CD8^+$ T cells and natural killer (NK) cells, as described in the following.

5.2.1. $CD8^+$ T cells & HLA class I antigen presentation

$CD8^+$ T lymphocytes are effector cells of the adaptive immune system and play a crucial role in anti-cancer immunity through their capacity to recognize and kill transformed cells upon recognition by T-cell receptor (TCR) of specific tumor-derived antigenic peptides presented in the context of HLA (*human leukocyte antigen*) class I molecules on the surface of target cells. In melanoma a variety of tumor-associated antigens have been described over the years (Table 1).

In addition to the so-called shared antigens (shared between different cancers, tissues and patients) such as differentiation antigens and oncofetal antigens, tumors can express, due to their high rate of somatic mutations, unique, patient- and tumor-specific neoantigens. These antigens are generally considered to be ideal targets for $CD8^+$ T cells, because they are exclusively expressed on tumor cells, and thus are not supposed to be affected by the negative selection in the thymus, a process, which typically leads to tolerance towards self-antigens through elimination of potentially auto-reactive immune cells (Alexandrov *et al.*, 2013; Yarchoan *et al.*, 2017).

Table 1: Tumor-associated antigens in melanoma (Robbins *et al.*, 1996; Parmiani, 2001; Hodi, 2006)

Melanocyte lineage/differentiation antigens: • Tyrosinase • Tyrosinase related protein-1, -2 (TRP1, TRP2) • Glycoprotein 100 (gp-100) • Melanocyte antigen (Melan-A/MART-1)
Oncofetal/cancer-testis antigens: • Melanoma associated antigen (MAGE) family • B melanoma antigen (BAGE) family • G antigen (GAGE) family • NY-ESO-1
Overexpressed antigens: • Preferentially expressed antigen in melanoma (PRAME) • Chondroitin sulfate proteoglycan 4 (CSPG4) • Survivin
Mutated antigens/neoantigens: • β-Catenin • CDK4 & CDKN2A • N-RAS

Upon binding of a specific TCR to a cognate HLA class I/peptide complex on the surface of a tumor cell the cytotoxic T cell becomes activated and is able to kill the target cell through various mechanisms (van der Bruggen *et al.*, 1991; Boon *et al.*, 2006). The major mechanism engages secretion of lytic granules containing the pore-forming protein perforin and serine proteases named granzymes by cytotoxic T cells towards target cells triggering apoptosis and subsequent cancer cell destruction (Chavez-Galan *et al.*, 2009; Jenkins and Griffiths, 2010). Another killing mechanism requires the direct interaction of a death receptor with a cognate ligand e.g. Fas ligand (FasL) that is induced on activated T cells and the Fas receptor expressed on tumor cells. The binding of the Fas ligand to the Fas receptor induces FADD-mediated activation of Caspase-8 and thus apoptosis of target cells (Strasser, Jost and Nagata, 2009). In the third 'scenario', the pro-inflammatory cytokine TNF-α released by activated cytotoxic T cells binds to the TNF receptor 1 (TNFR1) localized

on the tumor cell inducing a multitude of apoptotic signaling pathways inside the target cell (Chavez-Galan *et al.*, 2009).

Apart from TNF-α, activated cytotoxic $CD8^+$ T cells produce and release also Interferon-γ (IFN-γ), which essentially contributes to the efficacy of T cell-mediated tumor control due to its pro-apoptotic and anti-proliferative effects (Ikeda, Old and Schreiber, 2002; Chawla-Sarkar *et al.*, 2003; Matsushita *et al.*, 2015) directed not only towards the proximate target cell but more importantly also acting on surrounding tumor cells in the microenvironment (Sanderson *et al.*, 2012). Binding of IFN-γ to its receptor on target cells induces signal transduction through the JAK1/2-STAT1-IRF1 pathway (Figure 1) and can lead to growth arrest and death in melanoma cells via different mechanisms (Chawla-Sarkar *et al.*, 2003; Gollob *et al.*, 2005; Matsushita *et al.*, 2015). Moreover, IFN-γ increases the immunogenicity of melanoma cells by inducing HLA class I (also referred to as *major histocompatibility complex* (MHC) class I) expression and therefore antigen presentation on tumor cells making them more susceptible to $CD8^+$ T cell-mediated recognition and destruction (Seliger, Ruiz-Cabello and Garrido, 2008). Accordingly, defects in IFN-γ signaling cascade of melanoma cells lead to impaired HLA class I-dependent antigen processing and presentation to $CD8^+$ T cells (Respa *et al.*, 2011).

Antigen processing and presentation is a multistep process that is executed by the antigen processing machinery (APM). In the first step, precursor epitopes are generated by proteasome-mediated degradation and further trimmed by cytosolic peptidases. Then the resulting peptides are transported to the endoplasmatic reticulum (ER) via TAP transporter proteins (TAP1 and TAP2) and eventually further processed by the ER peptidases ERAP1 and ERAP2. Matured peptides are finally loaded on HLA class I molecules. The assembly of the HLA class I heterodimer from a polymorphic heavy chain and a light chain β_2-microglobulin (β_2m) takes place in the ER lumen with the help of several ER chaperons and is completed by the binding of a specific peptide. Only complexes consisting of these three components are stable and can be forwarded from the ER to the Golgi and arrive at the cell surface for antigen presentation. Inversely, peptides and HLA class I molecules that fail to associate in the ER are transported back to the cytosol for degradation (van Endert *et al.*, 1994; Hughes, Hammond and Cresswell, 1997; Saric *et al.*, 2002; Neefjes *et al.*, 2011).

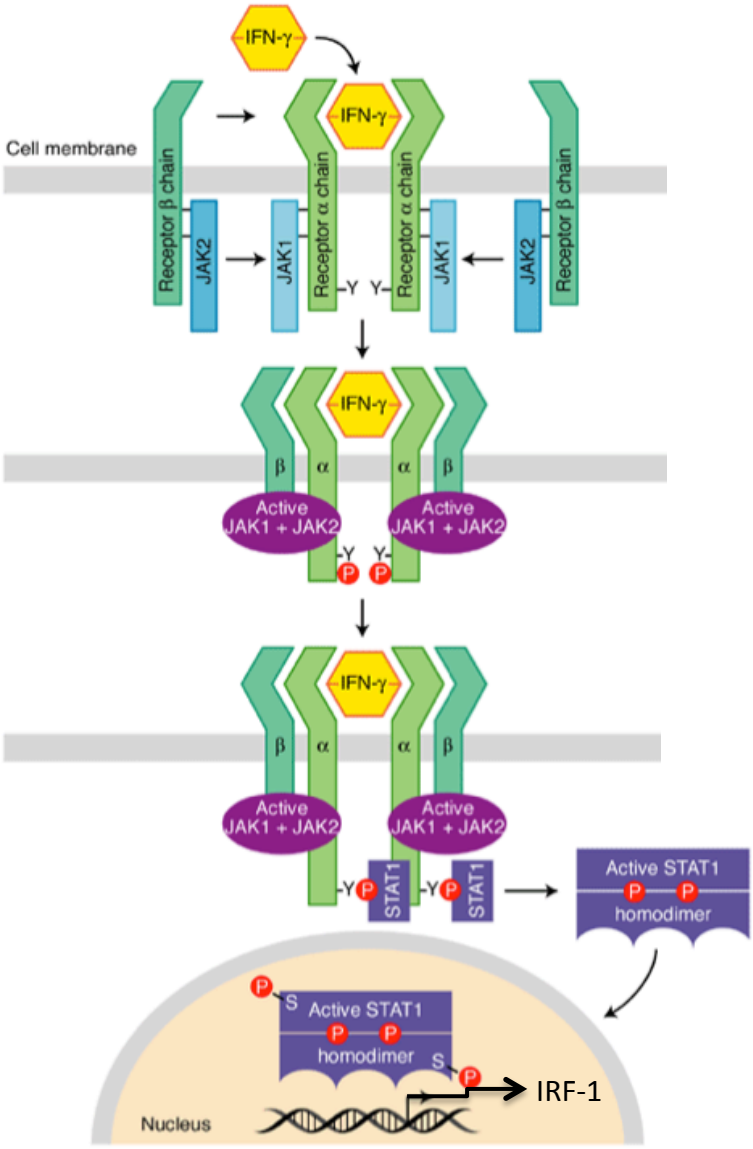

Figure 1: Scheme of the JAK1/2-STAT1 pathway. IFN-γ binds to the heterodimeric IFNGR1 (α unit)/IFNGR2 (β unit) receptor complex, leading to the activation of the receptor-associated kinases JAK1 and JAK2 that in turn recruit and phosphorylate the transcription factor STAT1. Phosphorylated STAT1 forms homodimers, which activate transcription of primary response genes (e.g. IRF1), adapted from (Newport, 2003).

The various mechanisms by which melanoma cells lose their sensitivity towards IFN-γ-mediated immunomodulatory, growth-restricting and pro-apoptotic effects as well as HLA class I expression and therefore escape from $CD8^+$ T cell recognition, will be discussed later.

5.2.2. NK cells & the NKG2D receptor/NKG2D ligand system

Tumor cells that down modulate or completely abolish HLA class I expression from the surface to escape $CD8^+$ T cell-mediated recognition, are potential targets for NK

cells – the effector cells of the innate immune system. NK cells are licensed to kill all cells lacking the 'self recognition' signal provided by HLA class I when additional activating signals are received (Aptsiauri *et al.*, 2007). In this context, especially melanoma cells have been shown to display frequently altered HLA class I expression patterns, which render them a suited target for NK cell-mediated killing (Mendez *et al.*, 2009).

NK cells are able to kill tumor cells trough the same mechanisms as $CD8^+$ T cells: 1. perforin/granzyme-mediated lysis, 2. apoptosis induction trough the engagement of death receptor ligands (e.g FasL) and 3. IFN-γ secretion (Morvan and Lanier, 2016). But in contrast to the $CD8^+$ T cells, NK cells need neither prior sensitization to exert cytotoxicity towards target cells nor a highly selective receptor recognizing a specific antigen (Morgado *et al.*, 2011). Instead, NK cells express several activating receptors (NKARs, NK cell activating receptors) and inhibitory receptors (NKIRs, NK cell inhibitory receptors) on the surface tightly regulating their cytotoxic properties. NKIRs block the effector functions of NK cells upon interaction with ligands expressed by normal and healthy cells to prevent autoimmune reactions ('self-tolerance'), while NKARs promote NK cell-mediated killing as they recognize a broad panel of ligands that are specifically upregulated on (pre)malignant or infected cells ('induced-self') (López-Soto *et al.*, 2017; Figure 2).

The best-studied activating receptors on NK cells are NKG2D (*Natural Killer Group 2, member D*), the natural cytotoxic receptors (NCRs): NKp46, NKp30 and NKp44 as well as DNAM-1 (*DNAX accessory molecule-1*). The group of inhibitory receptors is composed, among others, of KIRs (*killer cell immunoglobulin-like receptors*), CD94/NKG2A and CD85J recognizing HLA class I molecules and other inhibitory receptors like TIGIT or LAG3 (Lanier, 2005; López-Soto *et al.*, 2017). The susceptibility of melanoma cells to NK cell-mediated lysis depends thus on the expression of a sufficient amount of ligands for NK cell-activating receptors and the level of expression of HLA class I molecules. Casado and colleagues demonstrated that a high percentage of melanoma cell lines express ligands for NKG2D (85%) and DNAM-1 (95%)-activating receptors (Casado *et al.*, 2009). The ligands for the NKG2D receptor are called NKG2D ligands (NKG2DL) and include in humans the MIC (*histocompatibility complex class I chain related*) family ligands MICA and MICB as well as the ULBP (*UL16-binding protein*) family ligands ULBP1-6 (Raulet *et al.*, 2013). The surface expression of NKG2D ligands is strongly increased during

malignant transformation by activation of different cellular pathways like the DNA damage response pathway or by oncogene expression, serving to alert the immune system to possible pathological conditions of cells (Unni, Bondar and Medzhitov, 2008; Raulet *et al.*, 2013).

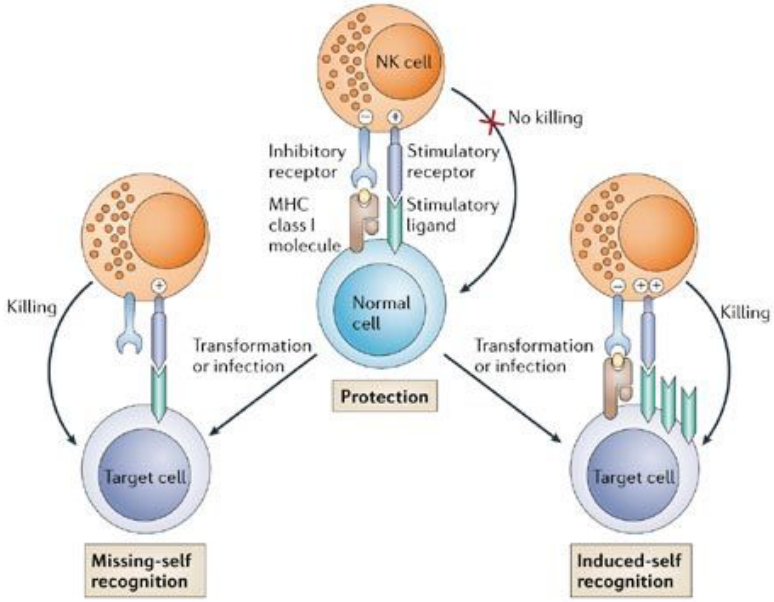

Figure 2: Scheme of NK cell-mediated killing of target cells (Raulet and Vance, 2006)**.**

The relevance of the NKG2D receptor/ligand system in controlling tumor initiation and progression in melanoma and other cancer entities has been well demonstrated in numerous mouse studies (Cerwenka, Baron and Lanier, 2001; Diefenbach *et al.*, 2001; Smyth *et al.*, 2005; Guerra *et al.*, 2008). In the process, it has been shown that tumor cells engineered to express NKG2DL and transplanted into mice were successfully rejected while cells lacking NKG2DL on the surface caused tumor growth (Cerwenka, Baron and Lanier, 2001; Diefenbach *et al.*, 2001). Furthermore, mice with NKG2D-deficiency have been shown to spontaneously develop malignant lesions more often than wild type animals, demonstrating the importance of an early NKG2D-mediated immune surveillance of cancer (Guerra *et al.*, 2008). Moreover, NKG2D is not only expressed on NK cells but also on other cytotoxic lymphocytes including $CD8^+$ and $\gamma\delta$T cells as well as NKT cells, where it exerts immune-stimulatory functions, thus indicating its wide-ranging role in the regulation of immune responses against malignant cells (Raulet, 2003; Maccalli *et al.*, 2007; Zafirova *et al.*, 2011). The expression of the ligands for the NKG2D activating receptor have been found on different human cancer cell lines (Pende *et al.*, 2002) with MICA and

ULBP2 frequently detected on melanoma cells *in vitro* and *in situ* (Vetter *et al.*, 2002; Paschen *et al.*, 2009; Schwinn *et al.*, 2009). However, tumor cells of different origins have been shown to release NKG2D ligands from the surface by proteolytic shedding to escape NK cell-mediated immune recognition (Waldhauer and Steinle, 2006; Chitadze *et al.*, 2013; Raneros *et al.*, 2015). Consistently, elevated levels of soluble NKG2DL (sNKG2DL) have been reported in numerous hematological malignancies as well as in several solid tumors including malignant melanoma (Raneros *et al.*, 2015). Increased levels of soluble ULBP2 (sULBP2) in metastatic melanoma patients have been associated with poor prognosis (Paschen *et al.*, 2009) and shown to be of predictive relevance in clinical outcome to the therapy with immune checkpoint blockade (Maccalli *et al.*, 2017). Immune checkpoint inhibitor therapy is introduced in the next chapter.

5.3. Therapeutic options

5.3.1. Targeted therapy

A major breakthrough in the therapy of metastatic melanoma was achieved with the identification of frequently occurring activating mutations in the MAPK signaling pathway (Davies *et al.*, 2002), and the subsequent development of selective inhibitors counteracting the malignant growth promoted by the hyper-activation of the mutant $BRAF^{V600E/K}$ kinase (Tsai *et al.*, 2008). Vemurafenib and dabrafenib are two $BRAF^{V600E/K}$-specific inhibitors (BRAFi) that have been approved by the Food and Drug Administration (FDA) in 2011 and 2013, respectively, for the treatment of patients with metastatic melanoma displaying mutated $BRAF^{V600E/K}$ (Najem *et al.*, 2017). In phase III clinical trials, the application of both inhibitors revealed prolonged median progression free survival (PFS) of 5.3 months (vemurafenib) and 5.1 months (dabrafenib) compared to 1.6-2.7 months for patients receiving chemotherapy consisting of dacarbazine. The response rates represented 48 % and 50 % for vemurafenib- and dabrafenib-treated patients respectively, compared to 5-6 % in the dacarbazine-group (Chapman *et al.*, 2011; Hauschild *et al.*, 2012). Furthermore, two selective inhibitors - trametinib and cobimetinib have been developed to target the MAPK pathway at the level of the *mitogen-activated, extracellular signal-regulated kinase* (MEK) that acts downstream of BRAF. Trametinib showed similar clinical effects as the BRAF inhibitors, increasing the PFS from 1.5 months (chemotherapy)

to 4.8 moths (trametinib) (Flaherty *et al.*, 2012). Unfortunately, despite profound clinical response rates, resistance to BRAFi and MEKi occurs in a majority of patients within 6-8 months resulting in disease progression and relapse (Sullivan and Flaherty, 2013). Currently, combination therapies involving simultaneous delivery of BRAFi and MEKi became a new standard of care in treatment of metastatic melanoma showing superior effects in terms of response and survival time as well as delayed resistance onset, when compared to mono-therapy (Larkin *et al.*, 2014; Long *et al.*, 2015).

Other targeted approaches include inhibition of ERK1/2 which showed in *in vitro* studies anti-melanoma activity against BRAF mutant, NRAS mutant and BRAF wild type melanoma cells (Wong *et al.*, 2014). Furthermore, the ERK1/2 inhibitor GDC-0994 is currently tested in clinical trials in combination with MEKi and as single agent in patients with advanced solid tumors (NCT01875705; NCT02457793) (Najem *et al.*, 2017). However, resistance to targeted therapy seems to remain for now a major obstacle in the treatment of melanoma and demonstrates the need for alternative therapeutic strategies with longer lasting anti-tumorigenic effects.

5.3.2. Immunotherapy

One such alternative encompasses immunotherapeutic approaches especially immune checkpoint blockade (ICB), which is said to have revolutionized the treatment of cancer (Couzin-Frankel, 2013). Immune checkpoints are essential negative regulators of T cell activity and their task is to prevent overshooting immune responses leading to enduring inflammation and autoimmunity. The best studied immune checkpoint receptors are *cytotoxic T-lymphocyte-associated protein-4* (CTLA-4) and *programmed cell death protein-1* (PD-1) (Fife and Bluestone, 2008). CTLA-4 and PD-1 act on different stages of an immune response. The CTLA-4 receptor inhibits potentially autoreactive T cells in the initial phase, called T cell priming, in the lymph nodes, while PD-1 blocks the effector function of already activated T cells in the peripheral tissue (Figure 3). Monoclonal antibodies (mAbs) against CTLA-4 and PD-1 (anti-CTLA-4 and anti-PD-1) disrupt checkpoint-mediated signaling and release T cells from inhibition leading to enhanced T cell activation, proliferation and effector function (Fife and Bluestone, 2008; Ribas, 2012).

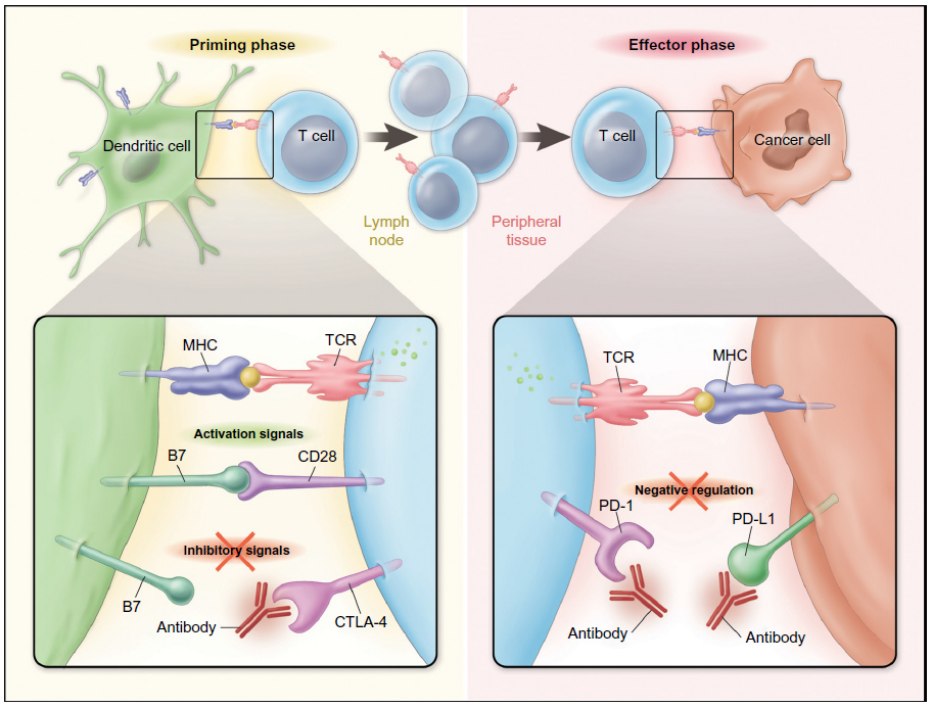

Figure 3: Checkpoint blockade in immunotherapy of cancer. CTLA-4 competes with the activating costimulatory CD28 molecule for binding of CD80/86 (B7-1/B7-2) expressed on dendritic cells. Binding of CTLA-4 to B7 molecules provides inhibitory signals and prevents T cell priming in lymph nodes. Administration of anti-CTLA-4 antibodies prevents this interaction and leads to T cell activation and proliferation. Binding of PD-1 to its ligand PDL-1 expressed among others on tumor cells, causes inhibition of T cell effector function in the peripheral tissue. Blocking antibodies against PD-1 or PD-L1 allow in particular activation of tumor-specific T cells (Ribas, 2012).

To date, the FDA has approved three monoclonal antibodies targeting immune checkpoints for the treatment of malignant melanoma: ipilimumab (anti-CTLA-4), nivolumab (anti-PD-1) and pemprolizumab (anti-PD-1), with ipilimumab being the first (Sadozai *et al.*, 2017). In the first phase III study previously treated patients with unresponsive tumors received ipilimumab or a melanoma-specific vaccinating peptide. The results demonstrated that patients receiving anti-CTLA-4 mAb showed an increased overall survival (OS) of 10.1 months compared to 6.4 months in the vaccine group. The overall response rate (ORR) was also strongly increased for ipilimumab (10.9 %) compared to the vaccine group (1.5 %) (Hodi *et al.*, 2010). Another study described by Robert et al. with treatment-naïve patients showed similar results for anti-CTLA-4 blockade plus chemotherapy with OS of 11.2 months and ORR of 15.2 % (Robert *et al.*, 2011). Further, a meta-analysis of anti-CTLA-4 trials showed long-term survival of 3 years in approx. 20 % patients (Schadendorf, Hodi, *et al.*, 2015). The application of anti-PD-1 mAb (nivolumab) achieved even better effects. In phase III trials with melanoma patients who failed responding to anti-CTLA-4 and BRAFi therapy nivolumab treatment showed an ORR of 31.7 % in

patients with and without BRAF mutations (Weber *et al.*, 2015). In treatment-naïve patients with wild-type melanoma nivolumab treatment displayed an ORR of 40 % and 1-year overall survival in 72.9 % of patients (Robert *et al.*, 2015). The combination of anti-CTLA-4 and anti-PD-1 mAbs increased the PFS from 6.9 months for nivolumab single treatment to 11.5 months for the combination and achieved a formidable ORR of 57.6 % (Larkin, Hodi and Wolchok, 2015). Another promising immunotherapeutic strategy is the administration of *ex vivo* expanded tumor specific T cells - adoptive T cell transfer (ACT). Here, tumor-infiltrating lymphocytes (TILs) are isolated from patient's tumor mass, grown to large numbers *ex vivo* and subsequently re-infused into a patient (Rosenberg *et al.*, 2008). ACT have been shown to induce objective response rates against melanoma reaching from 49-72 % in three sequential trials with 22 % of patients showing a complete remission (Rosenberg *et al.*, 2011). In summary, immunotherapy is able to induce long-lasting responses against melanoma in a considerable subset of patient however there is still a big proportion of cases, which do not experience any or just shortened treatment benefit. Currently it is of great interest to identify biomarkers, which could predict responsiveness of patients to ICB in order to minimize immune-related side effect as well as identify patients who might need more aggressive combination therapies (Rausch and Hastings, 2017). Also the exploration of NK cells as immunotherapeutic targets is an important research field, which might provide novel treatment options for patients with malignant melanoma, as discussed later in this work.

5.4. Resistance to therapy

The tremendous therapeutic progress that have been made in recent years in the treatment of metastatic melanoma implementing novel therapies such as MAPK inhibition and immunomodulatory antibodies, improved patient outcomes by prolonging the median overall survival from approx. 9 months to at least 2 years (Luke *et al.*, 2017). Unfortunately, many patients do not respond to the medication or develop resistance to the treatments. So far, great efforts have been made on investigating molecular mechanisms leading to evolution of resistance.

5.4.1. MAPKi resistance

Multiple mechanisms of resistance to MAPK pathway inhibition have been described to date with a major proportion of alterations causing a reactivation of the MAPK pathway itself or MAPK-redundant signaling (Rizos *et al.*, 2014; Shi, Hugo, *et al.*, 2014; Johnson *et al.*, 2015). The mechanisms reported include upregulation of kinases e.g. mutated BRAF, CRAF or COT1 (Montagut *et al.*, 2008; Johannessen *et al.*, 2010; Shi *et al.*, 2012), activating mutations in genes encoding *NRAS*, *MEK1/2*, or *AKT1* (Nazarian *et al.*, 2010; Wagle *et al.*, 2011; Shi, Hong, *et al.*, 2014) as well as abnormal splicing variants of *BRAF* (Poulikakos *et al.*, 2011). In addition, the loss of PTEN activity (Paraiso *et al.*, 2011) as well as overexpression and sustained activation of several receptor tyrosine kinases (RTKs) including EGFR, PDGFRβ or IGF-1R have been shown to confer resistance to MAPKi (Nazarian *et al.*, 2010; Villanueva *et al.*, 2010; Girotti *et al.*, 2013). Beside the tumor cell-intrinsic mechanisms of resistance discussed above, the tumor microenvironment as a potential extrinsic driver has been demonstrated to impact on the response to MAPKi through stromal secretion of HGF (*hepatocyte growth factor*), which activates another receptor tyrosine kinase MET expressed on tumor cells leading to reactivation of MAPK and PI3K-AKT pathways and thus to early insensitivity towards RAF inhibitors (Straussman *et al.*, 2012).

In a meta-analysis of BRAFi progressive patient samples one or more genomic resistance mechanisms have been found in 58 % of analyzed cases including *NRAS/KRAS* mutations in 20 %, *BRAF* splice variants in 16 %, *BRAF* amplification in 13 %, *MEK1/2* mutations in 7 %, and non-MAPK-pathway alterations (e.g. PI3K-AKT pathway modifications, *MITF* amplifications or RTK overexpression) in 11 % (Johnson *et al.*, 2015). In BRAFi/MEKi progressive samples very similar mechanisms of resistance have been described (Long *et al.*, 2014; Wagle *et al.*, 2014). The question arising from these findings concerns the residual approx. 40 % of cases without evidenced genomic contribution to resistance development. In their publication from 2015 Hugo and colleagues demonstrate the involvement of highly recurrent non-genomic and immune-related alterations in the acquisition of MAPKi resistance by a comparative transcriptomic and methylomic analysis of matched melanoma biopsies taken before treatment and during disease progression (Hugo *et al.*, 2015). Their main findings include frequent alterations in CpG methylation sites in resistant tumors leading to expressional changes in *c-MET*, *LEF1* and *YAP1*

designated by the group as drivers of acquired resistance, as well as decreased antigen presentation and impaired T cell effector function. Based on their results the group estimates that 38 % of resistance mechanisms to MAPKi are non-genomic while 56 % can be attributed to both genomic and non-genomic events (Hugo *et al.*, 2015). Better understanding of non-genomic and immune-related alterations during targeted therapy and resistance development is of tremendous value for future combination treatments including MAPK inhibition and immune checkpoint blockade.

5.4.2. IFN-γ resistance

Recently, clinical and experimental data has accumulated demonstrating a crucial role of the IFN-γ signaling pathway in evolution of resistance to antibody therapy against immune checkpoints (Benci *et al.*, 2016; Gao *et al.*, 2016; Zaretsky *et al.*, 2016). IFN-γ is produced by tumor-specific T cells upon recognition of antigens presented on tumor cells. In this process it plays an important role in anti-tumor responses mediated by cytotoxic $CD8^+$ T cells by inducing HLA class I expression on tumor cells and therefore increasing their immunogenicity as well as by displaying direct anti-proliferative and pro-apoptotic effects against malignant cells (Ikeda, Old and Schreiber, 2002; Seliger, Ruiz-Cabello and Garrido, 2008). However, long-lasting IFN-γ signaling may lead to a process called tumor immunoediting, which is a selective enrichment of escape variants due to the activity of immune cells, resulting in tumor cell evasion from T cell recognition (Ikeda, Old and Schreiber, 2002; Mittal *et al.*, 2014; Benci *et al.*, 2016). Several mechanisms have been described by which melanoma cells can escape from T cell-derived IFN-γ-mediated immune surveillance. One of them includes downregulation or complete loss of HLA class I expression on the surface in order to reduce antigen presentation to $CD8^+$ T cells. For human melanomas it could be shown that the loss of beta-2-microglobulin (β-2m) expression caused by a coincidence of chromosomal aberrations of chromosome 15 to which the *B2M* gene maps, on one allele, combined with mutations or small deletions in the *B2M* gene in the second allele, are causative for permanent HLA class I absence from the surface of melanoma cells. Moreover, these events have been demonstrated to cause loss of melanoma immunogenicity and T cell resistance in the course of metastatic disease (Paschen *et al.*, 2006; Sucker *et al.*, 2014; Zhao *et al.*, 2016). Thus, deficiency of β-2m causes an irreversible loss of HLA class I molecules,

which cannot be restored by IFN-γ signaling any more. Accordingly, loss of β-2m has been recently associated with resistance to anti-CTLA-4 and anti-PD-1 checkpoint blockade (Zaretsky *et al.*, 2016; Roh *et al.*, 2017). Another possibility for melanoma cells to evade T cell surveillance is HLA class I haplotype loss, which renders melanoma cells insensitive to certain tumor-specific $CD8^+$ T cell clones (Zhao *et al.*, 2016). Recently, it has been demonstrated that such a partial loss of HLA class I expression caused by chromosomal aberrations can apply to alleles presenting mutated antigens, thus allowing tumor escape from neoantigen-specific T cells, which are dealt as a particularly attractive target for therapy with immune checkpoint antibodies (Schrors *et al.*, 2017).

The most direct way for tumor cells to escape IFN-γ-mediated anti-tumorigenic effects is the acquisition of defects in the IFN-γ signaling cascade itself. In this process, tumor cells become concurrently insensitive to IFN-γ-mediated growth arrest and apoptosis as well as to induction of genes involved in antigen presentation. Moreover, $CD8^+$ T cell-derived IFN-γ is also known to upregulate the inhibitory PD-1 ligand (PD-L1) on the surface of tumor cells which is technically a protective mechanism of malignant cells prohibiting $CD8^+$ T cell attack (Mandai *et al.*, 2016). A recent study demonstrated that the $CD8^+$ T cell-associated IFN-γ-mediated upregulation of PD-L1 on tumor cells indicates a pre-existing anti-tumor response and is associated with clinical responses to anti-PD-1 immunotherapy (Tumeh *et al.*, 2014). Conversely, tumors with genetic defects in IFN-γ signaling pathway have been shown to lack cytokine-inducible PD-L1 expression and are therefore resistant to anti-PD-1 immune checkpoint blockade (Shin *et al.*, 2017). Genetic aberrations in the IFN-γ signaling pathway causing partial or complete insensitivity to this cytokine have been found in melanoma patient samples. Inactivating mutations in genes encoding the IFN (interferon) receptor-associated Janus kinases 1 and 2 (JAK1 and JAK2) have been implicated in the mediation of primary and acquired resistance to anti-PD-1 immunomodulatory antibodies (Zaretsky *et al.*, 2016; Shin *et al.*, 2017). Similar findings have been demonstrated for anti-CTLA-4 therapy resistance where significantly higher numbers of genetic defects in genes involved in IFN-γ signaling have been found in non-responders compared with patients who have shown response to anti-CTLA blockade. In addition, the same study demonstrated that mice bearing IFNGR1 (*IFN-γ receptor 1*) knockout tumors displayed impaired tumor

rejection upon anti-CTLA-4 blockade further confirming the impact of INF-γ signaling deficiency in resistance to immunotherapies (Gao *et al.*, 2016).

6. Objective

The impressive progress in the treatment of malignant melanoma achieved in the past seven years encompasses the development of inhibitors targeting oncogenic aberrations in the MAPK signaling pathway as well as the exploitation of the immune system by blocking inhibitory checkpoints on cytotoxic T cells. Both strategies have shown tremendous effects on the survival of melanoma patients, however the frequent and often rapid emergence of therapy resistance remains a major problem. Thus, further efforts have to be attempted on the elucidation of resistance mechanisms in order to overcome or delay resistance onset. The combinatorial treatment with MAPKi and immune checkpoint blockade (ICB) is currently proposed to prevent resistance development and improve the long-term outcomes of patients, however the optimal sequencing of both therapies is unknown.

The aim of the thesis was to investigate the impact of MAPK inhibition on the immune surveillance of human melanoma cells mediated by autologous $CD8^+$ T cells and NK cells, in order to provide new insights and valuable suggestions concerning treatment options for malignant melanoma, as well as to explore mechanisms contributing to insensitivity of melanoma cells towards the immune effector cytokine IFN-γ. To achieve this, different melanoma patient models consisting of tissue, corresponding cell lines, autologous $CD8^+$ T cells and NK cells were used in the studies.

7. Articles

I. Acquired IFN-γ resistance impairs anti-tumor immunity and gives rise to T cell-resistant melanoma lesions.

Antje Sucker, Fang Zhao, **Natalia Pieper**, Christina Heeke, Raffaela Maltaner, Nadine Stadtler, Birgit Real, Nicola Bielefeld, Sebastian Howe, Benjamin Weide, Ralf Gutzmer, Jochen Utikal, Carmen Loquai, Helen Gogas, Ludger Klein-Hitpass, Michael Zeschnigk, Astrid M. Westendorf, Mirko Trilling, Susanne Horn, Bastian Schilling, Dirk Schadendorf, Klaus G. Griewank, Annette Paschen

Published in: Nature Communications, May 2017
doi: 10.1038/ncomms15440

II. Impaired NK cell recognition of vemurafenib treated melanoma cells is overcome by simultaneous application of histone deacetylase inhibitors.

Sheila López-Cobo, **Natalia Pieper**, Carmen Campos-Silva, Eva M. García-Cuesta, Hugh T. Reyburn, Annette Paschen & Mar Valés-Gómez

Published in: Oncoimmunology, October 2017
doi: 10.1080/2162402X.2017.1392426

III. Evolution of melanoma cross-resistance to $CD8^+$ T cells and MAPK inhibition in the course of BRAFi treatment.

Natalia Pieper, Anne Zaremba, Sonia Leonardelli, Franziska Noelle Harbers, Marion Schwamborn, Silke Lübcke, Barbara Schrörs, Jolanthe Baingo, Alexander Schramm, Sebastian Haferkamp, Ulrike Seifert, Antje Sucker, Volker Lennerz, Thomas Wölfel, Dirk Schadendorf, Bastian Schilling, Annette Paschen, Fang Zhao

Published in: Oncoimmunology, April 2018
doi.org/10.1080/2162402X.2018.1450127

7.1. Article I

nature COMMUNICATIONS

ARTICLE

Received 7 Sep 2016 | Accepted 29 Mar 2017 | Published 31 May 2017

DOI: 10.1038/ncomms15440 OPEN

Acquired IFNγ resistance impairs anti-tumor immunity and gives rise to T-cell-resistant melanoma lesions

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Melanoma treatment has been revolutionized by antibody-based immunotherapies. IFNγ secretion by $CD8^+$ T cells is critical for therapy efficacy having anti-proliferative and pro-apoptotic effects on tumour cells. Our study demonstrates a genetic evolution of IFNγ resistance in different melanoma patient models. Chromosomal alterations and subsequent inactivating mutations in genes of the IFNγ signalling cascade, most often *JAK1* or *JAK2*, protect melanoma cells from anti-tumour IFNγ activity. JAK1/2 mutants further evolve into T-cell-resistant HLA class I-negative lesions with genes involved in antigen presentation silenced and no longer inducible by IFNγ. Allelic *JAK1/2* losses predisposing to IFNγ resistance development are frequent in melanoma. Subclones harbouring inactivating mutations emerge under various immunotherapies but are also detectable in pre-treatment biopsies. Our data demonstrate that JAK1/2 deficiency protects melanoma from anti-tumour IFNγ activity and results in T-cell-resistant HLA class I-negative lesions. Screening for mechanisms of IFNγ resistance should be considered in therapeutic decision-making.

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Understanding the mechanisms of T-cell inhibition by melanoma cells allowed for the development of new agents with considerable activity against metastatic disease including antibodies targeting the PD-L1/PD1 axis. PD-L1 expressed on melanoma cells binds its inhibitory PD1 receptor on cytotoxic $CD8^+$ T lymphocytes generating a checkpoint signal dampening the T cell's effector function[1]. Release from checkpoint blockade by treatment with anti-PD1 antibodies yields clinical benefit in a substantial proportion of melanoma patients, experiencing durable disease stabilization, tumour regression as well as complete remission[2–4]. Response to anti-PD1 therapy is strongly associated with the expression of its ligand on melanoma cells and the presence of $CD8^+$ T cells in the margin or center of metastatic lesions[5]. How T cells mediate disease stabilization or regression of bulky tumour masses remained unclear so far.

Upon activation by cognate HLA class I antigen complexes, T cells release cytolytic granules, containing perforins and granzymes, onto their target cells and secrete interferon (IFN)γ acting on cells in the microenvironment[6]. Perforin/granzyme-mediated killing and induction of apoptosis by death receptor engagement have long been considered the major anti-tumour effector mechanisms of $CD8^+$ T cells. Accordingly, expression of cytolytic markers in pretreatment melanoma biopsies was found to be significantly associated with clinical benefit to antibodies targeting the T-cell checkpoint CTLA-4 (ref. 7). But evidence from different *in vivo* studies suggests that the anti-proliferative and pro-apoptotic activity of IFNγ on melanoma cells contributes essentially to the efficacy of T-cell-mediated anti-tumour immunity.

IFNγ binds to the heterodimeric IFNGR1/IFNGR2 receptor complex, leading to the activation of the receptor-associated kinases JAK1 and JAK2 that in turn phosphorylate STAT1. Phosphorylated STAT1 homodimers activate transcription of primary response genes including the transcriptional activator IRF1 that in turn coordinates the expression of secondary response genes[8]. Activation of the JAK1/2-STAT1-IRF1 signalling cascade in melanoma cells as well as other tumour cells can induce growth arrest and death via different pathways[9–12]. Recently, it was demonstrated that adoptively transferred tumour antigen-specific $CD8^+$ T cells infiltrating B16 melanoma lesions at low numbers arrested the growth of several times higher numbers of tumour cells in an IFNγ-dependent manner[11]. Furthermore, T-cell-derived IFNγ in combination with tumour-necrosis factor (TNF)α was found to be essential also for *in vivo* induction of tumour-cell senescence abrogating disease progression in a pancreatic tumour model[13,14].

Based on this knowledge, we postulated that melanoma cells from patients responding to immunotherapy should be sensitive to the anti-proliferative and pro-apoptotic effects of IFNγ and that continuous cytokine exposure should select for the outgrowth of IFNγ-resistant tumour subclones. Here we demonstrate that IFNγ-resistant melanoma clones with inactivating *JAK1/JAK2* mutations frequently evolve in patients receiving different types of immunotherapy. IFNγ-resistant tumour cells are protected from cytokine-induced growth inhibition and apoptosis. Additionally, JAK1/JAK2-deficient lesions become T-cell-resistant by silencing HLA class I antigen presentation, which can no longer be restored by IFNγ signalling. Our findings suggest sequential screening of tumour biopsies for genetic defects in the IFNγ signalling cascade will aid therapeutic decision-making in patients with advanced melanoma.

Results

Acquired mutations in genes of the IFNγ signalling pathway. Assuming that the growth-inhibitory and pro-apoptotic activity of T-cell-derived IFNγ acts selectively on tumour cells, the evolution of genetic variants in melanoma with impaired cytokine signalling was explored. In a first step, we evaluated available exome data of 46 melanoma cell lines[15], established from metastases of different patients in our laboratory, for aberrations in *IFNGR1, IFNGR2, JAK1, JAK2, STAT1* and *IRF1*. Mutations in *JAK1* ($n=3$), *JAK2* ($n=1$) and *STAT1* ($n=1$) were detected in 5 out of the 46 cell lines (Table 1). By Sanger sequencing we confirmed the mutations on freshly isolated DNA from the respective cell lines Ma-Mel-36, Ma-Mel-53, Ma-Mel-54a, Ma-Mel-85 and Ma-Mel-102. Independent of existing exome data, Sanger sequencing revealed a *JAK1* mutation in a cell line from melanoma patient Ma-Mel-61 (Table 1). The specific mutations present in the cell lines were also detected *in situ* in corresponding tumour tissue, with the exception of metastasis Ma-Mel-54a. As shown in Table 1, targeted sequencing revealed a homozygous status for the mutant allele in three of the six cell lines (Ma-Mel-54a, Ma-Mel-61g, Ma-Mel-102). To determine whether these mutations functionally impaired IFNγ signalling, the cell lines were treated with recombinant IFNγ for 48 h followed by protein expression analyses of pathway components and downstream targets.

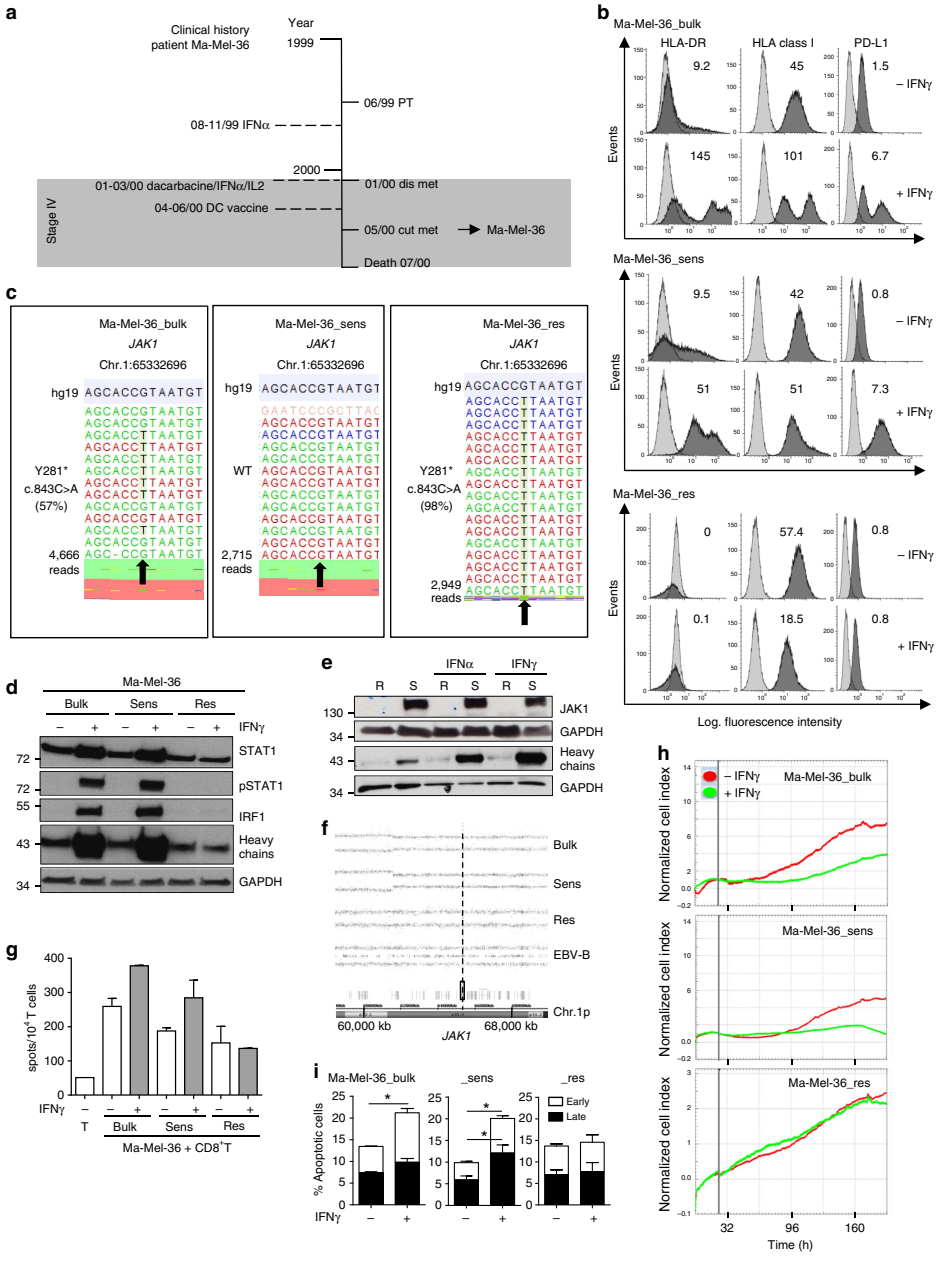
Despite a *STAT1* (c.947C>T) mutation frequency of approximately ~100%, Ma-Mel-102 cells still showed a slight induction of pSTAT1 and IRF1 in the presence of IFNγ (Supplementary Fig. 1a–c). However, the signals were weak and detectable only after long-term exposure of X-ray films, suggesting that the S316L exchange, located between the coiled-coil and DNA binding domains, strongly decreased STAT1 protein stability without necessarily leading to its complete inactivation. Parallel analyses on Ma-Mel-85 cells revealed a strong IFNγ pathway activation compared with Ma-Mel-102 cells, accompanied with an elevated surface expression of CD54, HLA class I and PD-L1 (Supplementary Fig. 1b–d). This is in line with a *JAK1* (c.1548C>A) mutation frequency of only 50% (Supplementary Fig. 1a), suggesting that wild-type *JAK1* was still active in Ma-Mel-85 cells. As expected, IFNγ signalling was detected also in Ma-Mel-53 cells showing a *JAK1* (c.2338G>A) mutation frequency of only 24% (Supplementary Fig. 1a–c).

Table 1 | Acquired mutations in genes of the IFNγ signalling pathway.

Patient	Metastasis excision	Disease stage	Cell line	Mutated gene	Allelic status	DNA	Protein	Mutation in tissue
Ma-Mel-36	05/2000	IV	Ma-Mel-36	*JAK1*	het*	c.843C>A	p.Y281*	Yes
Ma-Mel-53	03/2002	IV	Ma-Mel-53	*JAK1*	het	c.2338G>A	p.G780R	Yes
Ma-Mel-85	03/2004	IV	Ma-Mel-85	*JAK1*	het	c.1548C>A	p.F516L	Yes
Ma-Mel-54	09/2002	IV	Ma-Mel-54a	*JAK2*	hom	c.2876A>C	p.Q959P	n.d.
Ma-Mel-102	11/2004	III	Ma-Mel-102	*STAT1*	hom	c.947C>T	p.S316L	Yes
Ma-Mel-61	05/2005	IV	Ma-Mel-61g	*JAK1*	hom	c.1798G>T	p.G600W	Yes

n.d., not detectable.
*Consists in equal parts of homozygous wild-type and homozygous mutant subpopulations.

IFNγ resistance protects from cytokine-induced cell death. Targeted sequencing pointed to a heterozygous *JAK1* mutation in cell line Ma-Mel-36 (Table 1) that was established from a cutaneous patient metastasis (Fig. 1a). However, when treated with IFNγ, analyses of HLA-DR, HLA class I and PD-L1 surface expression on Ma-Mel-36 cells (hereafter referred to as Ma-Mel-36_bulk cells) demonstrated that the cell line consisted in equal parts of IFNγ-sensitive and IFNγ-resistant subpopulations (Fig. 1b). Both subpopulations were sorted from IFNγ-treated Ma-Mel-36_bulk cells based on their different



HLA-DR expression profiles. The IFNγ-sensitive subpopulation Ma-Mel-36_sens strongly upregulated HLA-DR and PD-L1 surface expression in response to cytokine treatment, whereas the IFNγ-resistant subpopulation Ma-Mel-36_res remained HLA-DR-negative and PD-L1-low under these conditions (Fig. 1b). By targeted sequencing we detected the *JAK1* mutation (c.843C > A) encoding a truncated non-functional JAK1-Y281* variant in ~60 and 100% of Ma-Mel-36_bulk and Ma-Mel-36_res cells, respectively, but not in Ma-Mel-36_sens cells (Fig. 1c). Accordingly, IFNγ treatment resulted in pSTAT1 and IRF1 detection in lysates from Ma-Mel-36_bulk and Ma-Mel-36_sens cells but not from Ma-Mel-36_res cells (Fig. 1d). In line with the sequencing results, JAK1 protein expression was detected only in Ma-Mel-36_sens but not in Ma-Mel-36_res cells (Fig. 1e).

Assuming that JAK1 deficiency in these cells was due to a gene mutation and concurrent allelic loss, we performed single-nucleotide polymorphism (SNP) array analyses on DNA obtained from the three tumour cell populations and autologous Epstein-Barr virus (EBV)-transformed B cells as a control. The same partial deletion on chromosome 1p, encompassing the region 1p36.3-1p13.1 (Chr.1:854,277-116,804,754) including the *JAK1* gene mapping at Chr.1p31.3 was detected in Ma-Mel-36_bulk, Ma-Mel-36_sens and Ma-Mel-36_res cells (Fig. 1f). This result demonstrated that the allelic *JAK1* loss occurred early in the course of disease and that a subsequent c.843C > A mutation in the remaining *JAK1* allele generated the JAK1-deficient Ma-Mel-36_res subpopulation. Consistently, JAK1 reconstitution by transient transfection of Ma-Mel-36_res cells with a *JAK1* expression plasmid restored IFNγ signalling (Supplementary Fig. 2a–c).

As shown in Fig. 1a, metastasis Ma-Mel-36 developed after the patient had been treated with recombinant IFNα and a combination of dacarbacine/IFNα/interleukin (IL) 2, suggesting activated tumour-reactive T cells selectively enriched the IFNγ-resistant cell subpopulation. Indeed, peripheral blood $CD8^+$ T cells from patient Ma-Mel-36 secreted IFNγ in the presence of autologous melanoma cells, as determined by ELISpot assay (Fig. 1g). Pretreatment of tumour cells with IFNγ slightly enhanced the activation of $CD8^+$ T cells by Ma-Mel-36_bulk and Ma-Mel-36_sens cells, whereas the T-cell-stimulatory capacity of Ma-Mel-36_res cells was not affected. Furthermore, impedance-based measurement of real-time proliferation in the xCELLigence system revealed a negative impact of IFNγ on the expansion of Ma-Mel-36_bulk and Ma-Mel-36_sens cells, while Ma-Mel-36_res cells efficiently proliferated (Fig. 1h). This was measurable also in terms of cell numbers: a considerable reduction in Ma-Mel-36_bulk and in particular Ma-Mel-36_sens cells was noted in the presence of IFNγ, due to an increase in apoptosis (Fig. 1i and Supplementary Fig. 2d,e). In contrast, cell numbers and spontaneous apoptosis of IFNγ-resistant Ma-Mel-36_res cells remained unaffected under these conditions (Fig. 1i and Supplementary Fig. 2d,e).

As shown in Fig. 1e, Ma-Mel-36_sens cells responded to IFNα treatment whereas JAK1-deficient Ma-Mel-36_res cells were also resistant to type I IFN. Considering patient Ma-Mel-36 received IFNα-based therapies before the development of resistant lesions, we hypothesized that type I IFN signalling by affecting cell survival might have contributed to the enrichment of JAK1-deficient cells. However, in contrast to IFNγ, IFNα treatment did not affect the survival of Ma-Mel-36_sens cells (Supplementary Fig. 2f).

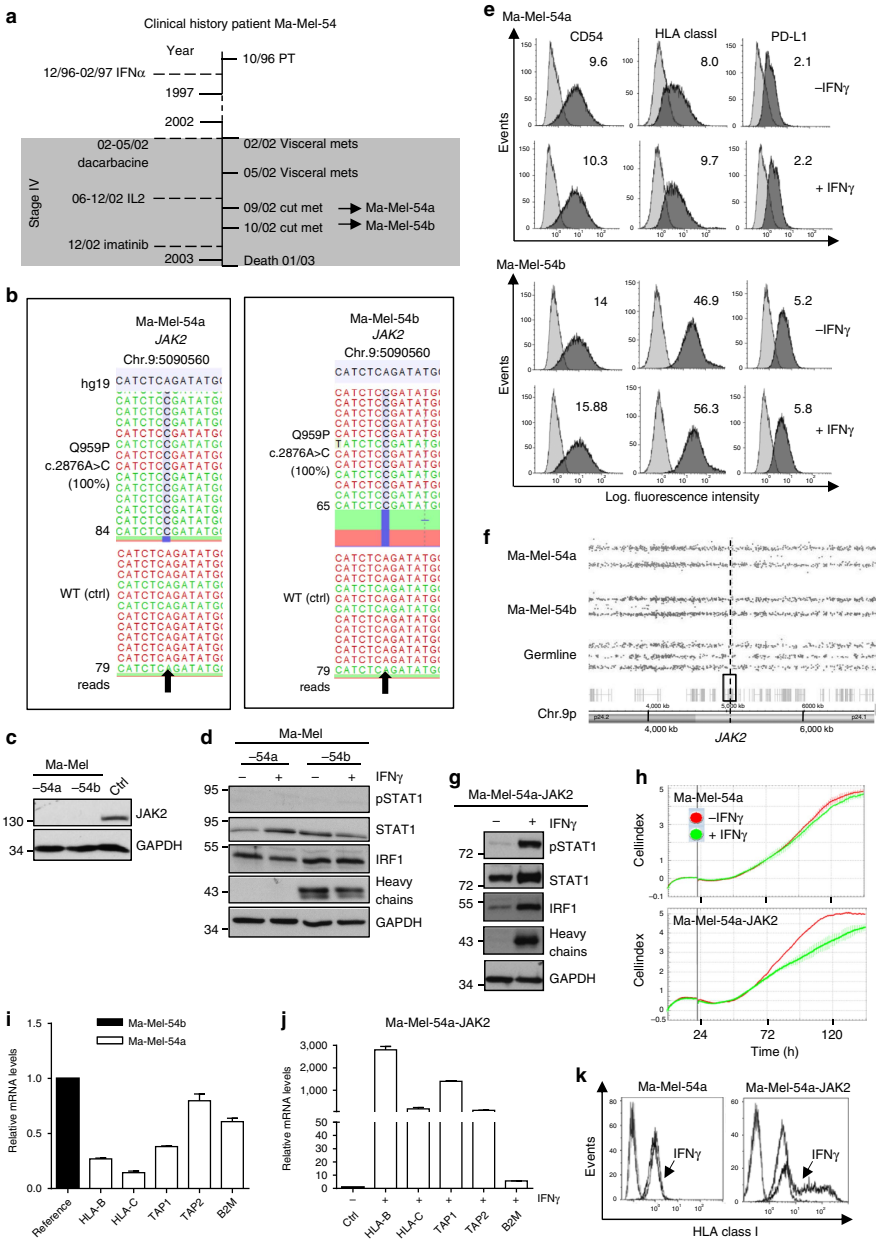
JAK2 deficiency blocks HLA class I upregulation by IFNγ. By Sanger sequencing we found a *JAK2* c.2876A > C exchange to be present in Ma-Mel-54a cells that, however, could not be detected in the corresponding tumour tissue (Table 1). To demonstrate that, in fact, the specific genetic alteration was acquired in the course of disease we sequenced DNA from a second cutaneous lesion (Ma-Mel-54b) of the patient, obtained one month after excision of metastasis Ma-Mel-54a (Fig. 2a). Indeed, tumour tissue Ma-Mel-54b and the corresponding cell line harboured the *JAK2* mutation already present in Ma-Mel-54a cells (Fig. 2b). Both cell lines showed a *JAK2* (c.2876A > C) mutation frequency of 100%, resulting in a Q959P exchange in the functionally important JAK2 JH1 kinase domain[16]. As shown in Fig. 2c, mutant JAK2-Q959P was no longer detectable by western blot. Accordingly, IFNγ signalling was completely abrogated in both cell lines, which no longer showed CD54, HLA class I and PD-L1 upregulation in response to cytokine treatment (Fig. 2d,e).

Assuming that JAK2 deficiency of Ma-Mel-54 cells was caused by the co-occurrence of a *JAK2* gene mutation and allelic *JAK2* loss, we performed SNP array analyses on DNA obtained from the two cell lines and autologous peripheral blood cells as a constitutive, normal control to detect aberrations of chromosome 9p to which the *JAK2* gene maps at Chr.9p24.1. The same deletion on chromosome 9p, encompassing the region 9p24.3–p13.2 (Chr.9:203,861-37,578,327) was detected in Ma-Mel-54a and Ma-Mel-54b cells (Fig. 2f), demonstrating the common origin of *JAK2* deficiency in both metastases. As shown in Fig. 2g, IFNγ sensitivity of Ma-Mel-54a cells was restored upon transient JAK2 re-expression as indicated by the induction of signalling pathway components. Furthermore, Ma-Mel-54a-JAK2

Figure 1 | Protection from cytokine-induced cell death by acquired IFNγ resistance. (**a**) Clinical history of patient Ma-Mel-36. Vertical line, time axis; left, therapeutic regimens; right, primary tumour (PT)/metastases development; arrow indicates cell line established from metastasis Ma-Mel-36; grey box, stage IV disease. (**b**) IFNγ-sensitive Ma-Mel-36_sens and IFNγ-resistant Ma-Mel-36_res cells sorted from IFNγ-treated (48 h) Ma-Mel-36_bulk cells based on their HLA-DR expression profile. Surface expression of indicated proteins measured by flow cytometry. Representative data from $n = 3$ independent experiments. (**c**) *JAK1* mutation defined by targeted sequencing on DNA from Ma-Mel-36_bulk and Ma-Mel-36_res cells. Plots of aligned sequencing reads in the location where the *JAK1* c.843C > A, p.Y281* mutation was identified, arrows highlight mutation or corresponding wild-type (WT) site. Number of sequencing reads notated on the left; %, frequency of mutation in reads. (**d**) Melanoma cells analysed by western blot for expression of STAT1, pSTAT1, IRF1 and HLA class I heavy chains after IFNγ treatment (48 h); GAPDH, loading control. Representative data from $n = 2$ independent experiments. (**e**) Ma-Mel-36_sens (S) and Ma-Mel-36_res (R) cells analysed for protein expression after IFNα and IFNγ treatment (48 h). Representative data from $n = 2$ independent experiments. (**f**) SNP results given as allelic distribution of chromosome 1p shown for DNA obtained from Ma-Mel-36_bulk, Ma-Mel-36_sens, Ma-Mel-36_res and autologous Epstein-Barr virus-transformed B cells as a control. Loss of one chromosomal allele in the region 1p36.3-1p13.1 (Chr.1:854,277-116,804,754; hg19) in all Ma-Mel-36 cell populations. Dashed line indicates *JAK1* location at Chr.1p31.3. (**g**) IFNγ release by autologous $CD8^+$ T cells in the presence of IFNγ-treated (24 h) Ma-Mel-36 cell populations measured by ELISpot assay. Mean values (+s.e.m.) from $n = 2$ measurements. (**h**) Real-time proliferation of Ma-Mel-36 cell populations in the presence/absence of IFNγ. Bold grey vertical lines indicate addition of IFNγ. Representative data from $n = 3$ independent experiments. (**i**) IFNγ-induced (7 days) apoptosis in Ma-Mel-36 cell populations determined by AnnexinV/PI staining. Percentage of early (AnnexinV +/PI −) and late apoptotic (AnnexinV +/PI +) cells depicted. Mean values (+s.e.m.) from $n = 3$ independent experiments. Only statistical significant differences defined by paired Student's *t*-test are indicated, $^*P < 0.05$.

transfectants proved to be sensitive towards the anti-proliferative activity of IFNγ in contrast to non-transfected Ma-Mel-54a cells (Fig. 2h).

Interestingly, western blot analyses suggested a lack of HLA class I heavy chain expression in Ma-Mel-54a compared with Ma-Mel-54b cells (Fig. 2d). Indeed, Ma-Mel-54a cells only weakly expressed HLA class I surface molecules (Fig. 2e). By quantitative reverse transcription–PCR we demonstrated low level expression of specific messenger RNAs (mRNAs) involved in antigen presentation in Ma-Mel-54a cells (Fig. 2i). Expression of



HLA-B, HLA-C, TAP1, TAP2 and *B2M* genes as well as HLA class I surface molecules were strongly upregulated solely by IFNγ-treated Ma-Mel-54a-JAK2 transfectants (Fig. 2j,k). Thus, JAK2 deficiency protected Ma-Mel-54a cells not only from anti-tumour IFNγ activity but also conserved their HLA class I-low phenotype that in turn might have hampered effective T-cell recognition of the tumour cells. Due to a lack of cryopreserved autologous T cells from this patient, the functional significance of the HLA class I-low phenotype could not be investigated in more detail.

Chromosome 1 alterations predispose to JAK1 deficiency. Similar to Ma-Mel-54, multiple cell lines were established from distinct melanoma metastases of patient Ma-Mel-61 collected over a period of 2.5 years, allowing us to follow the development of IFNγ resistance in the course of disease. The patient presented in the clinic with stage IV melanoma at the end of 2002 and received IFNα treatment for more than 2 years from December 2002 to January 2005 (Fig. 3a). From three of his metastases, excised under IFNα treatment, the cell lines Ma-Mel-61a, Ma-Mel-61b and Ma-Mel-61c were established. Additional three lesions were resected in 2005 after IFNα therapy, giving rise to the cell lines Ma-Mel-61e, Ma-Mel-61g and Ma-Mel-61h (Fig. 3a). Treatment with IFNγ and subsequent analyses of CD54, HLA class I and PD-L1 surface expression by flow cytometry revealed an upregulation of these markers only for cell lines established before Ma-Mel-61g (Fig. 3b, and Supplementary Fig. 3a).

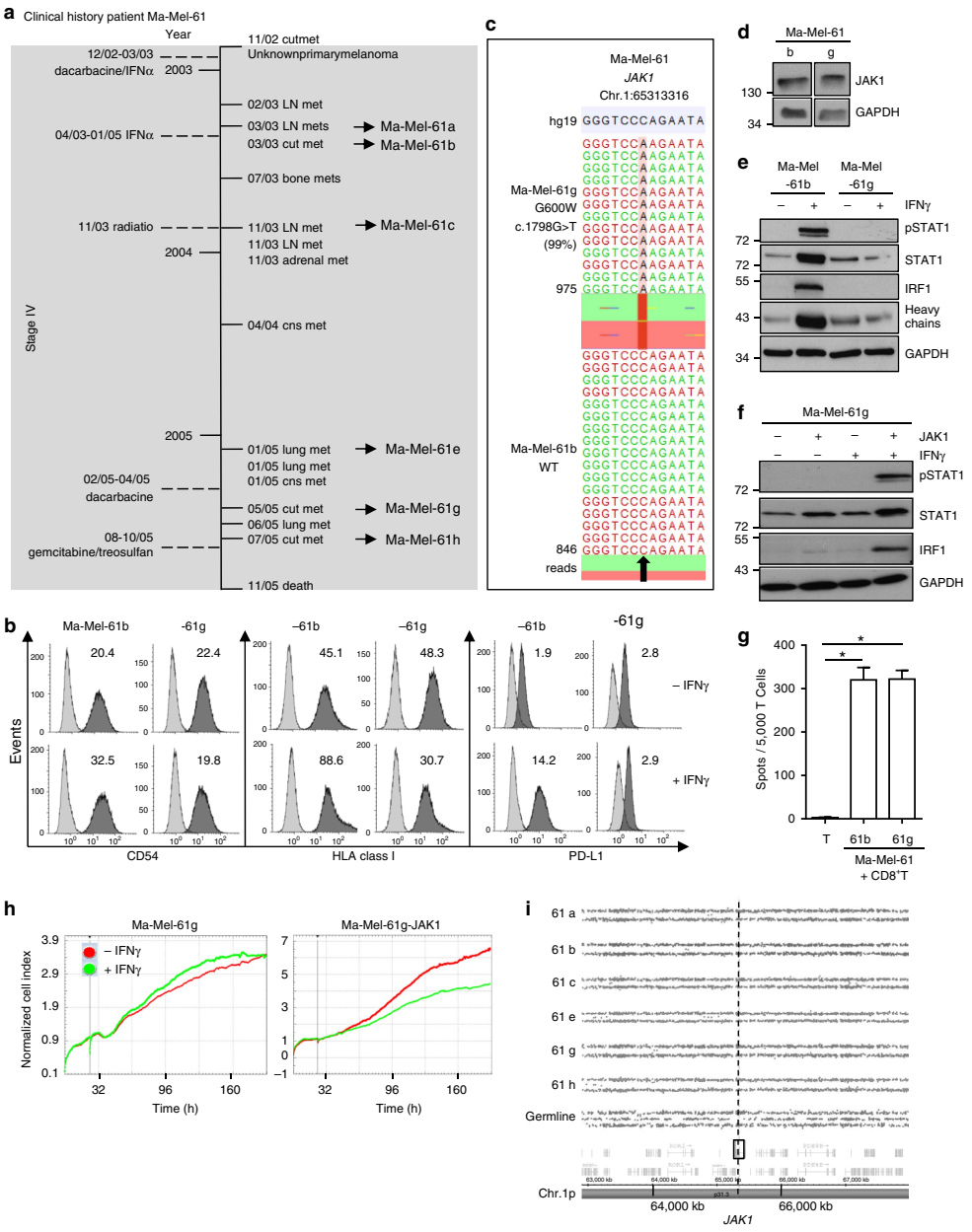
By targeted sequencing on DNA from Ma-Mel-61g and blood cells as a constitutive normal control we detected a *JAK1* c.1798G>T, JAK1-G600W mutation in 99% of the cells (Fig. 3c, Supplementary Fig. 3b), with the JAK1-G600W exchange affecting a conserved amino acid in the auto-inhibitory pseudokinase domain[17]. None of the IFNγ-sensitive Ma-Mel-61 cell lines showed a *JAK1* mutation (Fig. 3c, Supplementary Fig. 3b). The JAK1-G600W mutant protein was still detectable by western blot (Fig. 3d), but was found to be completely inactive, as no upregulation of pSTAT1, STAT1 and IRF1 was detected in Ma-Mel-61g cells in response to IFNγ treatment (Fig. 3e). Consistently, transfection of Ma-Mel-61g cells with an expression plasmid encoding wild-type JAK1, in contrast to JAK1-G600W, restored IFNγ signalling (Fig. 3f and Supplementary Fig. 3c). Again we detected autologous $CD8^+$ T cells secreting IFNγ in the presence of Ma-Mel-61g cells in the patient's peripheral blood, whose activity might have selected for the outgrowth of IFNγ-resistant cells (Fig. 3g). Accordingly, while proliferation of Ma-Mel-61g cells was not affected by IFNγ, the cells became sensitive upon JAK1 re-expression (Fig. 3h). Analysing the genetic evolution of JAK1 deficiency, we found a deletion on chromosome 1p, encompassing the region 1p34.3–1p12 (Chr.1:40,061,699-118,932,325) including the *JAK1* gene, to be present in all Ma-Mel-61 cell lines (Fig. 3i). This demonstrated that allelic *JAK1* loss was an early event in the course of disease progression in this patient, predisposing to IFNγ-resistance development, and that a subsequent inactivating *JAK1* point mutation led to complete abrogation of type II IFN signalling in Ma-Mel-61g cells.

Evolution of T-cell-resistant lesions. Similar to Ma-Mel-61g, IFNγ signalling was abrogated in Ma-Mel-61h cells due to the homozygous *JAK1* c.1798G>T, JAK1-G600W mutation (Fig. 4a,b). In contrast to Ma-Mel-61g, however, Ma-Mel-61h cells demonstrated a stable HLA class I-negative phenotype. Lack of HLA heavy chains and HLA class I surface expression was observed by western blot and flow cytometry, respectively (Fig. 4b,c). Staining of Ma-Mel-61g and Ma-Mel-61h tissue sections revealed the presence of HLA class I-negative tumour cells in both lesions (Fig. 4d and Supplementary Fig. 3d). Quantification of mRNAs involved in antigen presentation indicated a complete lack in the expression of *HLA-B, HLA-C, TAP1* and *TAP2* in Ma-Mel-61h cells compared with the control cells Ma-Mel-61b (Fig. 4e). Transient JAK1 re-expression and subsequent IFNγ treatment induced *de novo HLA-B, HLA-C, TAP1* and *TAP2* mRNA expression demonstrating a reversible silencing of antigen presentation in Ma-Mel-61h cells (Fig. 4e). This could not be observed for Ma-Mel-61h cells expressing JAK1-G600W, confirming functional inactivity of the mutant protein. Consistently, enhanced expression of specific mRNAs could also be measured for IFNγ-treated Ma-Mel-61g-JAK1 but not for Ma-Mel-61g-JAK1-G600W transfectants (Fig. 4e). As shown in Fig. 4f, the subpopulation of Ma-Mel-61h-JAK1 transfectants demonstrated *de novo* HLA class I surface expression after IFNγ-treatment, resulting in detection of the previously ignored tumour cells by autologous $CD8^+$ T cells (Fig. 4g). Overall, these data demonstrated that JAK1 deficiency in Ma-Mel-61h cells was followed by silencing of antigen presentation, generating a T-cell-resistant melanoma phenotype. Similar results were obtained for JAK2-deficient Ma-Mel-54a cells (Fig. 2g,i,k), suggesting the broader significance of our findings.

Genetic alterations defined in different melanoma data sets. The detection of IFNγ-resistant melanoma metastases in our patient cohort led us to assess the presence of alterations in type II IFN signalling pathway components in independent sample

Figure 2 | JAK2 deficiency blocks HLA class I upregulation by IFNγ. (**a**) Clinical history of patient Ma-Mel-54. Vertical line, time axis; left, therapeutic regimens; right, primary tumour (PT)/metastases development; arrows indicate cell lines established from metastases Ma-Mel-54a and Ma-Mel-54b; grey box, stage IV disease. (**b**) Mutations defined by targeted sequencing on DNA from melanoma cells and autologous blood cells as wild-type (WT) control (ctrl). Plots of aligned sequencing reads in the location where the *JAK2* c.2876A>C, p.Q959P mutation was identified. WT sequences shown on the bottom, arrows highlight mutation sites. Number of sequencing reads notated on the left; %, frequency of mutations in reads. (**c**) Lysates from Ma-Mel-54a, Ma-Mel-54b and control cells (ctrl) analysed by western blot for JAK2 expression. (**d**) Lysates from IFNγ-treated (48 h) Ma-Mel-54a and Ma-Mel-54b cells analysed for expression of STAT1, pSTAT1, IRF1 and HLA class I heavy chains. (**c**,**d**) GAPDH, loading control. Representative data from $n=2$ independent experiments. (**e**) Expression of CD54, HLA class I and PD-L1 on IFNγ-treated (48 h) melanoma cells, measured by flow cytometry. Representative data from $n=3$ independent experiments. (**f**) SNP results given as allelic distribution of chromosome 9p shown for DNA obtained from Ma-Mel-54a, Ma-Mel-54b and autologous peripheral blood cells as normal control (germline). Loss of one chromosomal allele in region 9p24.3–p13.2 (Chr.9:203,861-37,578,327; hg19) present in both cell lines. Dashed line indicates *JAK2* location at Chr.9p24.1. (**g**) Lysates from IFNγ-treated (48 h) Ma-Mel-54a-JAK2 transfectants analysed by western blot for expression of indicated proteins. (**h**) Real-time cell proliferation in the presence or absence of IFNγ. Bold grey vertical lines indicate addition of IFNγ. (**g**,**h**) Representative data from $n=2$ independent experiments. (**i**) Ma-Mel-54a and Ma-Mel-54b cells analysed for specific mRNA expression by quantitative reverse transcription–PCR. (**j**) Ma-Mel-54a-JAK2 transfectants analysed for specific mRNA expression by quantitative reverse transcription–PCR in the presence or absence of IFNγ (48 h). (**i**,**j**) Relative expression levels given as means (+s.e.m.) from $n=2$ independent experiments. (**k**) HLA class I expression on IFNγ-treated (48 h) Ma-Mel-54a cells and Ma-Mel-54a-JAK2 transfectants, measured by flow cytometry. Representative data from $n=2$ independent experiments.

collections. We screened 287 TCGA (The Cancer Genome Atlas) melanoma tissue samples for mutations in *IFNGR1, IFNGR2, JAK1, JAK2, STAT1* and *IRF1* (ref. 18). In 12.6% (36 of 287) of the samples alterations were identified, including single-nucleotide variations (SNV), small insertions and deletions (Indel) and homozygous deletions, that in most cases were mutually exclusive (Fig. 5a; Supplementary Data 1, Supplementary Fig. 4). Interestingly, a remarkable fraction of the biopsies (44%, 16 of 36) showed homozygous deletions in the different genes indicating abrogation of type II IFN signalling in tumour cells. Alterations



affecting genes of the IFNγ signalling cascade were more frequent in metastatic samples compared with primary tumours (Fig. 5b) and were detectable in metastases from patients receiving neo-adjuvant IFNα and from patients without treatment (Supplementary Data 1). Tumour biopsies with mutations did not show elevated expression of *IFNG* or *CD8A* mRNA in comparison to biopsies without mutations, suggesting a comparable activity of T cells in both types of lesions (Supplementary Fig. 5).

Support of our finding of recurrent genetic alterations in type II IFN signalling pathway genes was also obtained from additional published data sets. A mutation frequency of 22% (11 of 49), based on SNV/Indels and homozygous deletions, was detected in melanoma cell lines studied by the Cancer Cell Line Encyclopedia (CCLE)[19]. Considering only SNV/Indels, the frequency in melanoma tissue samples was 7% (20 of 287) for the TCGA melanoma collection (Fig. 5a)[18], 3% (3 of 91) for melanoma biopsies studied by Krauthammer *et al.*[20] (Fig. 5c), 6% (3 of 49) for melanoma cell lines from CCLE[19] (Fig. 5d) and 10% (12 of 121) for the cell lines analysed by Hodis *et al.*[15] (Fig. 5e). Overall these analyses found mutations to be present in a considerable fraction of melanoma cells.

Our studies in the three melanoma patient models identified allelic *JAK1* and *JAK2* losses as initial genetic alteration predisposing to IFNγ-resistance development. This led us to screen available SNP microarray data from 59 'in-house' melanoma cell lines for loss of heterozygosity (LOH) in the *JAK1* and the *JAK2* locus[21]. As shown in Fig. 5f, LOH for *JAK1* was detected in 25% (15 of 59) and for *JAK2* in 76% (45 of 59) of the cell lines suggesting a high risk of resistance development in the course of an effective anti-tumour T-cell response.

Based on the above sequencing results and functional data, we asked for the impact of alterations in IFNγ signalling genes on the control of disease progression. When assessed in the largest available TCGA melanoma cohort with survival data (479 samples) these alterations were found to have a statistically significant negative impact on patient survival (Fig. 6a). On the other hand, elevated mRNA levels for *STAT1* as well as its downstream target *IRF1*, indicating active IFNγ signalling, were strongly associated with improved overall survival (Fig. 6b). The direct correlation between *IFNG* and *CD8A* mRNA expression pointed to $CD8^+$ T cells as the major cytokine source (Supplementary Fig. 6a,b).

Mutations emerge before checkpoint blocking therapy. As resistance to IFNγ signalling could impact therapy efficacy, we screened 59 formalin-fixed, paraffin-embedded (FFPE) tumour samples from patients receiving anti-PD1 therapy for corresponding mutations by targeted sequencing of DNA isolated from macrodissected tumour cells and autologous control blood cells (Table 2). In 19% of the biopsies (11 out of 59) mutations were identified, affecting *IRF1* ($n=1$), *JAK1* ($n=5$) and *JAK2* ($n=5$), some of them clearly inactivating as the stop codon mutation in sample 15-12774 (IRF1 W195*), and the frame shift mutations in specimens D5923-13 (JAK2-D710fs) and 18298-15 (JAK2-T376fs). Furthermore, we determined the mutation frequency in pre-treatment biopsies from patients having received anti-CTLA-4 therapy, in this case evaluating existing exome sequencing data[7]. Mutations in type II IFN signalling pathway genes were identified in 9% of samples (10 out of 110) (Table 2).

Overall, these sequencing results demonstrate that genetic alterations in type II IFN signalling pathway components are present in a considerable number of melanomas. Although we could not detect significant differences in responses to anti-PD1 or anti-CTLA-4 treatment between patients with or without mutations (Supplementary Table 1), it is important to note that two of the three patients with clearly inactivating mutations (15-12774: IRF1 W195*; 18298-15: JAK2 D710fs) showed progressive disease under anti-PD1 treatment, while the third patient (18298-15: JAK2 T376fs) showed a partial response, suggesting that these mutations might have contributed to therapy resistance. Considerably larger cohorts of anti-PD1 and anti-CTLA-4 treated patients will be required to allow conclusive statistical analysis to be performed in future studies.

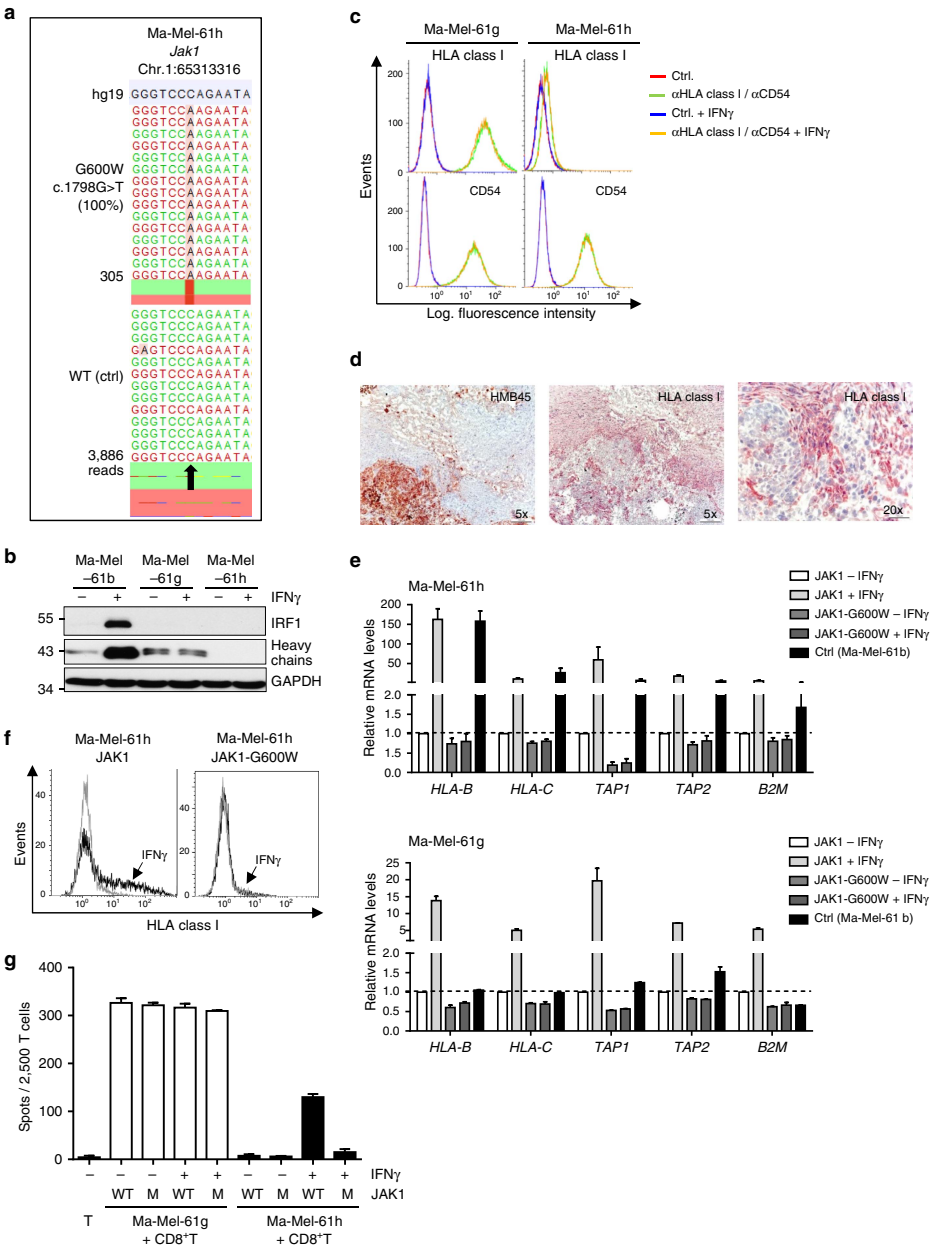
Discussion

Remarkable response rates in treatment of metastatic melanoma have been reported for adoptive T-cell transfer[22,23] and therapy with immune checkpoint-blocking antibodies, including anti-PD1 monotherapy[2,3] as well as anti-PD1 and anti-CTLA-4 combination therapy[4,24]. Long-term data from anti-CTLA-4 therapy suggest that a number of patients will show a durable complete response and may even be healed of metastatic disease[25]. Despite the considerable therapeutic potential, not all patients benefit equally well from immunotherapy. Primary as well as acquired therapy resistance is a major concern and the identification of resistance mechanisms is crucial for advancing treatment of melanoma and other malignancies.

The data presented in this work signify that under the selective pressure of an effective T-cell response tumour clones evolve that are considerably less susceptible or even resistant to T-cell effector mechanisms. As such the direct cytotoxic effects of $CD8^+$ T cells mediated by release of cytolytic granules or death receptor engagement are an essential but most likely insufficient part of the overall anti-tumour response that also depends on the secretion of IFNγ. By induction of growth arrest and cell death IFNγ has a broader impact on tumour cells and their

Figure 3 | Chromosome 1 alterations predispose to JAK1 deficiency. (**a**) Clinical history of patient Ma-Mel-61. Vertical line, time axis; left, therapeutic regimens; right, metastases development; arrows indicate cell lines established from metastases Ma-Mel-61a, Ma-Mel-61b, Ma-Mel-61c, Ma-Mel-61e, Ma-Mel-61g and Ma-Mel-61h; grey box, stage IV disease. (**b**) Surface expression of CD54, HLA class I, and PD-L1 on IFNγ-treated (48 h) Ma-Mel-61b and Ma-Mel-61g cells, measured by flow cytometry. Representative data from $n=3$ independent experiments. (**c**) Mutation defined by targeted sequencing on DNA from Ma-Mel-61g cells. Plots of aligned sequencing reads in the location where the *JAK1* c.1798G>T, p.G600W mutation was identified. Arrow highlights mutation site in Ma-Mel-61g or corresponding wild-type site (WT) in Ma-Mel-61b cells. Number of sequencing reads notated on the left; %, frequency of mutation in reads. (**d**) Lysates from Ma-Mel-61b and Ma-Mel-61g cells analysed by western blot for JAK1 expression; GAPDH, loading control. Representative data from $n=3$ independent experiments. (**e**) Lysates from IFNγ-treated (48 h) melanoma cells analysed by western blot for expression of STAT1, pSTAT1, IRF1 and HLA class I heavy chains; GAPDH, loading control. Representative data from $n=3$ independent experiments. (**f**) Lysates from IFNγ-treated (48 h) *JAK1*-transfected Ma-Mel-61g cells analysed for expression of the indicated proteins. Representative data from $n=3$ independent experiments. (**g**) IFNγ release by autologous $CD8^+$ T cells in the presence of melanoma cells, measured by ELISpot assay. Means and s.e.m. (error bars) from $n=4$ independent measurement. Statistical significant differences defined by paired Student's *t*-test are indicated, $^*P<0.05$. (**h**) Ma-Mel-61g and Ma-Mel-61g-JAK1 cells subjected to impedance-based real-time measurement of proliferation in the presence or absence of IFNγ. Addition of IFNγ indicated by bold grey vertical lines. Representative data from $n=3$ independent experiments. (**i**) SNP results given as allelic distribution of chromosome 1p shown for DNA obtained from the different Ma-Mel-61 cell lines and autologous blood cells as normal control (germline). Loss of one chromosomal allele in the region 1p34.3–1p12 (Chr.1:40,061,699-118,932,325; hg19) present in all cell lines. Dashed line indicates location of *JAK1* at Chr.1p31.3.

microenvironment[9–11,13,14]. Accordingly, analyses on TCGA melanomas revealed a strong association between patient survival and elevated mRNA levels of *STAT1* and its downstream target *IRF1*, indicating IFNγ-dependent pathway activation. Furthermore, the strong correlation between *IFNG* and *CD8A* mRNA in melanomas, both known as favourable prognostic markers[26–29], argues for CD8$^+$ T cells as a major IFNγ source. However, a significant contribution by other lymphocytes such as CD4$^+$ T cells of the Th1 phenotype or natural killer cells cannot be excluded[13,30].



Assuming that IFNγ exerts a strong selective pressure on tumour cells we screened an 'in-house' collection of short-term cultured melanoma cell lines for mutations in genes of the IFNγ signalling pathway and detected *JAK1, JAK2* and *STAT1* alterations in cells and corresponding tumour tissue from 6 out of 47 patients. In two heterozygous *JAK1* mutants IFNγ signalling was still active but was strongly impaired and no longer detectable in homozygous STAT1 and JAK1/2 mutants, respectively. JAK1/2-deficient tumour cells emerged in disease stage IV metastases under/after immunotherapy. The different treatments, including IFNα, IL2 and combinations thereof, might have induced or boosted the effector functions of tumour-reactive $CD8^+$ T cells, favouring mutant outgrowth. Indeed, we demonstrated that JAK1/2 loss protected melanoma cells from anti-proliferative and pro-apoptotic IFNγ activity. Since JAK1 is a component also of the type I IFN signalling pathway, an additional selective pressure of IFNα on tumour cells cannot be excluded[8,31]. In contrast to the JAK1/2-deficient melanoma cells, *STAT1* mutant cells were established from a treatment-naive stage III lymph node metastasis suggesting that in this case spontaneous anti-tumour T-cell responses enriched these cells. In addition to melanoma, inactivating mutations in genes related to IFNγ signalling, in particular *JAK1*, have recently been described for microsatellite instable endometrial and colorectal cancers, arguing for a contribution to disease progression also in other malignancies[32–37].

In our patient models, JAK1 deficiency originated from an initial chromosome 1p aberration causing mono-allelic *JAK1* loss in melanoma cells and a subsequent mutation inactivating the remaining *JAK1* allele. Losses of the short arm of chromosome 1 are not uncommon in cutaneous and uveal melanoma with larger deletions occurring in around 10% of cutaneous melanomas[38,39]. More focal deletions as well as copy number neutral losses of heterozygosity may occur, that we detected at a high frequency in a cohort of 59 melanoma cell lines[21], showing also a very high frequency of allelic *JAK2* losses (76%). All of these alterations in addition to gene mutations would predispose to *JAK1/JAK2* inactivation and IFNγ-resistance in tumours if put under selective pressure by the immune system.

Interestingly, our data demonstrate that IFNγ-resistant JAK1/2-deficient melanoma cells progress to a 'higher level' of immunotherapy resistance. We provide evidence for the first time that IFNγ-resistant HLA class I-positive metastases can evolve into HLA class I-negative lesions thereby gaining complete $CD8^+$ T-cell resistance. The HLA class I-negative phenotype is caused by a coordinated silencing of genes involved in antigen presentation (*HLA-B, HLA-C, TAP1, TAP2, B2M*). Downregulation of this set of genes has previously been reported for melanoma and other tumour entities. The underlying molecular silencing mechanisms remain unclear but are most likely of epigenetic nature[40–42]. IFNγ is well known for its role in upregulating antigen processing and presentation thereby augmenting the detection and elimination of malignant cells by tumour antigen-specific $CD8^+$ T cells[43,44]. However, in case of JAK1/2 deficiency IFNγ-induced restoration of antigen presentation in tumour cells is abrogated. Phenotypically HLA class I-negative JAK1/2-deficient metastases share features with tumours lacking HLA class I surface expression due to inactivating *B2M* mutations as described by us and others[33,45–48]. HLA class I-negative metastases will be resistant towards any type of immunotherapy that is dependent on the activity of HLA class I-restricted tumour antigen-specific $CD8^+$ T cells, including adoptive cell therapy and checkpoint modulators. However, in contrast to *B2M* mutants, melanoma cells of the regulatory HLA class I-negative phenotype can regain HLA class I expression to adapt to specific environmental conditions such as metastatic sites (for example, lung, liver) enriched for natural killer cells that are specialized in recognition and killing of HLA class I-negative malignant cells[49].

Recently, resistance to anti-PD1 and anti-CTLA-4 therapy has been associated with sustained IFNγ signalling upregulating ligands for multiple inhibitory receptors on T cells, as well as IFNγ resistance protecting from cytokine-induced cell cycle arrest/apoptosis[33–35,50]. It will be of importance to determine how far the alterations we detected in pretreatment biopsies will undergo positive selection in tumours recurring upon anti-PD1 therapy or whether mutations will evolve *de novo* as recently described[33]. Of equal importance will be the identification of novel IFNγ-resistance mechanisms. Epigenetic factors as well as altered expression of negative IFNγ pathway regulators in tumour cells or microenvironmental influences could have an additional relevant role in conferring resistance or reduced sensitivity to IFNγ[51,52]. Furthermore, the resistance mechanisms could go beyond IFNγ and apply to other cytokines such as TNFα[13,14]. In this regards, the combined action of IFNγ and TNFα has been demonstrated to destroy tumour cells and their stroma thereby essentially contributing to the eradication of established mouse tumours[53].

It will be a considerable future challenge to identify all mutations associated with IFNγ resistance and to define the coevolution of HLA class I expression in longitudinal melanoma biopsies. Only this strategy will ensure that patients receive the most promising treatment options and be switched to other therapeutic regimens if IFNγ resistance develops, for example, oncogenic pathway inhibitors that could eliminate resistant tumour clones and allow the patients to reinitiate immunotherapy.

Figure 4 | IFNγ-resistant melanoma evolves into a T-cell-resistant lesion. (**a**) Mutation defined by targeted sequencing on DNA from Ma-Mel-61h cells and autologous blood cells as wild-type (WT) control (ctrl). Plots of aligned sequencing reads in the location where the *JAK1* c.1798G>T, p.G600W mutation was identified. WT sequence shown on the bottom, arrow highlights mutation or corresponding wild-type (WT) site. Number of sequencing reads notated on the left; %, frequency of mutations in reads. (**b**) Lysates from IFNγ-treated (48 h) Ma-Mel-61b, Ma-Mel-61g and Ma-Mel-61h cells analysed by western blot for expression of IRF1 and HLA class I heavy chains; GAPDH, loading control. Representative data from $n=3$ independent experiments. (**c**) HLA class I and CD54 surface expression on IFNγ-treated (48 h) Ma-Mel-61g and Ma-Mel-61h cells, measured by flow cytometry. Representative data from $n=3$ independent experiments. (**d**) Immunohistochemical staining of serial cryostat tissue sections from metastasis Ma-Mel-61g for melanoma marker HMB45 and HLA class I. (**e**) Ma-Mel-61h and Ma-Mel-61g cells, transfected with expression plasmids encoding wild-type JAK1 or mutant JAK1-G600W, analysed for specific mRNA expression by quantitative reverse transcription–PCR in the presence of absence or IFNγ (48 h). Ma-Mel-61b cells served as a control (ctrl). Relative expression levels given as means (+s.e.m.) from $n=2$ independent experiments. (**f**) HLA class I surface expression on IFNγ-treated (48 h) Ma-Mel-61h-JAK1 and Ma-Mel-61h-JAK1-G600W transfectants, measured by flow cytometry. Representative data from $n=2$ independent experiments. (**g**) Ma-Mel-61h and Ma-Mel-61g cells, transfected with expression plasmids encoding wild-type JAK1 (WT) or mutant JAK1-G600W (M), analysed for recognition by autologous $CD8^+$ T cells in the presence or absence of IFNγ (48 h). T-cell activation measured as IFNγ release by ELISpot assay. Representative data from $n=2$ independent experiments.

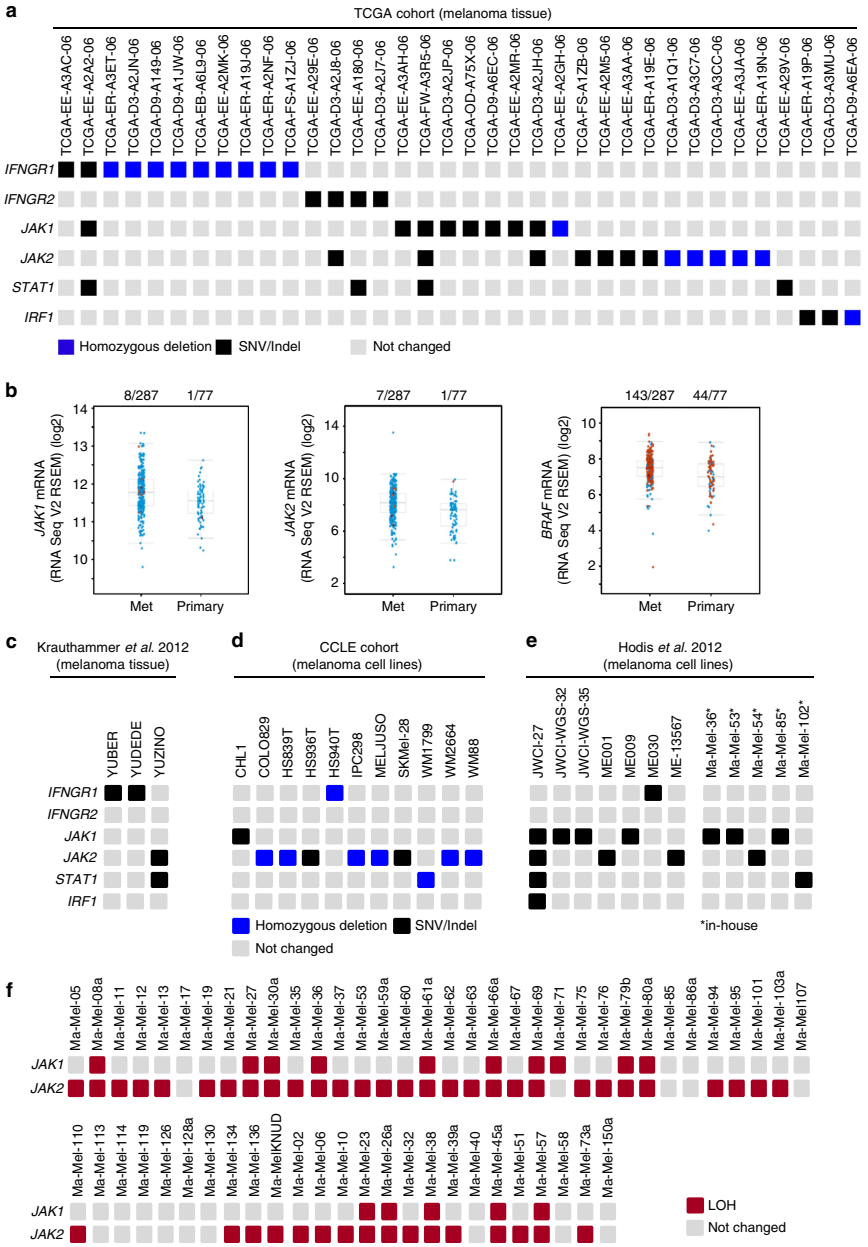


Figure 5 | Mutations in melanoma tissue samples and cell lines. (**a**) Genetic alterations (homozygous deletions, single-nucleotide variants [SNV], small insertions and deletions [Indel]) in components of the type II IFN signalling pathway in TCGA melanoma samples ($n=287$, accessed 31/12/2016). (**b**) Mutations in *JAK1, JAK2* and *BRAF* in the TCGA melanoma dataset stratified by tissue origin (primary tumours vs metastases). *y* axis, indicates expression of corresponding mRNA. Each circle represents a sample, samples harbouring SNV or Indels are shown in red ($n=287$, accessed 30/12/2016). (**c**–**e**) Recurrent mutations of type II IFN signalling pathway genes in published datasets[15,19,20]. (**f**) SNP array data from 59 melanoma cell lines[21] show LOH at the *JAK1* (Chr. 1p31.3) and *JAK2* (Chr. 9p24.1) locus.

Methods

Patients samples. Peripheral blood samples and tumour tissues were collected after written informed patient consent with institutional review board approval. Melanoma cell lines were established from excised metastatic lesions. 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). Melanoma cells were cultured in RPMI1640 or DMEM medium with L-glutamine (Gibco/Life technologies) and 10% fetal calf serum. Cells were seeded and rested overnight followed by addition of IFNγ (500 U ml^{-1}, Boehringer Ingelheim) or IFNα2b (1,000 U ml^{-1}, Essex Pharma) and incubation for indicated periods.

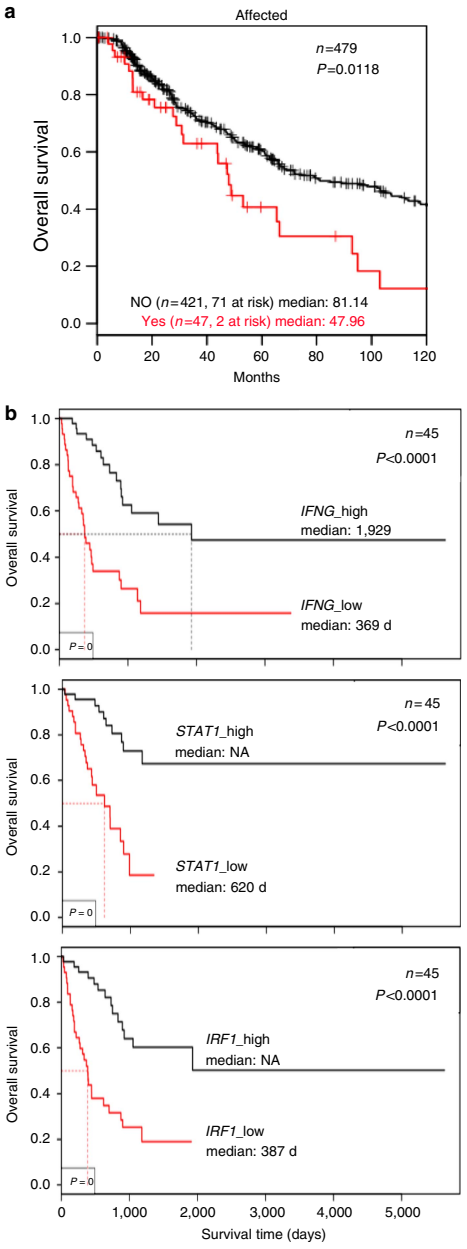


Figure 6 | Alterations in IFNγ pathway signalling impact on patient survival. (**a**) Truncating mutations, homozygous deletions and low protein levels of *IFNGR1, IFNGR2, JAK1, JAK2, STAT1* and *IRF1* define a subset of melanoma patients in the TCGA data set with decreased survival. Of 479 patients with data on aberrations, 468 had available survival data. Log-rank *P* value shown. (**b**) Kaplan–Meier survival curves for *IFNG, STAT1* and *IRF1* expressing TCGA melanomas[56], *P* values shown from Log-rank tests. Multivariate *P*values corrected for age: *IFNG* $P=1.79e\text{-}05$, $n=90$, 47 events; *STAT1* $P=3.82e\text{-}05$, $n=90$, 33 events; *IRF1* $P=2.52e\text{-}05$, $n=90$, 44 events.

Immunohistochemistry. Serial cryostat tissue sections were stained with antibodies specific for HLA-DR,-DP,-DQ, kindly provided by S. Ferrone[54], HMB-45 (Dako), HLA class I antigen complexes (W6/32; Dianova) in combination with a Polymer Kit containing an AP-coupled secondary antibody (ZytoChem-Plus AP Polymer Kit, Zytomed).

Isolation of genomic DNA. Five to ten 10 µm-thick sections of FFPE tissue were deparaffinized according to the following protocol: 2 steps of 10 min xylene, 5 min 100% ethanol, 5 min 95% ethanol, 5 min 70% ethanol, rinsing in water. After drying, tumour tissue was manually macrodissected from the sections. Genomic DNA from tissue as well as 200 µl of whole blood (normal control) was isolated using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The same kit was used for DNA isolation from pelleted cultured tumour cells, Epstein-Barr virus-transformed B lymphocytes and peripheral blood mononuclear cells.

Targeted sequencing. A custom amplicon-based sequencing panel covering 12 genes of the interferon pathway (Supplementary Table 2) was designed and prepared applying the GeneRead Library Prep Kit from QIAGEN according to the manufacturer's instructions. Individual samples were barcoded using a kit from New England Biosciences and 24 samples sequenced in parallel on an Illumina MiSeq Next Generation Sequencer. Sequencing analysis was performed applying the CLC Cancer Research Workbench from QIAGEN. After trimming the primer sequences, the sequence reads were aligned to the human genome assembly 19 (hg19). Analysis for both in/dels and SNVs followed. SNPs were filtered out by cross-referencing the dbSNP database, the 1,000 genomes database and in individual cases manually. Mutations affecting the coding region of the gene were considered if predicted to result in non-synonymous amino acid changes, overall coverage of the mutation site was ≥30 reads, >5 reads reported the mutation variant and the frequency of mutated to unmutated reads was ≥10%.

SNP array analysis. SNP arrays were performed using the CytoScan HD Array from Affymetrix. Hybridization was done according to the manufacturer's protocol and data analysis performed applying the program Chromosome Analysis Suite from Affymetrix.

Quantitative real-time PCR. Total mRNA was isolated from tumour cells using the RNeasy plus Mini Kit (Qiagen), in combination with RNase-free DNase Set (Qiagen) according to the manufacturer's instructions. Reverse transcription, TaqMan-based real-time PCR and calculation of relative expression were performed as described previously[55]. Taqman assay systems specific for *HLA-B, HLA-C, TAP1, TAP2, B2M, GAPDH* were purchased from Thermo Fisher. In all experiments the amount of specific mRNA was normalized to endogenous *GAPDH* mRNA levels.

Analyses of published melanoma data sets. Kaplan–Meier survival plots, log-rank tests and multivariate Cox-regressions based on differentially expressed genes from TCGA melanoma samples were assessed using the UZH cancer browser samples[56], using the 10% percentile for comparing samples with the lowest and the highest mRNA expression for *IFNG*, *STAT1*, *IRF1* and *CD8A*. Information on mutations, including single-nucleotide variants (SNV), small insertions/deletions (Indels) and copy number variants (considering only homozygous deletions) in genes of the type II IFN signalling pathway *(IFNGR1*, *IFNGR2*, *JAK1*, *JAK2*, *STAT1*, *IRF1)* were obtained from TCGA skin cutaneous melanoma (SKCM) samples with complete mutation data ($n=287$, accessed 31/12/2016). Survival analyses based on mutations and aberrant protein expression were based on the extended SKCM TCGA data set ($n=487$) and clinical data obtained from cBioPortal, generating

Table 2 | Mutations in biopsies from patients receiving anti-PD1 ($n=59$) and anti-CTLA4 ($n=110$) immunotherapy.

Material	Sample	Gene	AA change	cDNA change	Treatment	Response
Patient	15-12774	*IRF1*	W195*	584G>A	Nivo	PD
Patient	D5923-13	*JAK2*	D710fs	2123_2124insC	Pemb	PD
Patient	31968-14	*JAK1*	R681Q	2042G>A	Pemb	PD
Patient	14-28049	*JAK1*	E501K	1501G>A	Nivo	CR
Patient	D788-15	*JAK1*	E903K	2707G>A	Nivo	PD
Patient	13-12191	*JAK2*	H103Y	307C>T	Nivo	PR
Patient	32616-13	*JAK2*	S15F	44C>T	Nivo	PR
Patient	18298-15	*JAK2*	T376fs	1125delA	Pemb	PR
Patient	5870-14†	*JAK2*	P121S	361C>T	Nivo	SD
Patient	D2788-15	*JAK1*	K249N	747G>T	Nivo	Unknown
Patient‡	343-16§	*JAK1*	L149F, T147I	445C>T, 440C>T	Nivo	PD
Patient	132	*IFNGR1*	D465N	1393G>A	Ipilimumab	PR
Patient	4	*JAK1*	R110_splice	e5-1	Ipilimumab	PR
Patient	14	*JAK1*	G590R	1768G>C	Ipilimumab	PD
Patient	151	*JAK1*	V715M	2143G>A	Ipilimumab	PD
Patient	151	*JAK1*	G655D	1964G>A	Ipilimumab	PD
Patient	37	*JAK1*	G182E	545G>A	Ipilimumab	PD
Patient	4	*JAK2*	R138Q	413G>A	Ipilimumab	PR
Patient	163	*JAK2*	G164A	491G>C	Ipilimumab	PD
Patient	110	*IRF1*	R314W	940C>T	Ipilimumab	PD
Patient	82	*IRF1*	S221F	662C>T	Ipilimumab	PD

AA, amino acid; cDNA, complementary DNA; CR, complete response; fs, frameshift; ins, insertion; Nivo, Nivolumab; PD, progressive disease; Pemb, pembrolizumab; PR, partial response; SD, stable disease; splice, splice site mutation.
*Nonsense—stop codon mutation.
†Additionally a *IFNGR1*, E195K, 685G>A mutation.
‡Relapse under treatment.
§Additionally a *IFNGR1*, G129E, 461G>A and *IFNGR2*, T187I, 503C>T mutation.

Kaplan–Meier survival plots and log-rank tests in R (R Development Core Team; http://www.R-project.org). Coexpression plots were obtained for TCGA SKCM samples using cBioPortal[57]. Mutation calls, including SNV and Indels, from 110 patients before anti-CTLA-4 antibody therapy were reported previously[7]. Additional mutation data for melanoma tissues and cell lines with SNV/Indels[15,20] as well as for melanoma cell lines including also homozygous deletions, as part of the CCLE project[19] were assessed using cBioPortal[57].

SNP array data from 59 melanoma cell lines analysed with the 250 k StyI SNP array of the Affymetrix GeneChipV 500 K array set (Affymetrix, Santa Clara, CA), GEO accession number GSE17534 (ref. 21) were assessed for LOH using Affymetrix genotyping console software. In 44 cases, SNP data from corresponding germline DNA were available for paired analysis. In the remaining cases, the SNP data from tumour samples were compared with baseline values obtained from combined analysis of the SNP data from the 44 available germline cases.

Western blot. Proteins from tumour cell lysates were separated by SDS–polyacrylamide gel electrophoresis, blotted on nitrocellulose membranes and probed with the following primary antibodies: anti-STAT1 (Santa Cruz, clone M-22, 1:1000) and anti-pSTAT1 (Cell Signaling, clone 58D6, 1:1,000), anti-IRF1 (Santa Cruz, clone H-205, 1:500) and anti-GAPDH (Cell Signaling, 14C10, 1:5,000). HC10 (1:1,000) was used for detection of β2m-free HLA heavy chains[58,59]. After washing, membranes were incubated with the appropriate secondary antibodies linked to horseradish peroxidase. Antibody binding was visualized with the enhanced chemiluminescence (ECL) system. Full scans of western blots are depicted in Supplementary Figs 7–9.

Flow cytometry. The following directly labelled antibodies were used for staining of cellular surface markers: anti-HLA-ABC-APC (eBiosciences, clone W6/32; 1 µl), anti-CD54-PE (Beckmann Coulter, clone 84H10; 2.5 µl), anti-PD-L1-PE (Biolegend, clone 29E2A3; 5 µl) and anti-HLA-DR-PC7 (Beckmann Coulter, clone Immun-357; 2.5 µl). After fixation, stained cells were analysed by flow cytometry on a Gallios flow cytometer (Beckmann Coulter) and Kaluza (Beckman Coulter) software, respectively, for data analysis. In order to isolate specific Ma-Mel-36 subpopulations, cells were stained with anti-HLA-DR-PC7 and sorted based on the specific expression of the surface markers by flow cytometry on an Aria II cell sorter and the FACS Diva software (BD Biosciences).

Plasmid generation and transfection. Wild-type *JAK1* was amplified using Phusion High-Fidelity DNA Polymerase (NEB) and the following primers: JAK1-SPAfo: 5′-ATCGTCCTCGAGATGCAGTATCTAAATATAAAA-3′ and JAK1-SPAre: 5′-ATTGCTCATATGTTTTAAAAGTGCTTCAAATCC-3′. After restriction digest with *Xho*I and *Nde*I, the PCR product was ligated into the PMZ3F vector (kindly provided by the laboratory of Jack Greenblatt, University of Toronto)[60]. Protein expression was verified by immunoblotting using a Flag-specific antibody (Sigma). The point mutation was introduced using Quikchange mutagenesis (Agilent) according to the manufacturer's protocol and the following primers: JAK1G600Wfo: 5′-ACACACATCTATTCTTGGACCCTGA TGGATTA-3′ and JAK1G600Wre: 5′-TAATCCATCAGGGTCCAAGAATAGAT GTGTGT-3′). The intended point mutations were verified by DNA sequencing and protein expression was examined by immune blotting. Lipofectamine (Life Technologies) was used for plasmid transfection of melanoma cells. After 48 h, cells were harvested and subjected to further analyses or treated with G418 for enrichment of transfectants.

Real-time proliferation assay (xCELLigence). For background measurement 50 µl medium was added to an E-Plate 96 (Roche). Subsequently, melanoma cells were seeded in an additional volume of 100 µl medium. Cell attachment was monitored using the RTCA SP (Roche) instrument and the RTCA software Version 1.1 (Roche). After 20–24 h cells were treated with IFNγ (500 U ml^{-1}) or left untreated, followed by incubation for 7 d at 37 °C. All experiments were performed in duplicates. Changes in electrical impedance were expressed as a dimensionless cell index value, which derives from relative impedance changes corresponding to cellular coverage of the electrode sensors, normalized to baseline impedance values with medium only.

Expansion of autologous tumour-reactive T cells. Tumour-reactive T cells were expanded following a previously described protocol[47]. Briefly, CD8$^+$ T lymphocytes were isolated from cryopreserved peripheral blood mononuclear cells using anti-CD8 MicroBeads (Miltenyi Biotech). Isolated T cells (1×10^6) were co-cultured in 24-well culture plates with 1×10^5 irradiated (100 Gy or 120 Gy) autologous tumour cells per well in 2 ml of AIM-V (GIBCO/BRL) supplemented with 10% (vol/vol) human AB serum. Medium was supplemented with IL-2 (250 U ml^{-1}) on day 3. CD8$^+$ T cells were restimulated at weekly intervals with irradiated melanoma cells. After two rounds of restimulation, T cells were subjected to ELISpot assays.

IFNγ ELISpot assay. IFNγ enzyme-linked immunospot (ELISpot) assay was performed as previously described[61]. Briefly, multiscreen-HA plates (Millipore, Bedford, MA) were coated with 5 µg ml^{-1} anti-hIFNγ-mAb 1-D1K (Mabtech). T cells were seeded in RPMI medium and added at indicated numbers to 1×10^4 tumour cells per well. After 20–24 h incubation at 37 °C in 5% CO_2, a biotinylated secondary anti-hIFNγ antibody (1 µg ml^{-1}, clone 7-B6-1, Mabtech) was added and spots were developed by sequential addition of 1:1,000 diluted ExtrAvidin alkaline phosphatase and BCIP/NBT Liquid Substrate System (Sigma-Aldrich). Spot numbers were determined with the AID EliSpot reader (AID Diagnostika).

Data availability. SNP array data files are accessible at the NCBI GEO database. Cell lines used in this study can be obtained by MTA.

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Acknowledgements

Funding: This study was supported by the Deutsche Krebshilfe to A.P. and D.S. ('Translational Oncology' 111546) and to Su.H. (70111402); M.T. and A.M.W. receive funding from the DFG through TRR60 and GRK1949. The results shown here are in part based on data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

Author contributions

A.S. established patient models, performed targeted sequencing, analysed data; F.Z. carried out T-cell assays; C.H. performed T-cell assays and IHC; N.P. performed western blots, PCR, apoptosis and proliferation assays; R.M. carried out western blots, transfections, proliferation assays; N.S. supported targeted sequencing; B.R. helped with western blots; N.B. carried out IHC; Se.H. generated expression plasmids; B.W., R.G., J.U., C.L. and H.G. provided patient samples; L.K.-H. and M.Z. performed SNP arrays and data analyses; A.M.W. carried out cell sorting; M.T. supported plasmid constructions, edited the manuscript; Su.H. performed bioinformatics; B.S. collected patient samples, edited the manuscript; D.S. established patient models, contributed to conception and design of the study; K.G. contributed to conception and design of the study, carried out data analyses, edited manuscript; A.P. designed experiments, analysed data, wrote manuscript.

Additional Information

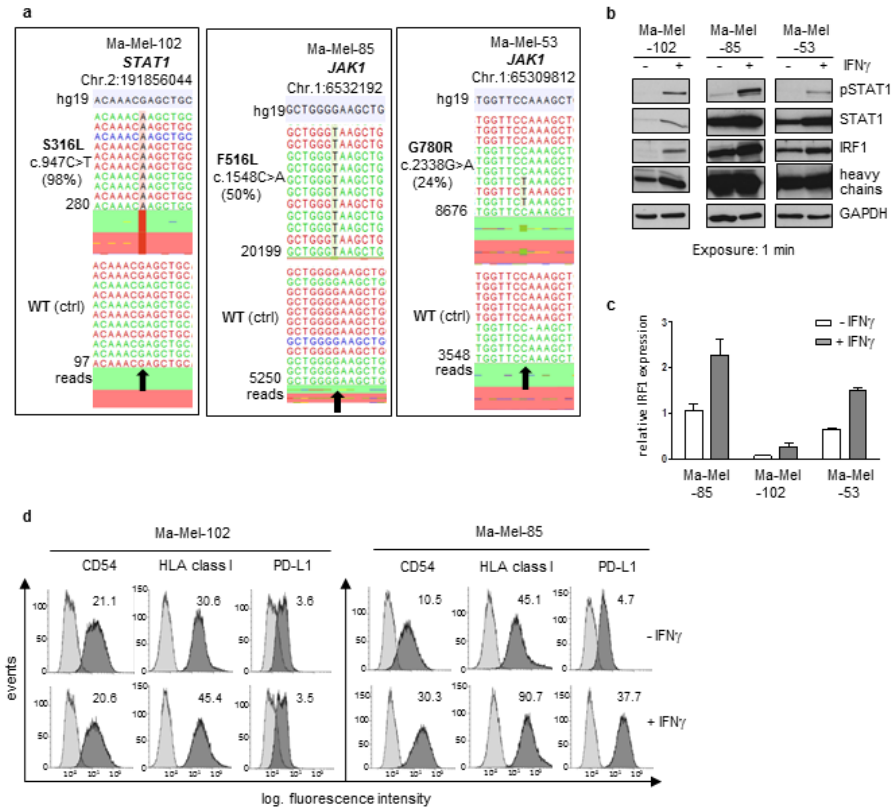
Accession code: SNP array data files are accessible at the NCBI GEO database under GSE96884.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

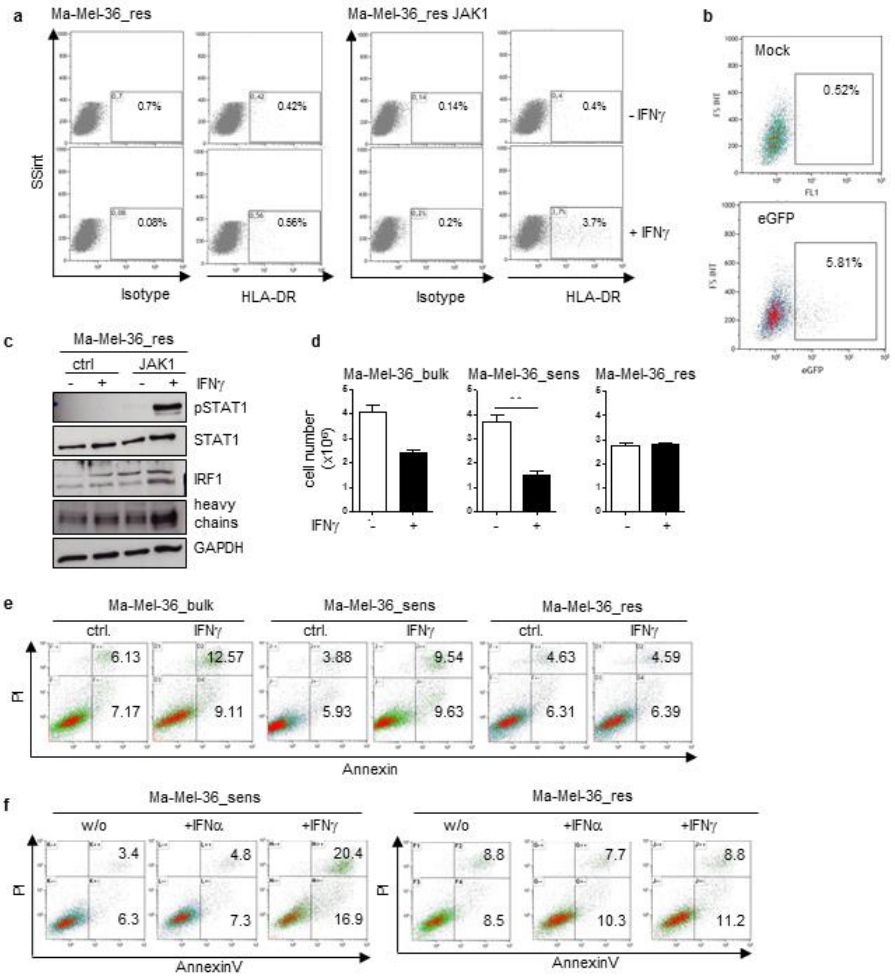
Competing interests: B.W.: grants from Merck/MSD, Bristol Myers Squibb, personal fees from Philogen, Roche. J.U.: advisory boards and on speakers bureaus of Amgen, BMS, GSK, MSD, Novartis, and Roche. R.G.: research support from Novartis, Pfizer, Johnson&Johnson; honoraia for lectures from Roche Pharma, Bristol-MyersSquibb, GlaxoSmithKline, Novartis, Merck Serono, MSD, Almirall-Hermal, Amgen, Galderma, Janssen, Boehringer Ingelheim; paid advisory role for Roche Pharma, Bristol-MyersSquibb, GlaxoSmithKline, Novartis, MSD, Almirall-Hermal, LEO, Amgen, Pfizer. C.L.: Advisory/Consultant or speakers fee from BMS, Roche, Amgen, Novartis, Pierre Fabre, Biontech, MSD. B.S.: advisory board or honoraria from Novartis, Roche, Bristol-Myers Squibb and MSD Sharp & Dohme, research funding from Bristol-Myers Squibb and MSD Sharp & Dohme, travel support from Novartis, Roche, Bristol-Myers Squibb and AMGEN. D.S.: advisory board or honoraria from Roche, Genentech, Novartis, Amgen, GlaxoSmithKline, Bristol-Myers Squibb, Boehringer Ingelheim, and Merck Sharp & Dohme. All other authors declare no competing financial interests.

How to cite this article: Sucker, A. *et al.* Acquired IFNγ resistance impairs anti-tumor immunity and gives rise to T-cell-resistant melanoma lesions. *Nat. Commun.* **8,** 15440 doi: 10.1038/ncomms15440 (2017).

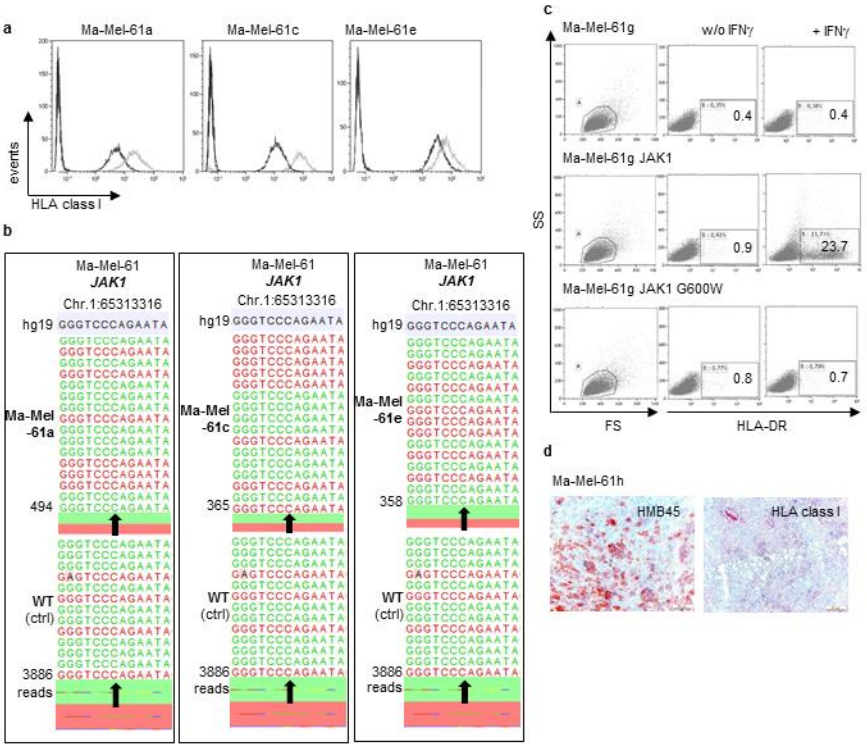
Supplementary Figures



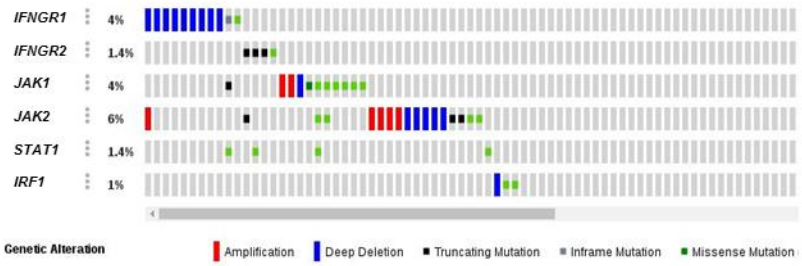
Supplementary Figure 1l *STAT1* mutation impairs IFNγ signaling. (**a**) Mutations defined by targeted sequencing on DNA from Ma-Mel-102, Ma-Mel-85 and Ma-Mel-53 cell lines and autologous blood cells as wild-type (WT) control (ctrl). Plots of aligned sequencing reads in the locations where *STAT1* c.947C>T, p.S316L, *JAK1* c.1548C>A, p.F516L and *JAK1* c.2338G>A, p.G780R mutations were identified. WT sequences shown on the bottom, arrows highlight mutation sites. Number of sequencing reads notated on the left; %, frequency of mutations in reads. (**b**) Lysates from IFNγ-treated (48 h) melanoma cells analyzed in parallel by Western blot for expression of pSTAT1, STAT1, IRF1 and HLA class I heavy chains; GAPDH, loading control. Representative data from n=2 independent experiments. (**c**) Quantification of IRF1 expression among all samples using the image processing program imageJ. IRF1 expression values were normalized to endogenous GAPDH. Mean values and SEM (error bars) obtained from n=2 independent experiments. (**d**) Surface expression of CD54, HLA class I and PD-L1 on IFNγ-treated (48 h) melanoma cells, measured by flow cytometry. Representative data from n=3 independent experiments.



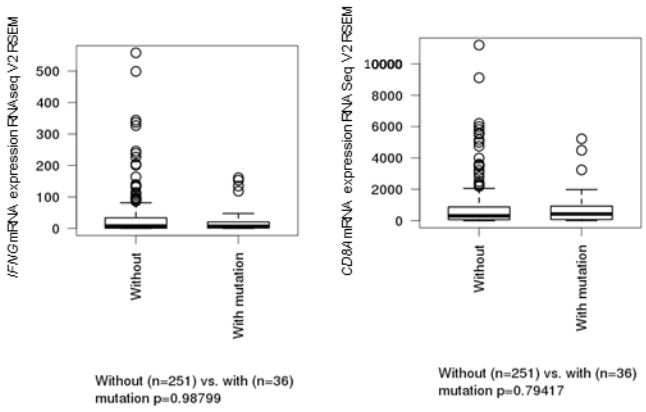
Supplementary Figure 2l JAK1 re-expression in Ma-Mel-36_res cells restores IFNγ signaling. (**a**) Melanoma cells transiently transfected with a *JAK1* expression plasmid and treated with IFNγ (500 U/ml) for 48 h. Non-transfected cells served as control. Surface expression of HLA-DR measured by flow cytometry. Representative data from n=2 independent experiments. (**b**) Transfection efficiency of Ma-Mel-36_res cells determined by eGFP plasmid transfection. Representative data from n=2 independent experiments. (**c**) Lysates from IFNγ-treated (48 h) *JAK1*-transfected and control Ma-Mel-36_res cells analyzed by Western blot for expression of indicated proteins; GAPDH, loading control. (**d**) Numbers of vital Ma-Mel-36_bulk, Ma-Mel-36_sens and Ma-Mel-36_res cells after IFNγ treatment (7 d). Only statistical significant differences defined by paired Student's t-test are indicated, **p<0.002. Mean values and SEM (error bars) obtained from n=3 independent experiments. (**e**) IFNγ-induced (7 d) apoptosis in melanoma cells determined by AnnexinV/PI staining in flow cytometry. (**f**) Comparison of IFNα and IFNγ treatment (7 d) for apoptosis induction in melanoma cells by AnnexinV/PI staining. (**e, f**) Representative dot plots from three (**e**) and two (**f**) independent experiments, indicating early (AnnexinV+/PI-) and late apoptotic (AnnexinV+/PI+) cells.



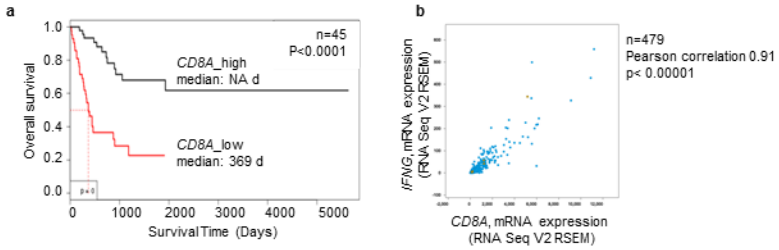
Supplementary Figure 3l JAK1 re-expression in Ma-Mel-61g cells restores IFNγ signaling. (**a**) Surface expression of HLA class I on Ma-Mel-61a, Ma-Mel-61c and Ma-Mel-61e cells treated with IFNγ (500 U/ml) for 48 h, measured by flow cytometry. Representative data from n=2 independent experiments. (**b**) No *JAK1* mutation in Ma-Mel-61a, Ma-Mel-61c and Ma-Mel-61e cells. Targeted *JAK1* sequencing on DNA from Ma-Mel-61a, Ma-Mel-61c and Ma-Mel-61e cells and autologous blood cells as wild-type (WT) control (ctrl). Plots of aligned sequencing reads, WT sequences shown on the bottom, arrows highlight the mutation site *JAK1* c.1798G>T, p.G600W detected in Ma-Mel-61g cells. Number of sequencing reads notated on the left. (**c**) Ma-Mel-61g cells transiently transfected with expression plasmids encoding JAK1 or JAK1 G600W treated with IFNγ (500 U/ml) for 48 h. Non-transfected cells served as control. Surface expression of HLA-DR measured by flow cytometry. Representative data from n=3 independent experiments. (**d**) Immunohistochemical staining of serial cryostat tissue sections from metastasis Ma-Mel-61h for melanoma marker HMB45 and HLA class I.



Supplementary Figure 4l Mutation co-occurrence in melanoma tumor samples. TCGA melanoma samples (n=287) with sequencing and CNV data were analyzed for co-occurence of genetic alterations. *JAK1* and *STAT1* mutations show a tendency for co-occurrence (p=0.008, log OR>3), although only 2 out of 4 *STAT1* mutations co-occur with 2 out of 11 *JAK1* mutations.



Supplementary Figure 5l *IFNG and CD8A* mRNA expression in tumors with and without mutations. TCGA melanoma samples (n=287) with available data for SNV/Indels and CNV (homdels) were studied for *IFNG* and *CD8A* mRNA expression. Two-sided Wilcoxon rank test revealed no differences in specific mRNA expression levels between tumor with and without mutations.



Supplementary Figure 6l *CD8A* mRNA correlates with survival and is co-expressed with *IFNG*. (**a**) Kaplan Meier survival curves for *CD8A* mRNA expressing TCGA melanomas, p-value shown from Log-rank tests. Multivariate p-value corrected for age: p=1.67e-05, n=90, 40 events; (**b**) *CD8A* and *IFNG* mRNA are co-expressed in TCGA samples (yellow points indicate samples mutated in either gene).

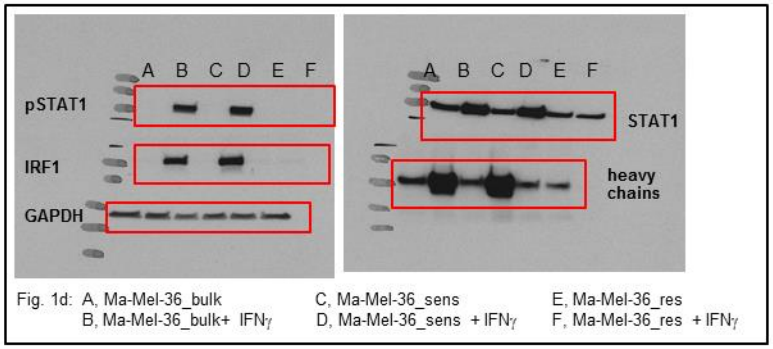


Fig. 1d: A, Ma-Mel-36_bulk
B, Ma-Mel-36_bulk+ IFNγ
C, Ma-Mel-36_sens
D, Ma-Mel-36_sens + IFNγ
E, Ma-Mel-36_res
F, Ma-Mel-36_res + IFNγ

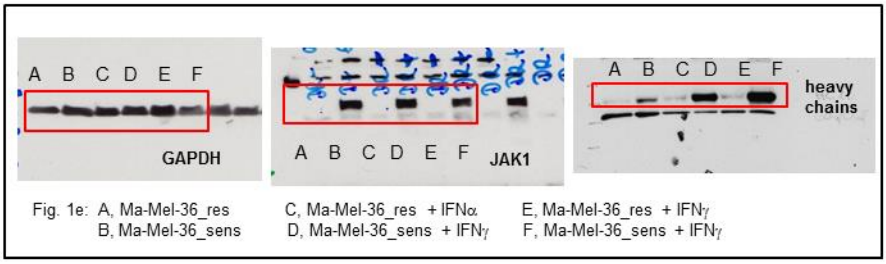


Fig. 1e: A, Ma-Mel-36_res
B, Ma-Mel-36_sens
C, Ma-Mel-36_res + IFNα
D, Ma-Mel-36_sens + IFNγ
E, Ma-Mel-36_res + IFNγ
F, Ma-Mel-36_sens + IFNγ

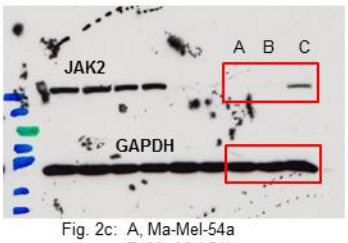
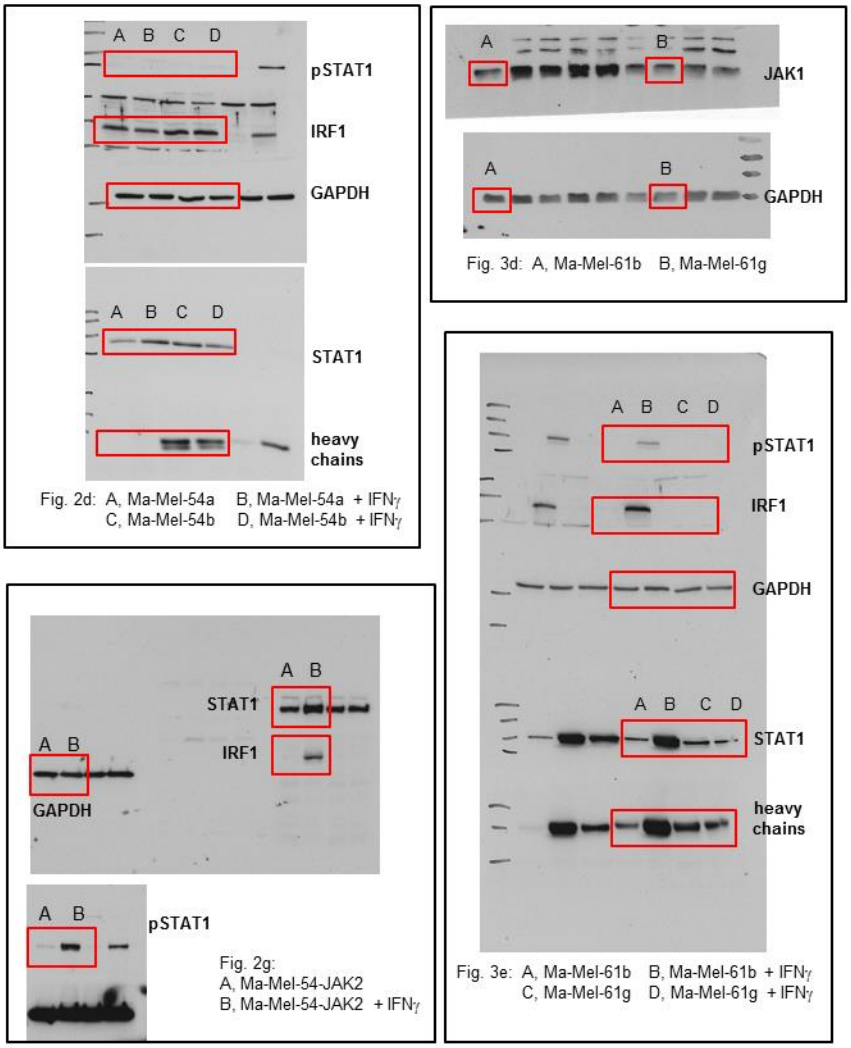


Fig. 2c: A, Ma-Mel-54a
B, Ma-Mel-54b
C, Ma-Mel-61b

Supplementary Figure 7l Full blots of immunoblots shown in the main figures of the manuscript. The respective main figures are indicated.



Supplementary Figure 8l Full blots of immunoblots shown in the main figures of the manuscript. The respective main figures are indicated.

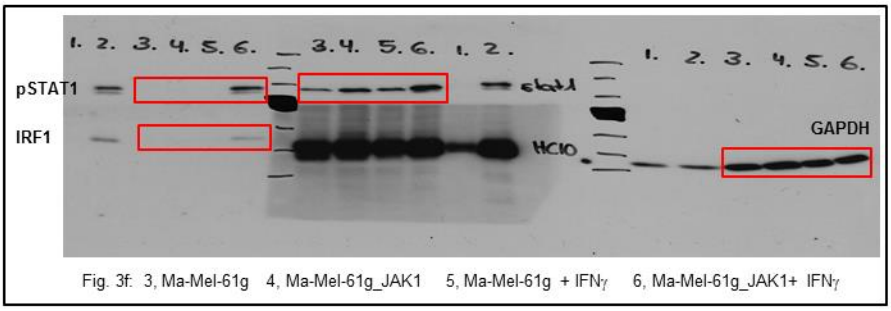


Fig. 3f: 3, Ma-Mel-61g 4, Ma-Mel-61g_JAK1 5, Ma-Mel-61g + IFNγ 6, Ma-Mel-61g_JAK1+ IFNγ

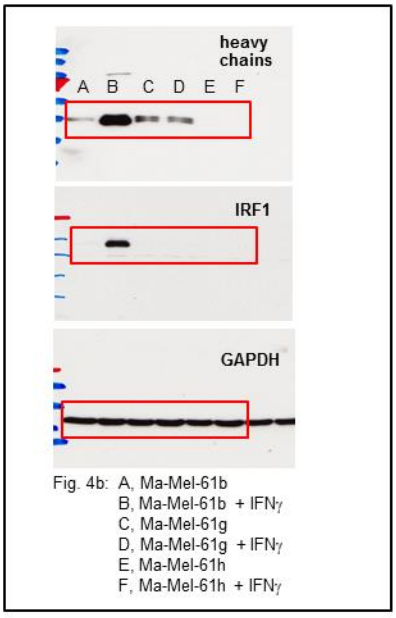


Fig. 4b: A, Ma-Mel-61b
B, Ma-Mel-61b + IFNγ
C, Ma-Mel-61g
D, Ma-Mel-61g + IFNγ
E, Ma-Mel-61h
F, Ma-Mel-61h + IFNγ

Supplementary Figure 9l Full blots of immunoblots shown in the main figures of the manuscript. The respective main figures are indicated

Supplementary Table 1: Treatment response in patient with and without mutations

Best response under anti-PD1 treatment

Clinical response, anti-PD-1 treatment	CR	PR	MR	SD	DC	PD	X-squared	df	p_chi2
Without mutations	1	9	4	7	21	20	NA	NA	NA
With mutations	1	3	0	1	5	6	2.59	4	0.63

Odds ratio for DC vs. PD (p=0.73, OR=1.25, CI=0.32-5.13)

Best response under anti-CTLA-4 treatment

Clinical response, anti-CTLA-4 treatment	CR	PR	SD	DC	PD	X-squared	df	p_chi2
Without mutations	2	12	10	24	67	NA	NA	NA
With mutations	1	2	2	5	9	1.31	3	0.73

Odds ratio for DC vs. PD (p=0.47, OR=0.64, CI=0.2-2.34)

7.2. Article II

ONCOIMMUNOLOGY
2017, VOL. 0, NO. 0, e1392426 (13 pages)
https://doi.org/10.1080/2162402X.2017.1392426

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ORIGINAL RESEARCH

Impaired NK cell recognition of vemurafenib-treated melanoma cells is overcome by simultaneous application of histone deacetylase inhibitors

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ABSTRACT

Therapy of metastatic melanoma advanced recently with the clinical implementation of signalling pathway inhibitors, such as vemurafenib, specifically targeting mutant $BRAF^{V600E}$. In general, patients experience remarkable clinical responses under BRAF inhibitor (BRAFi) treatment but eventually progress within 6–8 months due to resistance development. Responding metastases show an increased immune cell infiltrate, including also NK cells, that, however, is no longer detectable in BRAFi-resistant lesions, suggesting NK cell activity should be exploited to prevent disease progression. Here, we examined the effects of BRAFi on the expression of ligands targeting activating NK cells receptors immediately after treatment onset, prior to resistance development. We demonstrate that $BRAF^{V600E}$ mutant melanoma cells cultured in the presence of vemurafenib, strongly decreased surface expression of ligands for NK activating receptors including the NKG2D-ligand, MICA, and the DNAM-1 ligand, CD155, and became significantly less susceptible to NK cell attack. NKG2D-ligand protein downregulation was due to a significant decrease in mRNA levels, already detectable 24 h after drug treatment. Interestingly, vemurafenib-induced MICA downregulation could be counteracted by treatment of melanoma cells with the histone deacetylase (HDAC) inhibitor (HDACi) sodium butyrate, that also upregulated the DNAM1-ligand, Nectin-2. HDACi treatment enhanced surface expression of NKG2D-ligands in the presence of BRAFi, accompanied by recovery of NK cell recognition, but only upon simultaneous drug application. These results suggest that co-administration of BRAFi and HDAC inhibitors as well as having direct effects on melanoma cell survival, could also synergise to improve NK cell recognition and avoid tumour immune evasion.

ARTICLE HISTORY
Received 12 June 2017
Revised 9 October 2017
Accepted 11 October 2017

KEYWORDS
NK cell; NKG2D; BRAF inhibitors; melanoma; combination therapy; HDAC inhibitor; DNAM1

Introduction

Recent advances in the molecular characterization of melanoma cells have led to the development of new therapies for this aggressive, devastating type of skin cancer. Until 2009, the average survival time with metastatic melanoma was 6–12 months with a 5-year survival rate under 10%.[1] Since then, survival was significantly improved with the clinical implementation of new treatments, including immune checkpoint inhibition and specific chemotherapies.[2,3]

Nearly 50% of melanoma lesions have a valine to glutamic acid exchange at position 600 in the *BRAF* gene, known as $BRAF^{V600E}$.[4] This mutation leads to constitutive activation of the mitogen-activated protein kinase (MAPK) pathway that regulates cell proliferation and survival and so became a target for new inhibitors and therapeutic approaches.[5] Treatment with BRAF inhibitors (BRAFi), such as vemurafenib (PLX4032)[6] and dabrafenib (GSK2118436), induces tumour regression in a high proportion of metastatic melanoma patients with tumours bearing the $BRAF^{V600E}$ mutation and improves overall survival.[7] However, an important limitation of this therapy is the emergence of drug resistance after several months.[8] To prevent resistance development, combinations of MAPK inhibitors with immunotherapy are now being tested in clinical trials. This is based on the observations that lesions responding to BRAFi show an enhanced infiltration of immune cells. Indeed, BRAFi treatment of melanoma leads to increased tumour infiltration by $CD8^+$ T cells,[9,10] however it is also associated with increased expression in the tumour of markers such as TIM-3, PD1 and its ligand PDL1, that are associated with exhaustion and inhibition of effective immune responses. Interestingly, however, combination of BRAFi with checkpoint blockade produced only modest benefits when tested in murine models[11] and the combination of BRAFi with either anti-PD1[12] or anti-CTLA-4 immune checkpoint blocking antibodies[13] was associated with significant, severe toxicities in human patients, suggesting that there may be important difficulties associated with the use of these combinations of targeted therapy with immune checkpoint blockade in clinical practice. Despite this, the induction of long-lasting clinical responses by immunotherapy argues for the development of strategies that enhance the sensitivity of melanoma cells to immune cells under BRAFi treatment. Interestingly, mouse experiments suggest that besides T cells, also NK cells contribute to the therapeutic efficacy of

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Supplemental data for this article can be accessed on the publisher's website.

Table 1. Characteristics of cell lines established from metastatic melanoma.

	Ma-Mel-55	Ma-Mel-86c	Ma-Mel-86f	Ma-Mel-103b
BRAF	V600E	V600E	V600E	No mutation
Origin	Lymph node metastasis	Lymph node metastasis	Lymph node metastasis	Cutaneous/ subcutaneous metastasis
MICA alleles	A5.1/A5.1	A5.1/—	A5.1/A5	A5.1/A5
HLA	A2/A3	A01/—	β2-microglobulin$^-$	A2/A3
	B35/B7	B08/—		B8/B62

BRAFi in mouse melanoma models.[14,15] Since potentiation of cytotoxic T cell responses against malignant melanoma frequently faces the problem that melanoma cells readily acquire mutations that lead to evasion from T cell recognition,[16,17] the utilisation of Natural Killer (NK) cells as a component of immunotherapy for melanoma is of considerable interest.

NK cells respond to different targets depending on the balance of signals coming from a large array of receptor-ligand interactions.[18] It has been shown that NK cells can target melanoma cells for lysis via NKG2D, NKp46 and DNAM-1 and the absence of MHC Class I molecules only enhances this recognition.[19,20] NKG2D-ligands include, in human, two families of proteins, called MICA/B (MHC class I related Chain A/B) and ULBPs (UL16 Binding Proteins) that upon binding activate the NK cell cytotoxic machinery. DNAM1 participates in the adhesion between NK cells and their ligands which include CD155 (also called poliovirus receptor, PVR) and CD112 (Nectin-2) (Reviewed in.[21,22]) These ligands also regulate invasion and migration of tumour cells.[23] NK cells are known to accumulate in lymph nodes invaded by melanoma and can kill these tumour cells after cytokine activation.[24,25] Indeed, in murine models, NK cells have been shown to play a critical role in the control of melanoma metastasis achieved by BRAF inhibition,[14] however the effects of BRAF inhibition on the recognition of melanoma cells by human NK cells have not been explored systematically.

Published data indicate that the immune infiltrate changes in response to MAPK inhibitors and, again, on tumour progression.[26] Moreover, in an early phase of treatment the immune infiltrate contains NK cells while at progression the NK cells have disappeared.

We investigated the early effects of the BRAFi, vemurafenib, on the recognition of melanoma cells by NK cells. We show that, *in vitro*, treatment of human melanoma cells with the BRAFi leads to downregulation of ligands for activating receptors including NKG2D and DNAM-1, as well as upregulation of MHC Class I, a ligand for NK inhibitory receptors. These observations suggest that NK ligand expression strongly depends on the signaling route activated by oncogenic B-RAF, as recently observed by Frazao et al.[27]

Therefore, from a therapeutic perspective, although BRAF^{V600E} inhibition attacks melanoma growth directly, our data indicate that this treatment also contributes to tumour evasion from NK cell responses which could contribute to the development of resistance to BRAFi. Here, we also go on to show that the combination of BRAFi with drugs such as HDAC inhibitors promotes tumour cell death directly and increases immunogenicity for NK cells demonstrating the validity of the concept that combination therapy with BRAFi and other chemotherapeutic agents could enhance tumour destruction and immunogenicity.

Results

Characterization of ligands affecting NK activation on metastatic melanoma cell lines

To define the impact of BRAFi treatment on the recognition of melanoma cells by NK cells we selected four cell lines established from metastatic patient lesions, three BRAF-V600E mutant cell lines (Ma-Mel-55, Ma-Mel-86c and Ma-Mel-86f) and one BRAF-WT cell line (Table 1).[17,28] The cell lines Ma-Mel-86c and Ma-Mel-86f were established from two consecutive patient metastases with Ma-Mel-86c cells showing an HLA class I haplotype loss and Ma-Mel-86f cells being HLA class I negative due to a lack of β-2 microglobulin expression. The cells were also genotyped for their MICA alleles. In agreement with the loss of one HLA class I haplotype, Ma-Mel-86c expressed only one of the two MICA alleles detected in Ma-Mel-86f cells. In contrast, the cell lines Ma-Mel-55 and Ma-Mel-103 showed normal HLA class I and MICA expression (Table 1) (Figure 1). Surface expression of the NKG2D-ligands (NKG2D-L), MICA and ULBPs, was also analysed by flow cytometry. All the melanoma cell lines studied expressed MICA and ULBP2/5/6; ULBP3 was expressed by three out of four, while none of them expressed ULBP1. MICB was not present at the surface of these melanoma cell lines as assessed with a monoclonal antibody specific for MICB (data not shown). Since DNAM-1/CD155 interactions have been described to mediate NK cell recognition and lysis of melanoma cells,[19] the surface expression of the two DNAM-1 ligands, CD155 and CD112, was also analysed in this panel of cells. Interestingly, the expression of these adhesion molecules necessary for the formation of the NK immune synapse varied considerably between the different cell lines, in particular CD155.

Vemurafenib modulates the expression of NKG2D-ligands and CD155 on BRAF^{V600E} mutant melanoma cell lines

The effect of BRAF inhibition on the melanoma cells was analysed after 48 h of treatment with 1 μM vemurafenib. First, in MTT assays, the decrease in metabolic activity was confirmed to occur in BRAF mutant cells, but not in the non-mutant cell line; second, the decrease in pERK (Supplementary Figure 1A, B). Treatment with vemurafenib led to a decrease of surface MICA and ULBP3 on BRAF^{V600E} mutant cells, however, in the case of ULBP2/5/6, only the decrease in Ma-Mel-86f was statistically significant, although the amount of this molecule was rather low initially (Figure 2A, Supplementary Figure 1C). In contrast, MHC expression increased in cells carrying the V600E mutation. Interestingly, CD155 expression followed a similar pattern to MICA and

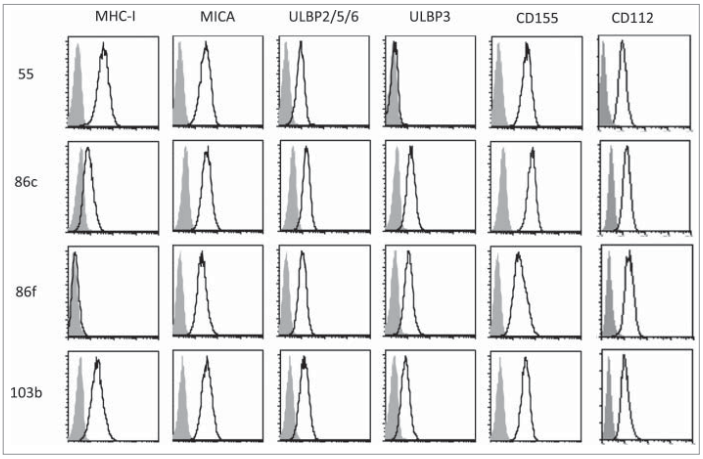

Figure 1. Cell surface expression of MHC, NKG2D-ligands and DNAM1-ligands on melanoma cells. The metastatic melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were stained with the indicated antibodies and analysed by flow cytometry. Isotype control appears in grey, and signal for each antibody as a black line. The panel shows one representative experiment out of 3–5.

decreased in $BRAF^{V600E}$ expressing cell lines after vemurafenib treatment, while CD112 was not significantly affected in most cell lines. To investigate the mechanism of the decreased cell surface expression of NK ligands caused by vemurafenib, the amount of total MICA protein in treated cells was analysed by Western Blot, showing a similar pattern to that of surface expression (Figure 2B). Moreover, the release of NKG2D-L did not seem to be involved in this phenomenon, since vemurafenib treatment did not increase the amount of soluble protein detected by ELISA (Figure 2C). Thus, changes in mRNA content were studied (Figure 2D). The decrease of MICA and CD155 cell surface expression after vemurafenib treatment was accompanied by reduced levels of mRNA in two (out of three) cell lines carrying the $BRAF^{V600E}$ mutation, while mRNA levels remained unchanged in the non-mutant cell line. ULBP2 mRNA did not change significantly after vemurafenib treatment. These results show that treatment with BRAFi of melanoma cells bearing the V600E mutation in *BRAF* reduced cell surface expression of ligands for activating NK receptors at the same time that the expression of MHC molecules, ligands for inhibitory receptors, was increased, suggesting that the ability of NK cells to mediate cytotoxicity against BRAFi treated melanoma cells could also be influenced.

Since the data obtained above pointed to the possibility that NK recognition could be affected early after treatment with the BRAFi, the kinetics of the effect of vemurafenib on surface expression of NK ligands was analysed 1, 24 and 48 h after the addition of the inhibitor (Figure 3A). The decrease of MICA and CD155 was not detectable after 1 h of treatment with the inhibitor. Although the trend could be observed at 24 h, it was only statistically significant for the line Ma-Mel-55. So, we conclude that, at 48 h, the effect of vemurafenib was evident for BRAF mutant melanoma cell lines but not for non-mutant cells. Interestingly, monitoring of real-time proliferation of melanoma cells in the Xcelligence system, demonstrated that ligand expression was related to cell proliferation: while significant changes in surface MICA were observed after 24 h of treatment in Ma-Mel-55, a significant decrease was also observed at 48 h in Ma-Mel-86c and Ma-Mel-86f, and this parallels the differences in proliferation curves between DMSO and vemurafenib treated cells for Ma-Mel-55, Ma-Mel-86c and Ma-Mel-86f (Figure 3B).

Vemurafenib affects the NK recognition of melanoma cells

The recognition by autologous and allogenic NK cells of melanoma cell lines treated with BRAFi for 48 h was analysed next using either NK cells from unrelated healthy donors or autologous NK cells from patient Ma-Mel-86 activated *in vitro*. The response of allogeneic NK cells against melanoma cell lines, either untreated or treated with BRAFi, was evaluated in degranulation assays (Figure 4). Treatment with vemurafenib led to a decrease in NK degranulation against the three metastatic melanoma cell lines with the V600E mutation in *BRAF* (Figure 4A). In these experiments, MHC class I was blocked to avoid donor-to-donor variation due to differences in inhibitory KIR expression of the donors. Even so, there was a decrease in NK recognition after treatment with vemurafenib which most likely is due to the decrease of activating ligands on melanoma cells. These data agree with reports showing that NK cells recognise melanoma and other tumour cells mainly through activating ligands such as NKG2D-L, NCR and DNAM-1[19,20,29]. Thus, enhanced expression of MHC class I by vemurafenib-treated melanoma cells is not the only factor that would facilitate NK evasion of vemurafenib-treated melanoma cells. Importantly, these data obtained in experiments with allogeneic NK cells could be confirmed in experiments using autologous NK cells from melanoma patient Ma-Mel-86, which showed that BRAF inhibition led to a reduction of around 40% in NK degranulation. In the autologous model, for comparison, NK cells were also incubated with vemurafenib. The decrease

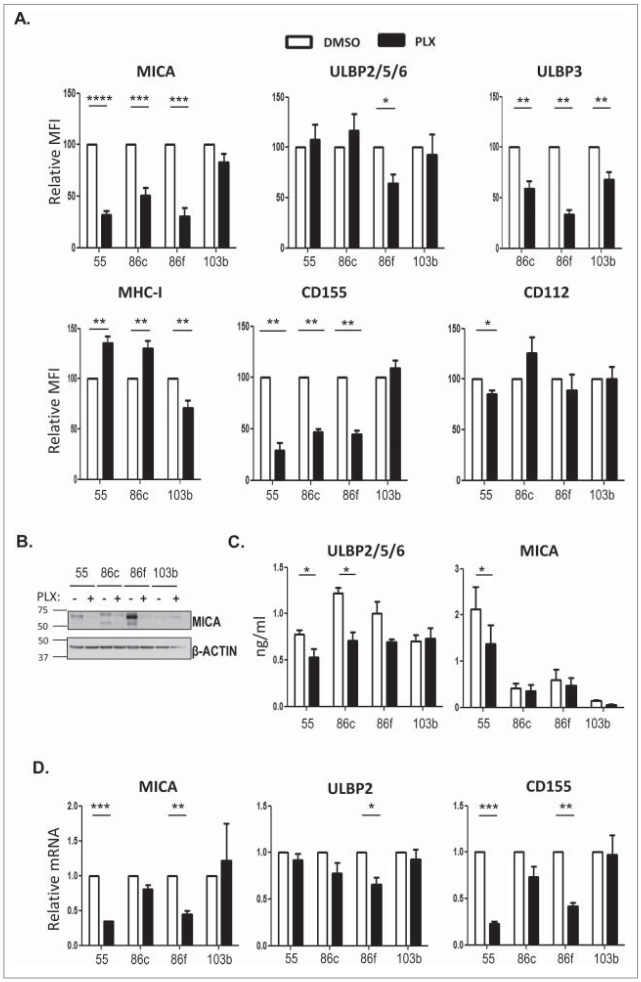

Figure 2. Effect of vemurafenib on NK ligand expression in melanoma cells. Melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with 1 μM vemurafenib (PLX) for 48 h (A,B, D) or 24 h (C) (control cells were treated with the carrier DMSO) A. Flow cytometry. Melanoma cells were stained for detection of the indicated markers by flow cytometry. The plots represent the change in the mean fluorescence intensity (MFI) of each marker, as the percentage of the molecule present in control (DMSO) cells. Data are the mean and SEM. Isotype MFI was subtracted (n≥3, a representative experiment is shown in Suppl. Fig. 1C). B. Western blot showing the total amount of MICA in whole cell lysates, using antigen affinity-purified biotinylated goat polyclonal anti-MICA antibody BAF1300. Actin was used as loading control (n = 4). C. Soluble MICA and ULBP2/5/6 released to supernatants of vemurafenib -treated metastatic melanoma cells was analysed by ELISA at 24 h post-treatment. Plots represent the mean and SEM of protein concentration (ng/ml) (n = 3). D. mRNA detected using qPCR. Cells were recovered to extract RNA. cDNA was prepared and used as template in qRT-PCR experiments. Data are the mean and SEM, relative to control (DMSO) cells. RPLP0 mRNA levels were determined for normalization ($^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

caused by vemurafenib on the recognition of autologous melanoma cells was similar for both BRAFi-treated and -untreated NK cells (Figure 4B), in agreement with prior data showing that NK cell activity was not affected by the use of vemurafenib.[14,30] Moreover, in the case of Ma-Mel-86f, no inhibitory signal could come from MHC-I because these cells showed a stable MHC-I-negative phenotype due to the loss β-2 microglobulin. Thus, the reduction in NK cell recognition of BRAFi-treated melanoma cells was most probably due to the decrease in ligands for activating NK receptors.

BRAF inhibitors in combination with HDACi could overcome NK resistance of melanoma cells

The above experiments show that treatment of malignant melanoma cells with BRAFi makes them less susceptible to NK cell recognition and attack. However, we and others, have shown that treatment with a number of other anti-cancer drugs can increase the immunogenicity of tumour cells by increasing expression of ligands for the NKG2D receptor.[31–35] Histone deacetylase inhibitors (HDACis) are currently under

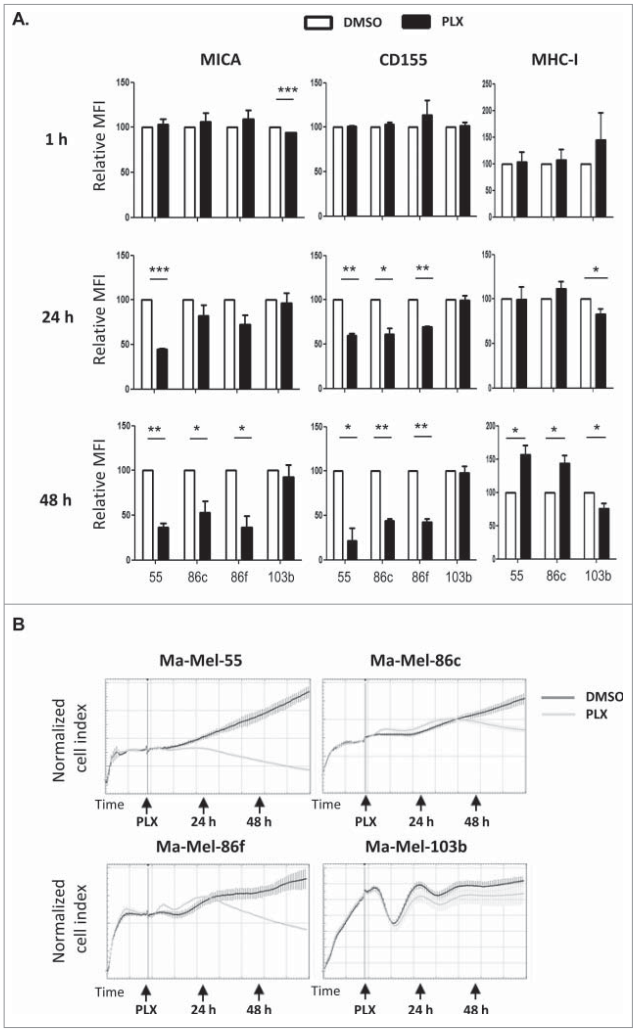

Figure 3. Kinetics of the effect of vemurafenib on surface NK ligand expression and proliferation in melanoma cells. A. Effect of vemurafenib on NKG2D-ligand surface expression. Metastatic melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with 1 μM vemurafenib for either 1 h, 24 h or 48 h (control cells with the carrier DMSO) and stained for detection of the indicated markers by flow cytometry. The plots represent the change in the mean fluorescence intensity (MFI) of each marker, as the percentage of the molecule present in control (DMSO) cells. Data show the mean and SEM. Isotype MFI was substracted ($^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$) (n = 3). B. Proliferation assay. Metastatic melanoma cells were allowed to adhere to E-plates and then treated with 1 μM vemurafenib (control cells with the carrier DMSO). Real time electrical impedance was monitored to quantify cell proliferation for 48 h using x-CELLigence. Cell index normalized to the treatment time is represented. Arrows indicate the time of vemurafenib addition (PLX), 24 and 48 h post-treatment. The plots show quadruplicates of one representative of 3 independent experiments.

development for use as anticancer agents (reviewed in[36,37]) and they are potent enhancers of NKG2D-L expression.[32] Initial experiments showed that the HDACi, sodium butyrate, consistently increased NKG2D-L surface expression in all the melanoma cell lines in a dose-dependent manner, the highest increase being observed with 5 mM and not affecting the viability of the V600E mutant melanoma cells (Figure 5A; Supplementary Figure 2). CD112 and MHC were affected with an 18 h exposure to the drug, but not CD155. Next, the effects on melanoma cells of treatment with the HDACi (sodium butyrate, 5 mM), in combination with vemurafenib (1 μM) were examined. MTT-based viability assays revealed that the combination of these drugs has a synergistic effect on the inhibition of the metabolic activity of the mutant melanoma cell lines (Figure 5B). In fact, cell numbers strongly decreased in the cultures after 48 h of incubation with the combination. Importantly, HDACi

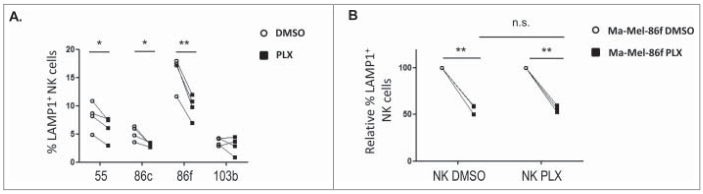

Figure 4. Effect of vemurafenib on NK cell recognition of melanoma cells. Metastatic melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated 48 h with either 1 μM vemurafenib or DMSO and used as targets in experiments of degranulation using NK cells. The plots represent the percentage of NK cells positive for surface CD107a (LAMP1) as a measure of degranulation. A. Healthy donor NK cells. Each symbol corresponds to an experiment with a different donor (n = 4). B. Autologous NK cells. Ma-Mel-86f were used as target cells. Autologous NK cell were also treated 48 h with DMSO or vemurafenib. Data represent the relative decrease as percentage of untreated cells. Three independent experiments are shown (n.s., non-significant; $^{*}p < 0.05$ $^{**}p < 0.01$).

treatment on melanoma cells lines also resulted in increased NKG2D-L surface expression and, strikingly, the effect of this drug was dominant when combined with vemurafenib for 24 h, both in cell surface and mRNA levels of the NK ligands (Figure 5C, D). MHC-I and DNAM1-L surface expression was also checked in these treatments at 24 h. It is worth noting that the increase in surface NK ligands caused by either butyrate or the combination of drugs was higher for MICA and ULBP2/5/6 than for the rest of the molecules studied. The drug combination did not significantly increase CD155, however, it consistently increased the amount of CD112 at the cell surface of melanoma cells. In contrast, no clear pattern was observed for MHC with the combination of drugs. Thus, these data demonstrate that combined sodium butyrate and vemurafenib treatment not only increases melanoma susceptibility to be recognised by NK cells, but also causes a marked decrease in cell viability of all the melanoma cell lines tested. In order to separate toxicity from NK cell recognition, NK cell degranulation experiments were carried out after only 24 h of treatment with this drug combination (Figure 6A). Melanoma cells exposed to the butyrate+ vemurafenib combination efficiently stimulated NK recognition and degranulation demonstrating that the diminished NK recognition of BRAFi-treated melanoma cells can be overcome by combining BRAF inhibition with the HDACi sodium butyrate. Antibody blocking confirmed a dominant role of NKG2D in NK recognition of melanoma and further demonstrated that the functional effect observed in the presence of the inhibitors was also mediated by the recognition of NKG2D ligands (Figure 6B). For consistency with the initial experiments, 48 h drug combination experiments were designed next but, to avoid the toxicity problem, melanoma cells were initially treated with vemurafenib only, then adding sodium butyrate for the last 18 h of the second day. Surprisingly, MICA downmodulation caused by vemurafenib was not rescued by a further incubation of melanoma cells with HDACi (Figure 7), implying that the inhibition of BRAF leads to a persistent blockade of MICA expression. Moreover, this combination treatment also provokes the decrease of other NK ligands in mutant cells such as ULBP2/5/6, ULBP3, CD155, CD112 and MHC-I, indicating that timing is an important factor to consider when designing a new combination therapy.

Discussion

In this paper, we explored whether treatment with BRAFi could influence the susceptibility of melanoma cells to NK cell recognition, in particular, via modulation of the expression of activating NK receptor ligands. NKG2D and its ligands constitute the best characterised system involved in NK surveillance of cancer[38] and melanoma cells are known to express various NKG2D-L affecting NK cell recognition.[20,39,40] Here, we demonstrate that exposure to the BRAFi, vemurafenib, modulates the surface expression of NKG2D-L, CD155 and MHC-I, all of which are important molecules regulating immune recognition by NK cells. The reduced cell surface expression of DNAM1-L and NKG2D-L were paralleled by decreases in the levels of DNAM1-L and NKG2D-L mRNA and were not due to an increase in the release of soluble proteins after $BRAF^{V600E}$ inhibition. This in turn indicates that expression of these ligands on melanoma cells is a consequence of downstream signaling via oncogenic $BRAF^{V600E}$. Importantly, the overall result of the altered expression of ligands for activating and inhibitory NK receptors was a decrease in the susceptibility of the metastatic melanoma cells to NK cell attack. We further demonstrate that treatment of the tumour cells with a combination of BRAFi and HDACi, known to increase cell surface expression of NKG2D-L, counteracted the loss of activating ligand expression, favoring NK cell recognition of the melanoma cells. We propose that, in addition to trying to further block the MAPK route, the use of combination therapy with drugs that potentiate the susceptibility of the tumour cell to NK cells could be a viable approach to overcome the problems of tumour resistance to BRAFi that arise in melanoma patients.

Although most melanoma cells express at least one NKG2D-L,[29] the characterization of NK activating and inhibitory ligand expression by several metastatic melanoma cells shown here revealed heterogeneity, as previously reported for melanoma and other cancer cell lines.[20,41] This observation led to the hypothesis that, although melanoma is generally considered an immunogenic cancer, different melanoma cells from different metastatic lesions might show different susceptibilities to immune attack. As expected, treatment with the BRAFi vemurafenib led to a decrease in proliferation of $BRAF^{V600E}$ mutant melanoma cells, as well as an increase of MHC Class I expression which was previously reported using the BRAFi dabrafenib.[42] Surprisingly, however, BRAFi treatment of these melanoma cells also triggered a marked decrease in the levels of NKG2D-L, as recently described by Frazao,[27] and CD155 at the cell surface, sufficient to seriously impair NK cell recognition of these BRAFi-treated melanoma cells. Exposure to vemurafenib affected neither the proliferation of $BRAF^{wt}$ melanoma cells

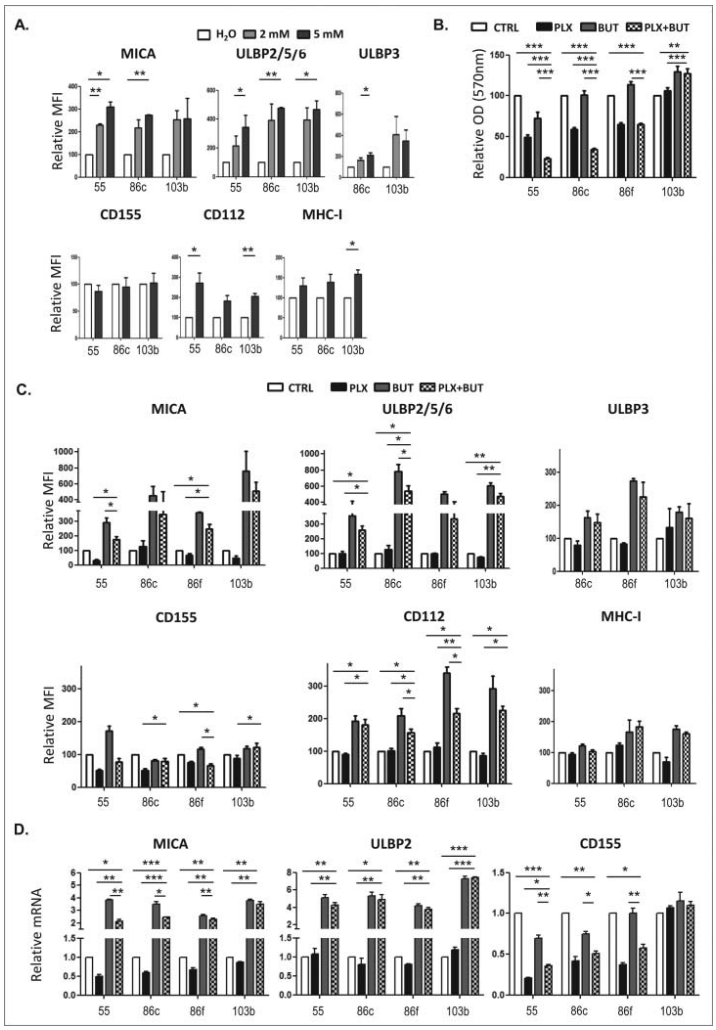

Figure 5. Effect of the combination of HDACi and BRAFi on the immunophenotype and proliferation of melanoma cells. A. Titration of sodium butyrate and surface expression of NK ligands. Metastatic melanoma cells Ma-Mel-55 (55), Ma- Mel-86c (86c) and Ma-Mel-103b (103b) were treated with the indicated amounts of sodium butyrate or water for 18 h and stained with antibodies for detection of the indicated markers by flow cytometry. The plots represent the change in the mean fluorescence intensity (MFI) of each marker, as the percentage of the molecule present in control (water) cells. Data show the mean and SEM corresponding to 3 experiments. B. MTT assay of melanoma cells treated with the drug combination. Metastatic melanoma cells Ma-Mel-55 (55), Ma- Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with either 1 μM vemurafenib, 5 mM sodium butyrate or the combination of both for 24 h (control cells with the carrier, DMSO and water, CTRL). Cells were assayed for metabolic activity by incubation with MTT and OD_{570} was measured. Data represented are the mean as percentage of untreated cells and SEM of four replicates, from one experiment out of two. C. NK ligands on melanoma cells treated with the drug combination. Cells were stained for detection of the indicated markers by flow cytometry. The plots represent the mean fluorescence intensity (MFI) of each marker, isotype subtracted and the relative change as the percentage of the molecule present in control (DMSO and water, CTRL) cells. Data show the mean and SEM corresponding to 3 experiments. D. mRNA detected using qPCR. Cells were recovered to extract RNA. cDNA was prepared and used as template in qRT-PCR experiments. RPLP0 mRNA levels were determined for normalization ($^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$). For simplicity, in B, C and D, only the p-values of each inhibitor related to the combination are shown.

nor the expression of ligands for NK cell receptors, except for a decrease in ULBP3.

The mechanism underlying the reduced levels of cell surface ligands for the NKG2D and DNAM-1 receptors after vemurafenib treatment of $BRAF^{V600E}$ mutant melanoma appears to be a decrease in synthesis of these proteins due to significantly lower levels of specific mRNA. These data demonstrate a connection between the specific inhibition of proliferative signals from the $BRAF^{V600E}$ mutant kinase and a decrease in NKG2D-L and CD155 mRNA, suggesting either transcriptional control of the expression of these molecules coupled to this MAPK route in humans or a decrease in RNA stability. Interestingly,

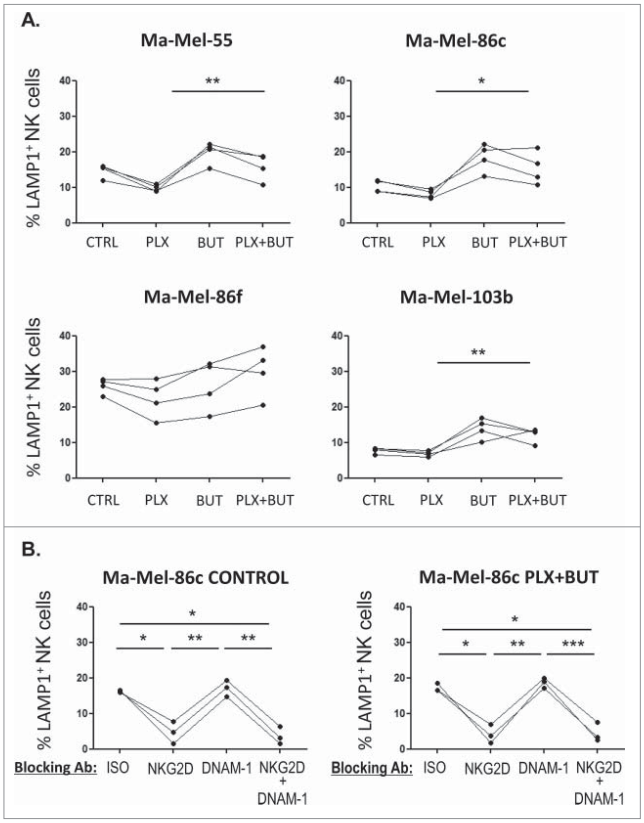

Figure 6. A. NK cell degranulation in the presence of HDACi- and BRAFi-treated melanoma cells. Melanoma cells Ma-Mel-55, Ma-Mel-86c, Ma-Mel-86f and Ma-Mel-103b cells treated with either 1 μM vemurafenib, 5 mM sodium butyrate or the combination of both for 24 h (control cells with the carrier, DMSO and water, CTRL) were used as targets in experiments of degranulation using NK cells from healthy donors PBMCs. The plots represent the percentage of NK cells positive for surface CD107a (LAMP1) as a measure of degranulation in 4 experiments ($^{*}p < 0.05$ $^{**}p < 0.01$). For simplicity, only the p-values of each inhibitor related to the combination are shown. B. Effect of the blockade of activating NK receptors in degranulation experiments in the presence of HDACi- and BRAFi-treated melanoma cells. Melanoma cells Ma-Mel-86c treated with carrier (either DMSO or water, control) and the combination of 1 μM vemurafenib and 5 mM sodium butyrate for 24 h were used as targets in experiments of degranulation using NK cells from healthy donors PBMCs. NK receptors (NKG2D, DNAM-1, or both) were blocked, as indicated, incubating PBMCs with the corresponding antibodies, or isotype (ISO) as negative control. The plots represent the percentage of NK cells positive for surface CD107a (LAMP1) as a measure of degranulation in 3 experiments ($^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}p < 0.001$).

another oncogene present in many human tumours, H-RasV12, has previously been related with upregulation of ULBP and, in certain cells, MICA.[43] Ras activates a number of signalling pathways, including RAF-MAPK/MEK and PI3K, related with proliferation. Indeed, exposure to the tyrosine kinase inhibitor sorafenib and inhibitors of the PI3K and MEK pathways can also modulate MICA/B and other NKG2D-L surface expression.[43,44] Thus, it seems plausible to speculate that transcriptional/post-transcriptional regulation of MICA could be under control of the proliferation signals initiated by BRAF.

That NKG2D-L are expressed in immortalised cells and proliferative situations has been long known[29] but, because proliferation not only occurs in tumour cells, the regulation of surface protein has to be very precise.[45] The finding of regulation by E2F transcription factors in the murine system provided the first molecular insight into how NKG2D-L regulation could be linked to proliferation.[46] However, NKG2D-L upregulation can also be ATM/ATR dependent in response to DNA-damage.[47] although this route does not seem to be involved in the expression of ligands in cells under other stresses. The effect of sequential treatment of melanoma cells with first vemurafenib and later sodium butyrate suggest that the regulation of expression is different for MICA and the other NKG2D-L as well as CD155 and CD112. The fact that, most probably, several pathways participate in driving the expression of NKG2D-L and other NK ligands makes biological sense because NKG2D-L have to be expressed only on target cells that suffer certain type of stress and should be eliminated.

The data presented here also support previous studies linking the biology of NKG2D-L with that of DNAM1-L, implying that there are likely common pathways in the regulation of these ligands for activating NK receptors, as previously proposed.[48] Since both MICA and CD155 decreased at the cell surface of melanoma cells treated with BRAFi, these data suggest

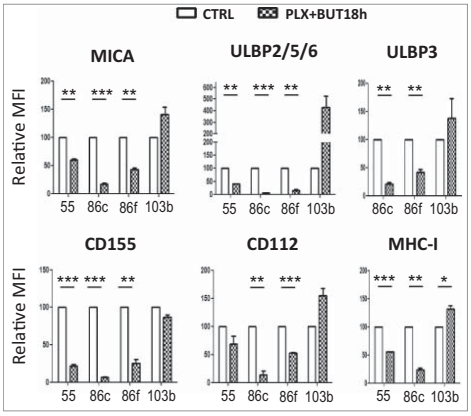

Figure 7. NK ligand expression on melanoma cells sequentially treated with vemurafenib (48 h) and sodium butyrate (last 18 h). Melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with 1 μM vemurafenib (PLX) for 48 h, for the last 18 h of culture, a final concentration of 5 mM sodium butyrate was added (control cells with the carrier DMSO or water). Cells were stained for detection of the indicated markers by flow cytometry. The plots represent the mean fluorescence intensity (MFI) of each marker, isotype subtracted and the relative change as the percentage of the molecule present in control (DMSO and water, CTRL) cells. Data show the mean and SEM corresponding to 3 experiments. ($^{*}p < 0.05$ $^{**}p < 0.01$, $^{***}p < 0.001$).

that signals mediated by activation of the oncogenic BRAF route play some role in the regulation of both molecules. It is interesting to note that inhibition of $BRAF^{V600E}$ signaling decreased the expression of NKG2D and DNAM ligands that drive activation of NK cells, and increased the expression of MHC class I molecules, that are ligands for inhibitory NK receptors. Strikingly, however, while $BRAF^{V600E}$ modulates the expression of NK activating ligands transcriptionally (this paper), the kinase acts to decrease MHC-I expression at the cell surface by altering the intracellular trafficking of these molecules, so that they are rapidly internalised from the melanoma cell surface and are sequestered intracellularly.[42] Simultaneous increase in MHC-I expression and decrease in ligands for NK activating receptors would be expected to inhibit NK recognition and, indeed, BRAFi treatment decreased NK cell reactivity against $BRAF^{V600E}$ mutant melanoma cells (Figure 4).

The most striking finding of this report was that the decrease of NKG2D-ligands that occurs in BRAFi-treated melanoma cell lines could be overcome by exposure to HDACi. Because HDACs have been suggested to contribute to tumorigenesis, due to the regulation of genes involved in DNA damage repair, and because this regulation has been co-related to patient prognosis, several FDA-approved HDACi are now used for the treatment of various types of cancer, the most successful being refractory cutaneous and peripheral T cell lymphoma.[37] However, the role of HDACi in cancer still has many unanswered questions and a possible contribution of immune system activation has been suggested only very recently.[36] For example, the use of HDACi has been shown to enhance T cell cytotoxicity towards BRAFi-treated melanoma which expressed more proapoptotic genes, although the involvement of NKG2D as co-receptor was not investigated in that system.[49] The expression of NKG2D in all human $CD8^{+}$ T cells is important in this context, since these cells can be found infiltrating tumours (TILs) and could play a critical role in immunosurveillance. Independent experiments have also shown that exposure to HDACi causes an increase in the expression of several NKG2D-L in tumour cells[32] and thus, we hypothesized that the decrease of NKG2D-L and NK cell recognition in BRAFi-treated cells could be reverted by the addition of HDACi or other drugs known to increase NKG2D-L expression. Here we show that treatment *in vitro* with the combination of both drugs, BRAFi and HDACi, can lead to the increase of NKG2D-L expression on melanoma cells. Blocking experiments confirmed that the expression of these ligands was the basis of the recovery of NK recognition. However, the addition of HDACi, after BRAFi-induced NKG2D-L downmodulation had already occurred, did not have the expected consequence of expression recovery. Instead, sodium butyrate needed to be incubated with vemurafenib simultaneously. Why NKG2D-L downmodulation by BRAFi could not be reverted when HDACi treatment was added later is not clear, but future experiments will investigate the hypothesis that increased phosphorylation of the translation initiation factor eIF2α (which would be expected to inhibit protein synthesis) induced by BRAFi pretreatment.[50] blocks the ability of HDACi to trigger increased NKG2D-L expression.

In any case our observation that simultaneous treatment with BRAFi and HDACi affected melanoma cell growth and immunogenicity more potently than treatment with either agent alone suggests the value of exploration of novel approaches of drug combinations aiming to potentiate distinct mechanisms of cancer elimination, i.e. direct drug cytotoxicity on the malignant cells and indirect increase of the susceptibility of the tumour to elimination by immune cells. Moreover, because different lesions from the same patient can either carry or not the $BRAF^{V600E}$ mutation, the effect of sodium butyrate on non-mutant cells could be of added value: the combination would avoid the undesirable decrease of NKG2D-L on BRAFV600E mutant cells, but also increase the activating NK ligands (including DNAM1-L) in non-mutant cells. In addition, in the $BRAF^{V600E}$ mutant lesions, the combination of HDACi and vemurafenib would synergise to decrease tumour cell viability. Our data demonstrate that combination of BRAFi and HDACi enhances NK cell recognition of melanoma cells. While the effect on T cell recognition still has to be addressed our data suggest that it would not be impaired because MHC class I expression is not significantly altered.

In aggregate, the data presented in this manuscript support the use of HDACi in combination with BRAFi to treat melanoma, based on the rescue of recognition by NK cells. The possibility of combining these two types of drugs has been suggested previously, however, in the context of their effect on cell death.[51] Although melanoma is generally considered very immunogenic, it is also quite heterogeneous at the level of genetic alterations that can lead to the lack of β2 m expression in one lesion of a patient and loss of heterozygosity in the MHC in a second one. Thus, it is very important to understand how the drugs directed to one type of lesion can affect other lesions in the same patient, as studied here in patient model Ma-Mel-86, and make sure that all of them can be controlled. Exploring the effect of chemotherapeutic drugs on tumor cell

recognition by immune cells, as shown here for NK cells, may help proposing new avenues for intervention and facilitate the selection of drugs for combination in clinical trials for cancer therapy. Since it is not feasible to assess all the possible combinations in patients, proposing drug synergies in preclinical assays, as we show here for BRAFi and HDACi, may prove to be a useful strategy in the design of new therapeutic regimes.

Materials and methods

Reagents

Reagents were purchased from Sigma, unless otherwise stated. Vemurafenib was obtained from Selleckchem and used at a final concentration of 1 μM.

Cell lines and peripheral blood mononuclear cell culture

The metastatic melanoma cell lines Ma-Mel-55, Ma-Mel-86c, Ma-Mel-86f and Ma-Mel-103b were obtained as described previously[17,28] and typed for MICA subfamily by satellite analysis[52] and using Peak Scanner v1.0 software (Applied Biosystems). 721.221, RPMI-8866, K562 cells (used for functional assays) and the melanoma cell lines were grown in RPMI medium supplemented with 10% FCS, 1 mM glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM Hepes, 100 U/ml penicillin and 100 U/ml streptomycin (Biowest).

Peripheral blood mononuclear cells (PBMCs) were purified from healthy volunteer buffy coats (Regional Transfusion Centre, Madrid) or from donations from patient Ma-Mel-86 (University Hospital Essen). Approval from local ethical committees and informed consent from all participants were obtained and experiments have been conducted according to the principles expressed in the Declaration of Helsinki. Activated NK cells were prepared as described previously.[53] modified by the inclusion of IL-12 and IL-18 stimulation at the beginning of the culture. Briefly, PBMC were isolated by centrifugation on Ficoll-HyPaque. Adherent cells were eliminated by adherence to tissue culture plates and the remaining PBMCs were then cultured in the presence of irradiated feeder cells (721.221 and RPMI-8866 B cell lines) in RPMI-1640 medium (Lonza) supplemented with 10% FBS, 10% male AB human serum, 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 U/ml streptomycin, 10 mM Hepes, 50 μM β-mercaptoethanol (Biowest), 10 U/ml IL-12(Peprotech) and 25ng/ml IL-18 (MBL). 5–6 d later, 50 U/ml of IL-2 (Peprotech) was added. After 7–8 d of culture, the proportion of NK cells was checked by flow cytometry using CD3 and CD56-specific mAbs, and used for functional experiments. If necessary, PBMCs were re-stimulated with IL-2.

Flow cytometry

Cells were incubated with the appropriate primary antibodies or isotype control. Monoclonal and PE-conjugated antibodies specific for ULBPs were purchased from R&D Systems (Abingdon, UK) (ULBP2/5/6, MAB1298 or FAB1298P; ULBP3, MAB1517); PE-conjugated antibody for MICA was purchased from R&D Systems (FAB1300P). 1H10 antibody specific for MICA[54] and HP1F7 antibody recognizing MHC-I[55] were described before; monoclonal antibody anti-CD112 and anti-CD155 FITC were purchased from Santa Cruz Biotechnology, and anti-CD155 from Abcam followed by either PE- or FITC-labelled F(ab')2 fragments of goat anti-mouse Ig (Dako). Cell death was evaluated by the analysis of 7-Aminoactinomycin D (7AAD) (Beckman Coulter) staining. Samples were analyzed using BD FACSCalibur (Becton Dickinson), Gallios Flow Cytometer or Cytomics FC 500 (Beckman Coulter). Analysis of the experiments was performed using Kaluza (Beckman Coulter) and FlowJo (Tree Star, Inc).

Viability/proliferation assays

Viability/Proliferation assays using MTT, were performed by plating 3000 cells per well in 96-well plates. After 16 h, either vehicle or inhibitor was added for 24 or 48 h, as indicated. Supernatants were then discarded, and MTT solution (20% complete medium, 70% PBS, 10% MTT stock solution 5 mg/ml in PBS) was added and incubated at 37°C for 4 h. MTT solution was discarded, and DMSO was added and OD at 554 nm was measured with TECAN Infinite M200 (Life Sciences). In combination treatments, the OD was measured at 570 nm with Thermo Scientific™ Multiskan™ FC Filter-based Microplate Photometer (Thermo Fisher Scientific). OD at 690 nm was used as reference measure. Four replicates per condition and cell line were done.

Proliferation assays using the xCELLigence® RTCA DP (Acea Bioscience) system were performed by plating 5000 cells in 16 well Eplates. Cells were allowed to adhere for 16 h and growth was followed for 2 d with the addition of vemurafenib or DMSO as control. Four replicates per condition and cell line were done. Blank was measured with complete RPMI medium. Data were analysed and normalized to time of treatment using the RTCA 2.0 Software.

Degranulation assays

PBMCs were co-cultured for 2 h with target cells at a final E:T ratio of 1 NK to 2 target cells. Surface expression of LAMP1 (CD107a) was analyzed by flow cytometry in NK cells $CD3^{-}CD56^{+}$ stained with directly conjugated antibodies (Biolegend). K562 cells were used as control for NK degranulation against a known NK target (internal experiment control). Basal degranulation was measured in PBMCs incubated alone (negative control). In the case of non-autologous PBMCs, target cells were pre-incubated 30 minutes at 25°C with 10 μg/ml of anti-MHC class I antibody HP1F7.[55] In experiments using autologous PBMCs, degranulation analyses were performed by incubating the effector cells for 3 h with monensin and anti-LAMP1 antibody. For blocking experiments PBMCs were incubated 30 minutes at RT with 5 μg/ml of the corresponding antibody (MOPC21, as isotype control, from Sigma; anti-NKG2D, MAB139 or anti-DNAM-1, MAB666, both from R&D Systems) before co-incubation with targets.

Quantitative PCR (qPCR)

After treatment, as indicated in the figure legends, melanoma cell lines were washed with PBS. Total mRNA was isolated

from tumour 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 instructions from the manufacturers. Real-time quantitative RT-PCR was carried out with specific TaqMan Gene Expression assays. Relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing expression levels of investigated mRNA to ribosomal protein lateral stalk subunit P0 (RPLP0) mRNA.

ELISA

NKG2D-L were detected using sandwich ELISAs on supernatants from cells cultured in the presence or absence of vemurafenib for 24 h. Plates were coated with purified mAb R&D for MICA (MAB13002) or goat polyclonal Ab for ULBP2/5/6 (AF1298) (R&D) in BBS (Borate Buffered saline). After incubation overnight at 4°C, the plates were blocked with 2% BSA-HBS (Hepes Buffered saline: 10 mM hepes pH 7.6, 150 mM NaCl) for 2 h at 37°C; then the samples were added and incubated overnight at 4°C. Bound proteins were detected using biotinylated goat anti-MICA (BAF1300) or biotinylated goat anti-ULBP2/5/6 (BAF1298), both from R&D, followed by streptavidin-HRP (Amersham) and developed using the peroxidase substrate system (ABTS; Roche). The absorbance was measured at 410 nm with a reference wavelength of 490 nm with Thermo Scientific™ Multiskan™ FC Filter-based Microplate Photometer (Thermo Fisher Scientific). Under these conditions, the cutoff for detection of recombinant soluble MICA (R&D Biosciences) (1300-MA) and ULBP2/5/6 (1298-UL) was around 100 pg/ml, and the ELISA absorbance values were directly proportional to the concentration of protein over the range of 90 pg/ml to 3 ng/ml.

Western blot

Cells were lysed in lysis buffer [50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP-40 with protease inhibitors (leupeptin and pepstatin A)] and centrifuged to pellet nuclei. Samples were resolved on 10% SDS-PAGE and transferred to Immobilon-P (Millipore). The membrane was blocked using 5% nonfat-dry milk or BSA in PBS-0.1% Tween 20. MICA proteins were detected by incubating the membrane with biotinylated goat polyclonal anti-MICA antibody (R&D) (BAF1300). Mouse anti- β-actin (AC-15) was from Sigma, rabbit polyclonal anti-ERK, rabbit anti-p-ERK (20G11), rabbit anti-GAPDH (14C10) (Cell Signaling). Secondary antibodies [goat anti-mouse Ig-HRP (Dako), donkey anti-goat Ig-HRP (Santa Cruz Biotechnology), goat anti-rabbit Ig-HRP (Cell Signaling)] or streptavidin-HRP were used prior to developing using the ECL Plus system (GE Pharmaceuticals).

Stastistical analysis

Student's t-test was done for paired samples using GraphPad Prism 5. p-value is shown in each figure, when not indicated is because differences were non-significant.

Disclosure statement

The authors declare no potential conflicts of interest.

Acknowledgments

SLC was the recipient of a mobility fellowship from the Spanish Education Ministry and a recipient of a FPU fellowship (MECD); EMG-C was a recipient of a PhD fellowship from "Fundación La Caixa", CC-S was a recipient of a JAE-Intro fellowship (CSIC).

Funding

This work was supported by the Madrid Regional Government under grant "Inmunothercan" S2010/BMD-2326; the Spanish Ministries of Economy and Health under grants SAF-2012-32293, SAF2015-69169-R (MINECO/FEDER) (MVG) and SAF2014-58752-R (HTR). AP received support from the "*Hiege-Stiftung gegen Hautkrebs*". SLC and EMGC were recipients of Fellowships from Spanish Ministry of Education (FPU) and La Caixa, respectively. CC-S was a recipient of a JAE-Intro fellowship (CSIC).

Author contributions

SLC, AP, MV and HTR contributed to the conception and design of the study, acquisition, analysis and interpretation of data and wrote the manuscript; CCS and NP performed experiments; EGC provided data and/or reagents for the study; AP, MV and HTR provided material support.

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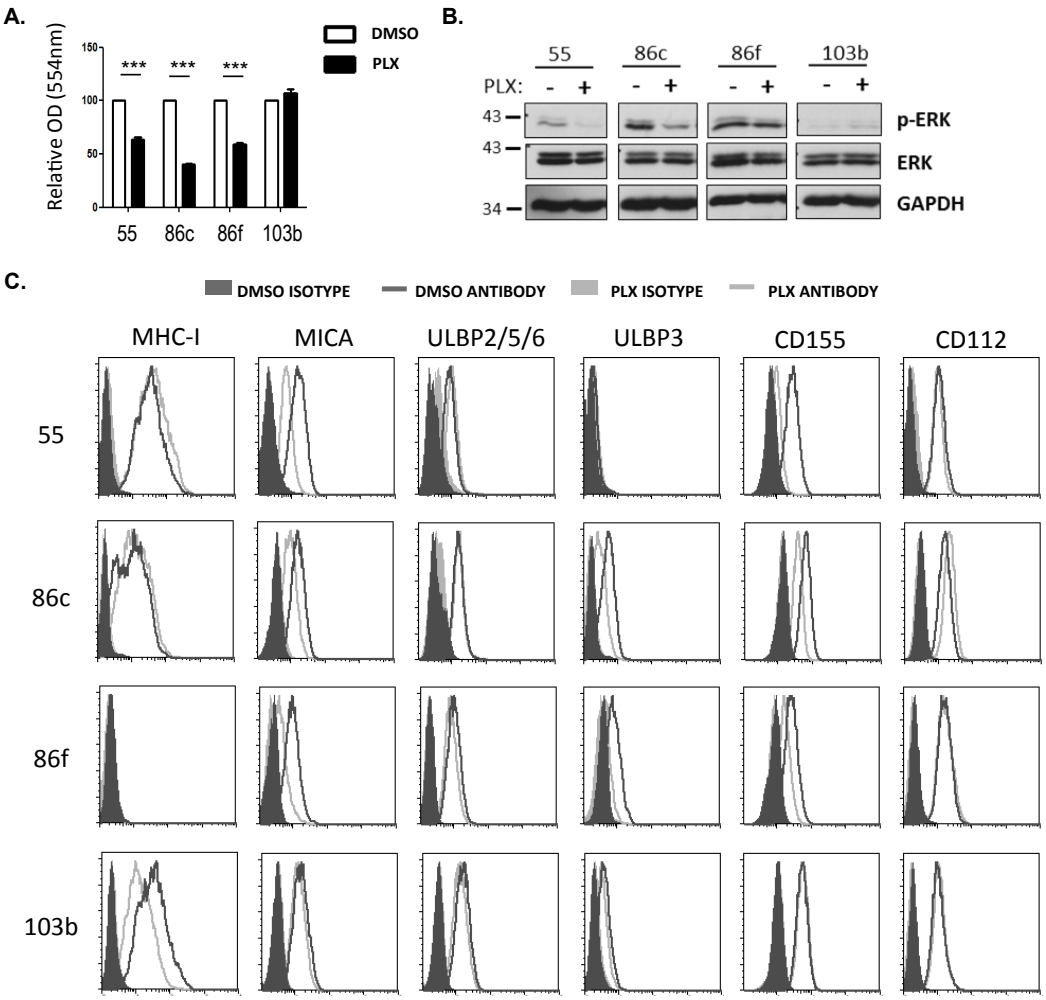
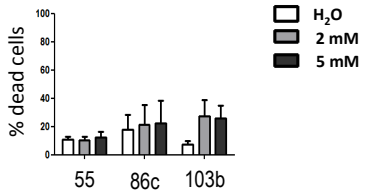
Supplementary Figure 1



Supplementary Figure 1. Effect of vemurafenib on viability, ERK levels and NK ligand expression in melanoma cells.
A. Cell viability. Metastatic melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with 1 μM vemurafenib for 48 h (control cells with the carrier DMSO). Then, MTT was added and cells were assayed for viability. OD_{554} was measured in supernatants for 4 h after addition of 0.5 mg/ml MTT. The mean and SEM (four replicates) of the percentage of the molecule related to untreated cells from one representative experiment, out of two, is shown. (***p<0.001). **B. Expression of pERK by vemurafenib-treated cells.** Melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with 1 μM vemurafenib (+) for 1h (control cells with the carrier DMSO, -), lysed and proteins analysed by Western Blot using the indicated antibodies. GADPH was used as loading control. Data show one representative experiment out of two. Molecular weights are indicated in kDa. **C. Cell surface expression of MHC, NKG2D-ligands and DNAM-1-ligands on melanoma cells treated with vemurafenib.** The metastatic melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c), Ma-Mel-86f (86f) and Ma-Mel-103b (103b) were treated with 1 μM vemurafenib (PLX) for 48 h (control cells with the carrier DMSO), stained with the indicated antibodies and analysed by flow cytometry. The figure shows one representative experiment of more than 3. Statistical analysis is shown in Figure 2.

Supplementary Figure 2



Supplementary Figure 2. Effect of butyrate on viability of metastatic melanoma cells
Melanoma cells Ma-Mel-55 (55), Ma-Mel-86c (86c) and Ma-Mel-103b (103b) were treated with the indicated amounts of sodium butyrate or water (carrier control) for 18 h and stained with 7AAD for detection of cell death. The plot represents the mean and SEM of the percentage of dead cells as 7AAD+ cells.

7.3. Article III

ONCOIMMUNOLOGY
2018, VOL. 0, NO. 0, e1450127 (9 pages)
https://doi.org/10.1080/2162402X.2018.1450127

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ORIGINAL RESEARCH

Evolution of melanoma cross-resistance to CD8$^+$ T cells and MAPK inhibition in the course of BRAFi treatment

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ABSTRACT

The profound but frequently transient clinical responses to BRAF^{V600} inhibitor (BRAFi) treatment in melanoma emphasize the need for combinatorial therapies. Multiple clinical trials combining BRAFi and immunotherapy are under way to further enhance therapeutic responses. However, to which extent BRAF^{V600} inhibition may affect melanoma immunogenicity over time remains largely unknown. To support the development of an optimal treatment protocol, we studied the impact of prolonged BRAFi exposure on the recognition of melanoma cells by T cells in different patient models. We demonstrate that autologous CD8$^+$ tumor-infiltrating lymphocytes (TILs) efficiently recognized short-term (3, 7 days) BRAFi-treated melanoma cells but were less responsive towards long-term (14, 21 days) exposed tumor cells. Those long-term BRAFi-treated melanoma cells showed a non-proliferative dedifferentiated phenotype and were less sensitive to four out of five CD8$^+$ T cell clones, present in the preexisting TIL repertoire, of which three recognized shared antigens (Tyrosinase, Melan-A and CSPG4) and one being neoantigen-specific. Only a second neoantigen was steadily recognized independent of treatment duration. Notably, in all cases the impaired T cell activation was due to a time-dependent downregulation of their respective target antigens. Moreover, combinatorial treatment of melanoma cells with BRAFi and an inhibitor of its downstream kinase MEK had similar effects on T cell recognition. In summary, MAP kinase inhibitors (MAPKi) strongly alter the tumor antigen expression profile over time, favoring evolution of melanoma variants cross-resistant to both T cells and MAPKi. Our data suggest that simultaneous treatment with MAPKi and immunotherapy could be most effective for tumor elimination.

ARTICLE HISTORY
Received 28 November 2017
Revised 27 February 2018
Accepted 2 March 2018

KEYWORDS
BRAF; CD8$^+$ T cells; MEK; antigens; inhibitor; melanoma; resistance

Introduction

The BRAF^{V600} mutation, expressed in approximately 50% of melanomas, mediates constitutive activation of the BRAF-MEK-ERK pathway and therefore tumor proliferation. Since 2011, two selective inhibitors vemurafenib and dabrafenib have been approved for treatment of BRAF^{V600} mutant melanoma. However, the rapid and high rate of clinical responses to BRAF inhibitor (BRAFi) therapy in melanoma patients is often abolished by disease progression due to acquired resistance in tumor cells.[1,2] Superior clinical responses can be achieved by BRAFi/MEKi combination therapies,[3-6] but still disease progresses in the majority of treated patients.

Salvage therapies for patients with disease progression on BRAFi or BRAFi/MEKi often involve immunotherapies, e.g. immune checkpoint blockade to reinvigorate tumor-reactive T cells and generate sustained clinical response. The rationale for this therapeutic strategy is that BRAFi treatment enhances melanoma recognition by differentiation antigen-specific T cells *in vitro* and increases T cell infiltration/clonality in responding lesions *in vivo*.[7-14] In addition, studies in mouse tumor models demonstrated a critical contribution of CD8$^+$ T cells to BRAFi responses.[15-17]

But, after the development of acquired resistance to BRAFi therapy, the density of T cell infiltrates returns to pretreatment level.[7,10,14] Moreover, recent analyses of the transcriptomes from targeted therapy-resistant lesions revealed molecular signatures which could result in cross-resistance to immunotherapy, such as deficiency of CD8$^+$ T cells and loss of antigen presentation.[18] So far only limited functional data is available on melanoma susceptibility towards autologous tumor-reactive CD8$^+$ T cells after prolonged BRAF^{V600} inhibition, which is of critical importance for the guiding of optimal combination protocols.

In the present work, by exploring the activation of autologous TILs and multiple antigen-specific T cell clones present within the TIL repertoire, we demonstrate that BRAFi strongly alters the tumor antigen expression profile over time, resulting

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in resistance against CD8$^+$ T cells specific for shared antigens and a neoantigen. Also long-term combinatorial BRAFi/MEKi treatment protected melanoma cells from T cell recognition. The fact that T cell-resistance coevolves with drug-resistance in melanoma cells argues against a sequential application of MAPKi and immunotherapy but favours simultaneous treatment regimens in order to prevent the development of immune evasive tumor variants.

Results

Melanoma cells lose their capacity to stimulate autologous CD8$^+$ TILs in the course of BRAFi treatment

First, melanoma cells treated with BRAFi for different intervals were studied for their recognition by *in vitro* expanded autologous TILs, including short-term treated (3 d, 7 d), long-term treated (14 d, 21 d) and BRAFi-resistant tumor sublines. Short-term BRAFi treatment induced significant apoptosis in BRAF^{V600E}-positive Ma-Mel-86c melanoma cells (Fig. 1A). Residual vital cells presented with senescence-like features,[19] as indicated by enlarged/flattened cell morphology and elevated ß-galactosidase activity (Fig. 1B). Prolonged treatment till day 21 did not further reduce cell numbers and cells remained in a senescence-like state. After approximately one month of continuous inhibitor exposure, a BRAFi-resistant proliferative Ma-Mel-86c variant (Ma-Mel-86c/Res) was established (data not shown). As shown in Fig. 1C, short-term treated tumor cells stimulated autologous CD8$^+$ TILs to release IFNγ as efficiently as untreated control cells. But, after 14 d of BRAFi treatment, the ability of melanoma cells to induce IFNγ release by CD8$^+$ TILs was significantly reduced. This effect was found to be most pronounced for Ma-Mel-86c/Res cells.

Next, surface expression of HLA class I and PD-L1 was analysed on BRAFi-treated Ma-Mel-86c cells. Flow cytometry data revealed that the ratio of HLA class I to PD-L1 molecules reverted from significantly increased for short-term treated cells back to the level of untreated control cells, excluding that the impaired T cell recognition of long-term BRAFi-treated Ma-Mel-86c cells was due to biased surface expression of HLA class I and PD-L1 (Fig. 1D, Fig. S1A and S1B).

Taken together, our data indicate that BRAFi can alter tumor immunogenicity in a time-dependent manner: short-term treated tumor cells efficiently activate the pre-existing CD8+ TIL repertoire, whereas long-term inhibition decreases T cell activation.

Melanoma cells acquire resistance against autologous shared antigen-specific T cells

Assuming that BRAFi treatment could influence the expression of antigens recognized by CD8$^+$ T cells, we took advantage of the knowledge about previously defined tumor antigens in patient model Ma-Mel-86, Lübcke et al., unpublished[20] including shared antigens and neoantigens (Fig. 2A). Using peptide-loaded autologous EBV-transformed B-cells as targets we detected CD8$^+$ TILs recognizing Tyrosinase- and CSPG4 (HMW-MAA)-

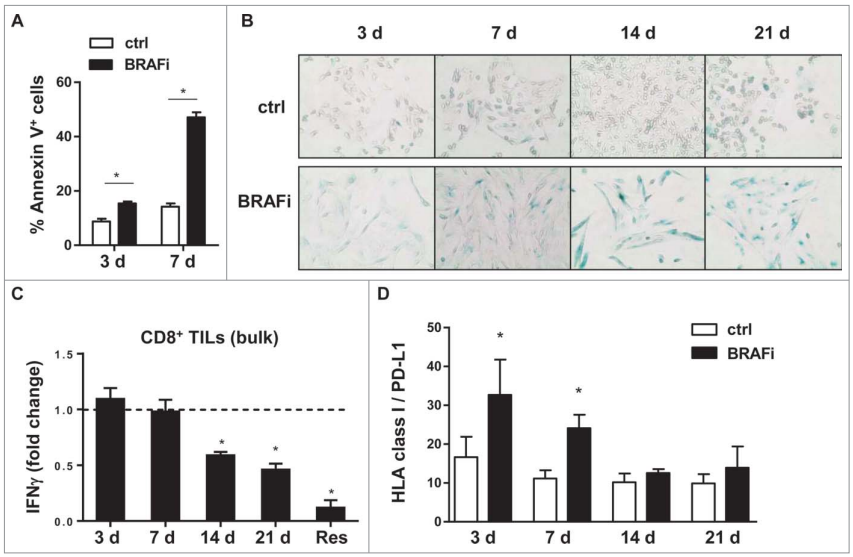

Figure 1. Melanoma cells lose their capacity to stimulate autologous CD8$^+$ TILs in the course of BRAFi treatment. (A) BRAFi (vemurafenib, 0.5 μM) induces apoptosis in Ma-Mel-86c tumor cells after 3 and 7 d of treatment, as measured by flow cytometry. Percentage of Annexin V$^+$ cells is depicted as mean+SEM (n = 3). *, $p < 0.05$. (B) Staining for senescence-associated β-galactosidase activity in Ma-Mel-86c cells after 3, 7, 14 or 21 d of BRAFi treatment and corresponding non-treated control cells (ctrl). Representative images from one of three independent experiments. (C) Activation of autologous bulk CD8$^+$ TILs by BRAFi-treated cells (3, 7, 14, 21 d) or BRAFi-resistant (Res) Ma-Mel-86c cells was determined by intracellular IFNγ staining. Results are shown as fold change of IFNγ^+ CD8$^+$ T cells stimulated by BRAFi-treated tumor cells relative to corresponding untreated tumor cells (n = 3). *, $p < 0.05$, BRAFi vs ctrl. (D) Surface expression of HLA class I and PD-L1 on Ma-Mel-86c cells after BRAFi treatment (0.5 μM). Data are depicted as ratio of mean fluorescence intensity of HLA-class I to PD-L1 (mean+SEM, n >3). *, $p < 0.05$, BRAFi vs ctrl.

A

CD8+ T cell clones	antigen specificity	peptide region	peptide sequence	HLA-restriction
5C/149	Tyrosinase	$TYR_{146-156}$	SSDYVIPIGTY	HLA-A*01:01
11C/73	CSPG4	$CSPG4_{554-562}$	HIIFPHGSL	HLA-C*07:01
1A/39	$HERPUD1^{G161S}$	$HERPUD1_{154-162}$	GLGPGFSSY	HLA-B*15:01
16C/106	$MMS22L^{S437F}$	$MMS22L_{429-438}$	YYSKNLNSFF	HLA-A*24:02

B

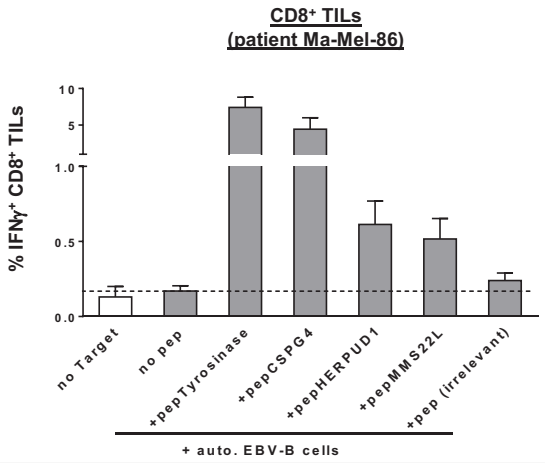

Figure 2. Melanoma antigen-specific $CD8^+$ T cell clones in patient model Ma-Mel-86. (A) Antigen specificity and HLA-restriction of $CD8^+$ T cell clones from patient Ma-Mel-86 used in this study. (B) Detection of T cells with known antigen specificity among TILs from patient Ma-Mel-86. Synthetic peptides-loaded (1 μg/ml) autologous EBV-transformed B cells (auto. EBV-B) were used as antigen-presenting cells and cocultured with TILs for 4 h. Percentage of IFNγ-producing $CD8^+$ TILs was determined by using intracellular cytokine staining and depicted as mean+SEM (n = 3). Non-loading or irrelevant peptide-loaded B cells were used as negative controls.

derived peptide epitopes (Fig. 2B). Expression of Tyrosinase was upregulated after short-term BRAFi treatment but gradually disappeared in the long-term treated cells (Fig. 3A). MITF, the master regulator for melanoma differentiation, followed a similar expression pattern, indicating a switch to a dedifferentiated cell phenotype (Fig. 3A). Accordingly, the enhanced recognition of short-term BRAFi-treated melanoma cells by the autologous Tyrosinase-specific $CD8^+$ T cell clone 5C/149 was followed by a significant decrease in case of long-term treated target cells (Fig. 3B). Activation of the CSPG4-specific T cell clone 11C/73 was significantly reduced in both short-term and long-term treatment settings (Fig. 3C), which correlated with the stable downregulation of CSPG4 expression under BRAFi treatment (Fig. 3D). To prove that protection from $CD8^+$ T cell recognition was indeed due to the decrease in antigen expression, and not caused by defects in antigen presentation, long-term (21 d) BRAFi-treated Ma-Mel-86c cells were pulsed with synthetic antigenic peptides before assaying their T cell-stimulatory capacity (Fig. 2A). As shown in Fig. 3E, peptide-loaded long-term BRAFi-treated tumor cells were efficiently recognized by the corresponding $CD8^+$ T cell clones. Furthermore, we analyzed the influence of BRAFi on the expression of specific components of the antigen processing machinery (APM) in melanoma cells. As shown in Fig. 3A, BRAFi exposure induced HLA heavy chain and ERAP1 protein expression, especially after long-term exposure. Meanwhile, mRNA levels of *B2M, TAP1,* and *TAPBP* remained unaffected upon BRAFi treatment, while *HLA-A* was significantly upregulated at all analyzed time points (Fig. S1C), arguing against an acquired defect in APM component expression in BRAFi-treated tumor cells.

Thus, Ma-Mel-86c cells acquired resistance against shared antigen-specific T cells due to the loss of differentiation antigen expression already two weeks after the onset of BRAFi treatment and an early-initiated steady downregulation of the CSPG4 antigen.

In a second patient model, Ma-Mel-63, we observed similar alterations in shared antigen expression, such as Tyrosinase, Melan-A and CSPG4 (Fig. 4A and 4B), indicating the broader applicability of our findings. Although Ma-Mel-63a cells upregulated HLA heavy chain and ERAP1 expression and showed a constantly enhanced ratio of HLA-class I to PD-L1 surface expression under BRAFi exposure (Fig. 4A and 4C), activation of a Melan-A-specific $CD8^+$ T cell clone was significantly impaired when stimulated with long-term treated Ma-Mel-63a cells (14d and 21d, Fig. 4D). Moreover, the dedifferentiated BRAFi-resistant subline Ma-Mel-63a/Res was no longer

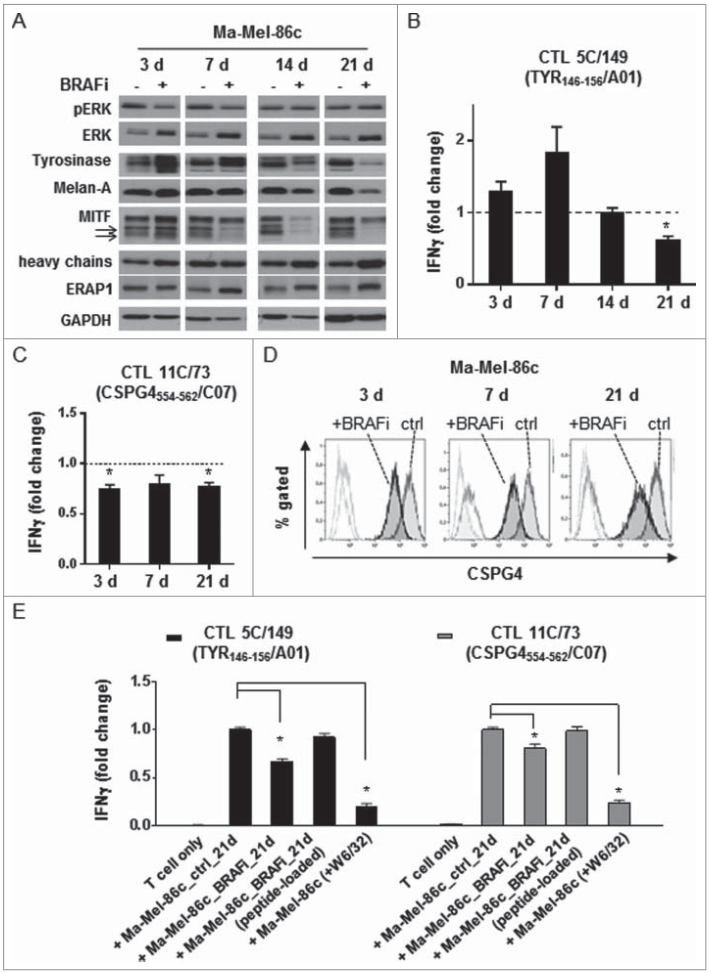

Figure 3. Activation of pre-existing shared antigen-specific CD8$^+$ T cells by BRAFi-treated autologous tumor cells in patient model Ma-Mel-86. (A) Expression of pERK, ERK, Tyrosinase, Melan-A, MITF, HLA heavy chains, and ERAP1 in BRAFi-treated and -untreated Ma-Mel-86c cells was analysed by Western blot. GAPDH served as loading control. Representative data from one of at least two independent experiments. (B) Activation of Tyrosinase-specific CD8$^+$ T cell (CTL) clone 5C/149 and (C) CSPG4-specific CD8$^+$ T cell (CTL) clone 11C/73 by autologous BRAFi-treated (0.5 μM) Ma-Mel-86c cells was determined by IFNγ ELISpot assay. Results are presented as fold change of IFNγ spots from T cells stimulated with BRAFi-treated relative to untreated tumor cells. Data is depicted as mean+SEM (n = 3). *, $p < 0.05$, BRAFi vs ctrl. (D) Surface expression of CSPG4 on BRAFi-treated Ma-Mel-86c cells was measured by flow cytometry. Unstained tumor cells were used as negative control (light-grey empty lines). Representative data from one of three independent experiments. (E) Activation of antigen-specific CD8$^+$ T cells by BRAFi-treated (0.5 μM for 21 d) Ma-Mel-86c cells was determined by IFNγ ELISpot. Loading of synthetic antigenic peptides (1 μg/ml) or blockade of HLA class I (W6/32, 50 μg/ml) on tumor cells was performed at 37°C for 30 min before T cell stimulation. Results are presented as fold change of IFNγ spot numbers relative to IFNg spots obtained by T cells stimulated with untreated (ctrl) tumor cells. Data is depicted as mean+SEM (n = 3). *, $p < 0.05$.

capable of stimulating Melan-A-specific T cells due to the complete loss of target antigen expression (Fig. 4E and F).

BRAFi differentially impacts on neoantigen-specific CD8$^+$ T cell recognition

Besides shared antigens, also neoantigens have been defined in patient model Ma-Mel-86.[20] CD8$^+$ T cells specifically targeting two neoantigens, HERPUD1^{G161S} and MMS22L^{S437F}, were readily detectable in TILs (Fig. 2B). While BRAFi treatment upregulated HERPUD1 mRNA expression in Ma-Mel-86c cells, it led to a reduction in MMS22L mRNA expression (Fig. 5A). Consistent with the antigen expression pattern, the HERPUD1^{G161S}-specific CD8$^+$ T cell clone 1A/39 showed enhanced activation by BRAFi-treated autologous tumor cells independent of treatment duration, whereas responsiveness of the MMS22L^{S437F}-specific CD8$^+$ T cell clone 16C/106 to BRAFi-treated tumor cells decreased over time (Fig. 5B). Thus, besides

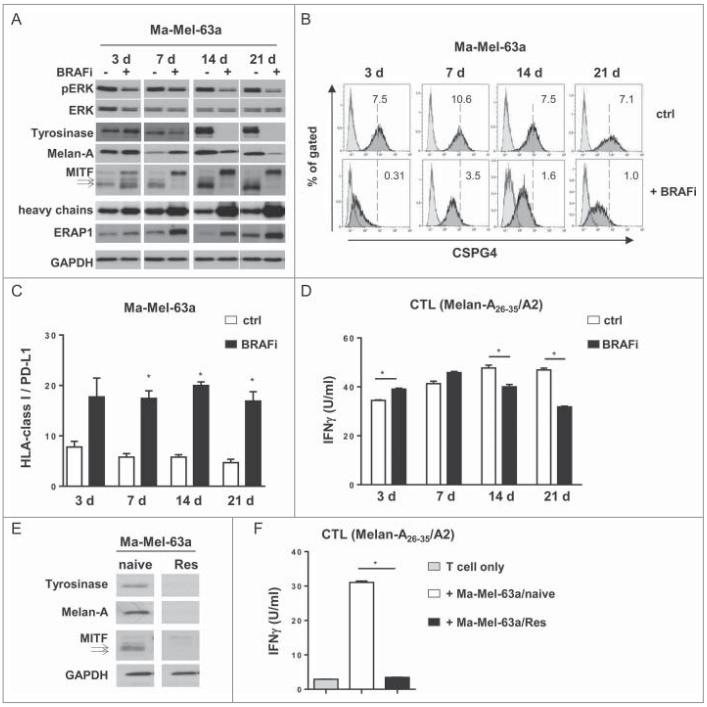

Figure 4. Regulation of antigen expression by BRAFi in tumor cells and its impact on Melan-A-specific $CD8^+$ T cell activation in patient model Ma-Mel-63. (A) Expression of pERK, ERK, Tyrosinase, Melan-A, MITF, HLA heavy chains, and ERAP1 in BRAFi (vemurafenib, 0.5 μM)-treated or -untreated $BRAF^{V600E}$-positive Ma-Mel-63a cells was determined by Western blot. GAPDH served as loading control. Representative data from one of at least two independent experiments. (B) Surface expression of CSPG4 on BRAFi-treated and -untreated (3, 7, 14 or 21 d, filled dark grey) Ma-Mel-63a cells was measured by flow cytometry. Unstained tumor cells were used as negative control (filled light grey). Representative data from one of three independent experiments. (C) Surface expression of HLA-class I and PD-L1 on Ma-Mel-63a cells after BRAFi treatment (0.5 μM). Data are depicted as ratio of mean fluorescence intensity of HLA class I to PD-L1 (mean+SEM, n = 4–5). *, $p < 0.05$, BRAFi vs ctrl. (D) IFNγ release by Melan-A-specific, HLA-A2-restricted $CD8^+$ T cell clone stimulated with BRAFi-treated or -untreated Ma-Mel-63a cells ($HLA\text{-}A2^+$) was determined by ELISA and one representative data from two independent experiments is shown as mean+SEM. (E) Expression of Tyrosinase, Melan-A and MITF in BRAFi-naive or -resistant (Res) Ma-Mel-63a cells was determined by Western blot. GAPDH served as loading control. Representative data from three independent experiments. (F) IFNγ release by Melan-A-specific, HLA-A2-restricted $CD8^+$ T cell clone stimulated with BRAFi-naive or -Res Ma-Mel-63a cells ($HLA\text{-}A2^+$) was determined by ELISA and one representative data from two independent experiments is shown as mean+SEM.

shared antigens, presentation of a neoantigen, generally considered as an ideal target in immunotherapy,[21] was impaired by long-term BRAFi treatment of Ma-Mel-86c cells.

Evolution of T cell resistance in the course of BRAFi/MEKi treatment

Combination therapy with BRAFi/MEKi is currently replacing BRAFi monotherapy as the standard of care treatment for $BRAF^{V600}$ mutant melanomas. Therefore, we further investigated the impact of combinatorial BRAFi/MEKi treatment on melanoma cell immunogenicity. As shown in Fig. 6A, short-term BRAFi/MEKi-exposed Ma-Mel-86c cells efficiently stimulated autologous $CD8^+$ T cells whereas the T cell-stimulatory capacity of long-term BRAFi/MEKi-treated tumor cells was strongly impaired and even abolished in case of double drug-resistant melanoma cells. Comparable to Ma-Mel-86c, long-term BRAFi/MEKi-treated as well as double drug-resistant Ma-Mel-63a cells showed a strong reduction in T cell stimulation (Fig. 6B). Also the regulation of melanoma differentiation antigens in BRAFi- and BRAFi/MEKi-treated tumor cells followed a similar pattern. Short-term exposure to BRAFi/MEKi upregulated Tyrosinase, Melan-A and MITF expression in the investigated melanoma cell lines, followed by their downregulation in long-term treatment settings (Fig. 6C and 6D).

Taken together, the effects of BRAFi single treatment on T cell recognition of melanoma cells were recapitulated by BRAFi/MEKi exposure with melanoma cells developing cross-resistance to both $CD8^+$ T cells and MAPKi after long-term treatment.

Discussion

Numerous clinical trials combining BRAFi and immunotherapy are currently ongoing to further enhance therapeutic responses against melanoma (http://www.clinicaltrials.gov). To support the development of a rationale for the optimal treatment protocol, we here used autologous human T cell/melanoma models to illustrate the impact of BRAFi on tumor immunogenicity over time. To our knowledge, this is the first study, which precisely evaluated the time-dependent alteration

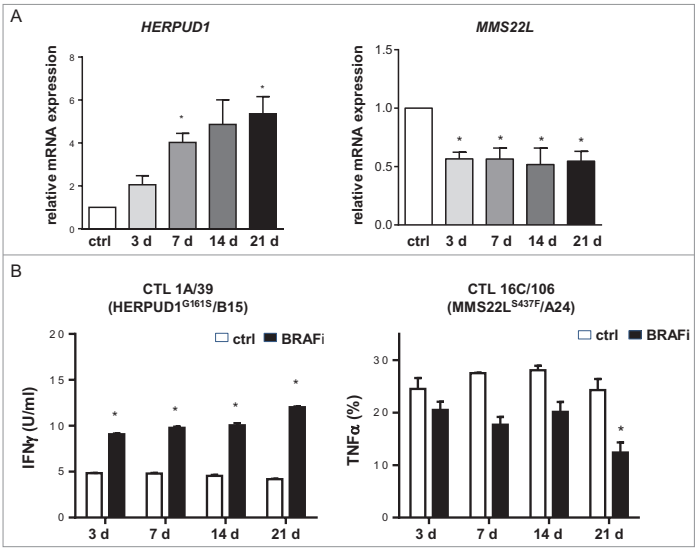

Figure 5. BRAFi treatment differentially impacts on neoantigen-specific CD8$^+$ T cell recognition. (A) mRNA levels of HERPUD1 and MMS22 L in Ma-Mel-86c cells after BRAFi treatment (vemurafenib, 0.5 μM) were quantified by qPCR and normalized to endogenous RPLP0 mRNA. Expression levels (mean+SEM, n = 3) are depicted relative to the expression in untreated cells (ctrl). *, $p < 0.05$, BRAFi vs ctrl. (B) IFNγ release by HERPUD1^{G161S}-specific CD8$^+$ T cell clone 1 A/39 stimulated with BRAFi-treated or -untreated Ma-Mel-86c cells (HLA-B*15:01 transfectant) was determined by ELISA and one representative data from two independent experiments is shown as mean+SEM. Percentage of TNFα^+ cells in MMS22L^{S437F}-specific CD8$^+$ T cell clone 16C/106 stimulated with BRAFi-treated or -untreated Ma-Mel-86c cells (HLA-A*24:02 transfectant) was determined by intracellular cytokine staining (mean+SEM, n = 2). *, $p < 0.05$, BRAFi vs ctrl.

of T cell activation by BRAFi-treated melanoma cells at the level of multiple antigens.

We found that BRAFi strongly altered the antigen expression profile in the investigated melanoma cell lines. Consistent with several published studies,[11-13] we observed that short-term BRAFi treatment in tumor cells enhanced the expression of shared melanoma differentiation antigens, such as Tyrosinase and Melan-A, and resulted in improved tumor recognition by corresponding antigen-specific T cells. In parallel, another shared antigen, CSPG4, and one mutated antigen, MMS22L^{S437F}, showed an early-suppressed expression, which was readily detectable after 3 days of inhibitor exposure. Of note, long-term BRAFi treatment led to downregulation of the initially upregulated differentiation antigens and, therefore, significantly decreased T cell activation. Moreover, BRAFi/MEKi treatment recapitulated the evolution of melanoma resistance to T cells detected under single BRAFi treatment.

Exposure of melanoma cells to BRAFi did not adversely affect the expression of specific APM components, arguing against the involvement of a BRAFi-induced deficient antigen processing and presentation in T cell resistance. But still, we cannot exclude that also the overexpression of specific antigen processing components in BRAFi-treated tumor cells such as the trimming peptidase ERAP1 might decrease rather than enhance the generation of specific peptide epitopes. Previously, it was demonstrated that ERAP1 trimming activity destroys the Melan-A$_{26-35}$ epitope,[22] suggesting that ERAP1 activity contributes to the low stimulatory capacity of long-term BRAFi-treated Ma-Mel-63a cells towards Melan-A$_{26-35}$–specific T cells.

Compared to BRAFi alone, BRAFi/MEKi treatment has significantly improved the clinical outcome of patients with BRAF^{V600} mutant melanoma.[5] In line with the clinical observation, Deken et al. demonstrated a more persistent infiltration of T cells into responding melanoma lesions of both BRAFi/MEKi-treated tumor-bearing mice and some melanoma patients.[17] However, to which extent tumor-specific T cells influenced this BRAFi/MEKi induced response remains unclear. Here, we observed similar pattern of antigen regulation and tumor-specific T cell activation between BRAFi/MEKi and BRAFi treated melanoma cells at all time points analyzed, suggesting that BRAFi/MEKi treatment is not superior to BRAFi single treatment in boosting and prolonging the activation of the pre-existing T cell repertoire.

Our finding that melanoma acquires cross-resistance to tumor-specific T cells after long-term BRAFi and BRAFi/MEKi treatment might partially explain why T cell infiltrates in resistant lesions were similar or even below to the levels in pretreatment biopsies.[7,10,14,18] Complementary to recent transcriptome studies on MAPKi-treated melanomas,[18,23] our functional investigation contributes a dynamic mechanistic insight into the scenarios how impaired CD8$^+$ T cell activation co-evolves with targeted therapy resistance.

Therefore, we assume that simultaneous or early sequential combination of BRAF^{V600}-directed treatment and immunotherapy could be most effective in preventing the development of immune-evasive tumor variants. Indeed, simultaneous administration of BRAFi or BRAFi/MEKi together with diverse immunotherapies prolonged survival and reduced tumor

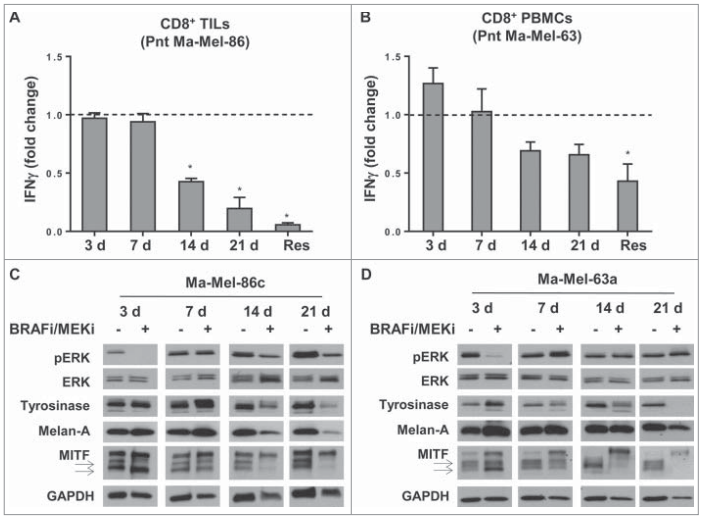

Figure 6. Evolution of T cell resistance in the course of combinatorial BRAFi/MEKi treatment. Activation of autologous bulk $CD8^+$ T cells by BRAFi/MEKi-treated (3, 7, 14, 21 d) or BRAFi/MEKi-resistant (Res) Ma-Mel-86c (A) and Ma-Mel-63a cells (B) was determined by intracellular IFNγ staining. Results are shown as fold change of $IFN\gamma^+$ $CD8^+$ T cells stimulated by BRAFi/MEKi-treated tumor cells relative to corresponding untreated tumor cells (n = 3). *, $p < 0.05$, BRAFi/MEKi vs ctrl. (C, D) Expression of pERK, ERK, Tyrosinase, Melan-A, and MITF in BRAFi/MEKi-treated or -untreated Ma-Mel-86c (C) and Ma-Mel-63a (D) cells was determined by Western blot. GAPDH served as loading control. Representative data from one of at least three independent experiments.

growth in mouse models.[13,15,16,23-25] In a pilot study, Deniger et al. demonstrated that administration of vemurafenib two weeks prior to TILs generated objective but non-synergistic clinical responses, similar to that of TILs or vemurafenib alone,[26] while a retrospective analysis indicates that patient outcomes of anti-CTLA4 therapy following BRAFi resistance are poor.[27] Furthermore, our data raise the need for more comprehensive analyses of tumor antigen alterations by MAPKi in future studies, in order to understand the precise influence of targeted therapies on cancer immunogenicity.

Materials and methods

Patient samples

Samples from melanoma patients, including tumour tissues and peripheral blood cells were collected after approval by the institutional review board and patient informed written consent. Tissues were mechanically dissected for generation of cell lines or expansion of TILs. Melanoma cell lines were tested for mycoplasma 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). Tumor cells were cultured in RPMI1640 medium (Gibco life technologies) supplemented with 10% FCS and penicillin/streptomycin at 37°C in a 5% CO_2 atmosphere. Expansion of TILs was achieved by culturing suspension cells from tumor tissue in RPMI 1640 supplemented with 10% FCS, penicillin/streptomycin, and 500 IU/ml rhIL-2 (Chiron Corporation) for 2–5 weeks.

$CD8^+$ T cell clones from Patient Ma-Mel-86 and Melan-A_{26-35} specific $CD8^+$ T cell clone are generated as previously described.[20,28]

Prolonged inhibitor treatment strategy

Briefly, melanoma cells were seeded at a density of 1×10^6 cells per 75 cm^2 culture flasks in RPMI1640 medium with 10% FCS and penicillin/streptomycin. After overnight resting, cells were treated with 0.5 μM vemurafenib (BRAFi, Selleckchem) alone or in combination with 50 nM trametinib (MEKi, Selleckchem) for the indicated days. Subsequent treatment with fresh inhibitors was performed twice a week until the termination of experiments.

Apoptosis assay

After 3 or 7 days of inhibitor treatment, all cells were harvested, washed with PBS and resuspended in binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$). For the staining 5 μl of Annexin V-APC (BD Biosciences) and 10 μl of propidium iodite (BD Biosciences) were added to 100 μl of 1×10^5 cells and incubated for 15 min at room temperature. After the incubation 400 μl of binding buffer were added and the samples were measured immediately by flow cytometry.

Senescence-associated ß-galactosidase assay

Cells were either left untreated or treated with 0.5 μM vemurafenib for indicated days followed by washing with phosphate-buffered saline (PBS) and fixed with 37% formaldehyde in PBS for 5 min at room temperature. After rinsing with PBS, cells were subjected to ß-Gal assay as described by Dimri et al.[29] and photographed under light microscopy.

Flow cytometry

Tumor cells were stained for surface expression with the anti-CSPG4-PE (Miltenyi Biotec., 5 μl/test), anti-HLA-class I-APC (eBiosciences, clone W6/32, 0.5 μl/test), and anti-PD-L1-PE antibodies (Biolegend, clone 29E2A3, 2 μl/test). Background fluorescence was determined by unstained samples. Cells were analysed after fixation in 4% Paraformaldehyde in a Gallios flow cytometer and the Kaluza software was used for data analysis (Beckman Coulter).

Western blot

Tumor cells were lysed in 1 x Cell Lysis buffer (Cell Signaling) supplemented with 1 mM PMSF. Lysates were prepared by centrifugation at 13000 rpm for 15 min at 4° C. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membranes and probed with the following primary antibodies: anti-ERK1/2 (Cell Signaling, 1:5000), anti-pERK1/2 (Cell Signaling, clone D13.14.4E, 1:2500), anti-MITF (Sigma-Aldrich, clone C5, 1:1000), anti-Tyrosinase (Santa Cruz, T311, 1:500), anti-Melan-A (Zytomed, clone M2-7C10, 1:5000), anti-HLA heavy chain (Nordic-MUbio, clone HC-10, 1:5000), anti-ERAP1 (R&D, AF2334, 1:1000) and anti-GAPDH (Cell Signaling, clone 14C10, 1:5000). After washing, membranes were incubated with the appropriate secondary antibodies linked to horseradish peroxidase. Antibody binding was visualized with the ECL chemiluminescence system (Thermo Fisher Scientific).

Assays of T cell activation

Intracellular cytokine staining was performed in TILs after co-culture experiment. Briefly, 1×10^5 TILs were stimulated for 4 h with the indicated tumor cells (1×10^5 cells) in AIM-V complete medium containing 10 μg/mL Brefeldin A (Sigma-Aldrich). Cells were then fixed/permeabilized using the Fixation/Permeabilization Concentrate and Diluent kit (eBioscience) followed by staining with antibody cocktail containing anti-human CD3-Brilliant Violet 421 (1 μl/test), CD8-APC-Cy7 (1 μl/test), CD4-APC (1 μl/test), and IFNγ-PE (5 μl/test) antibodies (Biolegend). Cells were analysed in a Gallios flow cytometer and the Kaluza software was used for data analysis (Beckman Coulter).

Activation of $CD8^+$ T cell clone 16 C/106 was demonstrated by intracellular TNFα staining using the anti-human CD8-APC-Cy7 (1 μl/test) and TNFα-PC7 (3 μl/test) antibodies (Biolegend), following the above described protocol.

Other T cell clones were measured as IFNγ release by ELISpot assay or ELISA. For ELISpot assay, multiscreen-HA plates (Millipore) were coated with 5 μg/mL anti-IFNγ mAb 1-D1 K (Mabtech). 2×10^3 – 5×10^3 T cells in AIM-V complete medium were seeded together with tumour cells (5×10^4 cells/well). After 20-hour incubation at 37°C in 5% CO_2, a biotinylated secondary anti-IFNγ antibody (1 μg/mL, clone 7-B6-1, Mabtech) was used and spots were developed by sequential addition of 1:1000 diluted ExtrAvidin alkaline phosphatase and BCIP®/NBT Liquid Substrate System (Sigma-Aldrich). Spot numbers were determined with the AID ELISpot reader (AID Diagnostika). For ELISA, supernatants were then collected from the U-bottom plate after 24 h coincubation of T cells and tumor cells and analysed by using following reagents: capture mAb 1-D1K (2 μg/mL) and biotinylated detection mAb 7-6B-1 (1 μg/mL) (Mabtech), Streptavidin-alkaline phosphatase complex (Vectastain Elite Kit; Vector Laboratories), p-nitro-phenyl-phosphate substrate solution (Sigma-Aldrich). The spectrophotometric absorbance at 405 nm was determined after suitable developing time, approx. 10–20 min.

Quantitative real-time PCR

Total mRNA was isolated from tumour cells using RNeasy Plus Kit (Qiagen) and polyadenylated 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. Relative RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing expression levels of investigated mRNA to ribosomal protein lateral stalk subunit P0 (RPLP0) mRNA.

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 software (GraphPad, version 6.02). Experimental groups were considered to be significantly different with $p < 0.05$ (indicated by *).

Disclosure of potential conflicts of interest

S. Haferkamp participated in advisory board from Roche Pharma, BMS, received speaker honoraria from Amgen, Novartis, BMS and received research funding from Novartis and BMS. B. Schilling reports receiving honoraria from Roche and travel support as well research funding from Bristol-Myers Squibb. D. Schadendorf is an advisory board member and receives honoraria from Roche, Genentech, Novartis, Amgen, GlaxoSmithKline, Bristol-Myers Squibb, Boehringer Ingelheim, and Merck Sharp & Dohme. No potential conflicts of interest were disclosed by the other authors.

Funding

This work was supported by the 'Hiege-Stiftung gegen Hautkrebs' to F. Zhao, the European Union's Horizon 2020 research and innovations program under Marie Sklodowska-Curie grant agreement No 641458 to S. Leonardelli, and the Else Kröner-Promotionskolleg Essen funded by the Else Kröner-Fresenius-Stiftung to F. N. Harbers.

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Supplementary Figure

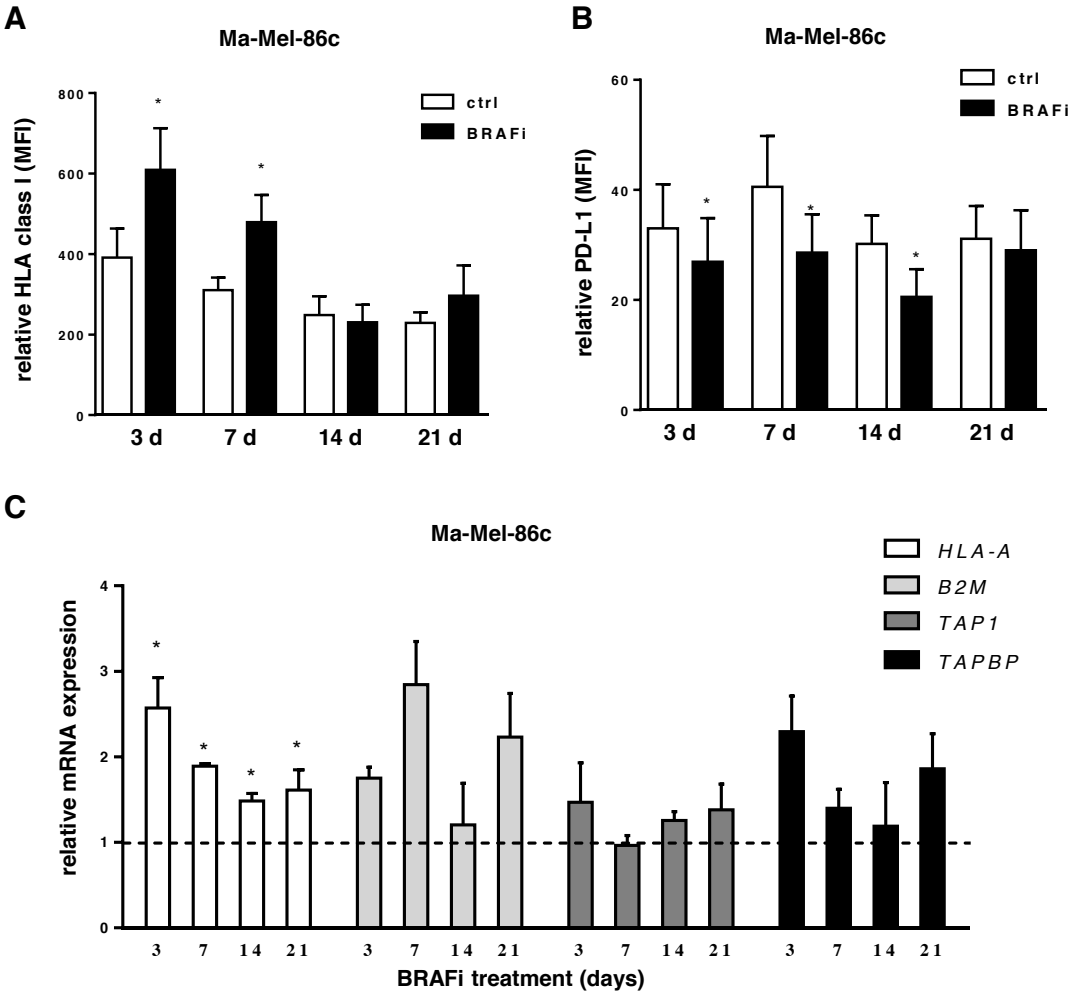

Figure S1.

Figure S1: Expression of HLA class I, PD-L1 and APM components under BRAFi treatment in Ma-Mel-86c cells

Surface expression of HLA class I **(A)** and PD-L1 **(B)** on Ma-Mel-86c cells after BRAFi treatment (0.5 µM vemurafenib). Data are depicted as mean fluorescence intensity of antibody stained cells normalized to unstained control cells (mean+SEM, n = 4-5). *, $p<0.05$, BRAFi vs ctrl. **(C)** *HLA-A, B2M, TAP1* and *TAPBP* mRNA levels in Ma-Mel-86c cells after BRAFi treatment (vemurafenib, 0.5 µM) were quantified by qPCR and normalized to endogenous RPLP0 mRNA. Expression levels (mean+SEM, n=3-4) are depicted relative to the expression in untreated cells (ctrl). *, $p<0.05$, BRAFi vs ctrl.

8. Discussion

8.1. Genomic heterogeneity of melanoma

It is well known that cancer is a very heterogeneous disease and consists of cells, which differ in their gene expression profile based on their genomes, epigenomes, transcriptomes and proteomes, but also in their invasive, proliferative, angiogenic and metastatic potential as well as in metabolic characteristics and immunogenic properties (Marusyk and Polyak, 2010).

In this context malignant melanoma is considered one of the most heterogeneous malignancies displaying the highest mutational load among different cancer types (Alexandrov *et al.*, 2013). In addition, only a couple of mutations have been identified as common 'disease drivers' in melanoma, while the majority of genetic aberrations are 'private', thus leading to the high genetic and phenotypic diversity not only within a single patient (intrapatient heterogeneity) but also between different patients (interpatient heterogeneity). Importantly, single lesions can also display marked heterogeneity (intratumor heterogeneity) (Grzywa, Paskal and Wlodarski, 2017).

Recently, evidence for intrapatient tumor heterogeneity has been demonstrated in treatment-naïve metastatic melanomas where divergent gene-expression pattern have been found between different lesions from a patient, in 50 % of the analyzed cases (Harbst *et al.*, 2014). Moreover, another study demonstrated that synchronous metastases might arise from different subpopulations found in the primary lesion and that one metastasis can be founded by more than one cell population present in the primary tumor, suggesting a mechanism by which genetic heterogeneity arises among different metastases and within single lesions (Sanborn *et al.*, 2015).

Consistently, Andor and colleagues provided evidence for the extensive genomic intratumoral diversity of melanoma in a pan-cancer analysis of exome sequences from paired tumor and normal cells of over thousand primary tumor samples of different cancer types available in The Cancer Genome Atlas (TCGA). The study demonstrated on the basis of somatic SNVs (*Single Nucleotide Variations*) and CNVs (*Copy Number Variations*) the coexistence of multiple subclones in the same sample in all analyzed primary melanomas (Andor *et al.*, 2016).

One of the driving forces in the development of intratumor heterogeneity is genomic instability which belongs to the hallmarks of cancer as described by Hanahan and Weinberg (Hanahan and Weinberg, 2011). In the case of malignant melanoma numerous genetic aberrations at multiple levels including chromosomal alterations,

e.g. allelic losses (LOH), point mutations, deletions and duplications have been attributed to different melanoma cell subpopulations arising during disease progression. The studies demonstrating the extensive genomic changes contributing to the intratumor diversity in melanoma have been reviewed recently (Grzywa, Paskal and Wlodarski, 2017).

The multi-clonal nature of cancer is subject to Darwinian-like selection process that promotes selective outgrowth of subpopulations, which have acquired genetic and phenotypic advantages to certain microenvironmental conditions (Nowell, 1976; Burrell *et al.*, 2013). One such condition is the presence of IFN-γ in the tumor microenvironment which is a consequence of an effective T cell response and is known to exert selective pressure on malignant cells leading to tumor immunoediting and to escape from T cell recognition (Ikeda, Old and Schreiber, 2002; Mittal *et al.*, 2014). Accordingly, we and others were able to demonstrate that IFN-γ-resistant melanoma clones evolve in melanoma patients receiving different types of immunotherapy (Zaretsky *et al.*, 2016; Shin *et al.*, 2017; Sucker *et al.*, 2017). In the process, chromosomal alterations and subsequent inactivating mutations in genes involved in type II IFN signaling (e.g. *JAK1*, *JAK2*) have been identified as causative for the loss of sensitivity towards IFN-γ-mediated antitumorigenic effects. Our analysis of the Ma-Mel-36 cell line established from a cutaneous patient metastasis revealed intratumor heterogeneity as this cell line was found to be composed of two genetic variants from which one was sensitive to IFN-γ while the other subpopulation did not respond to the treatment. The reason for the resistant phenotype of the subpopulation was the acquisition of JAK1 deficiency caused by a mono-allelic loss followed by a subsequent mutational inactivation in the remaining *JAK1* gene. Further, we demonstrated intrapatient heterogeneity in the melanoma patient model Ma-Mel-61 with two out of six cell lines established from different metastases displaying resistance to IFN-γ. Insensitivity in both mentioned cell lines was mediated by JAK1 deficiency, which was caused by an allelic loss and a subsequent mutational inactivation in the case of Ma-Mel-61g, and by a homozygous mutation of *JAK1* in Ma-Mel-61h. Interestingly, the mono-allelic loss of *JAK1* has been also detected in the sensitive cell lines of patient Ma-Mel-61 and in the sensitive subpopulation of Ma-Mel-36 suggesting a predisposition of this cells to *JAK1* deficiency and the possible outgrowth of IFN-γ-resistant clones (Sucker *et al.*, 2017). The role of genomic heterogeneity in melanoma development and resistance to

targeted therapy is well known (Shi, Hugo, *et al.*, 2014; Roesch, 2015; Sanborn *et al.*, 2015). However the influence of the genomic heterogeneity of melanoma on immune-mediated tumor control in the context of immunotherapy is less well explored. The data published by us and others provided evidence for the development of diverse genetic aberrations under the influence of an effective immune response and mechanistic insights how this genomic heterogeneity contributes to resistance against $CD8^+$ T cells and consequently also to immune-based therapies especially to immune checkpoint blockade (Zaretsky *et al.*, 2016; Shin *et al.*, 2017; Sucker *et al.*, 2017).

8.2. Combination therapies

The high level of heterogeneity in melanoma represents a major obstacle in the successful treatment of patients with mono therapies as only a subset of sensitive cells can be eradicated by a certain approach while the remaining cells might display varying extent of genetic and epigenetic alterations conveying therapy resistance. Thus, multiple therapeutic combination strategies have been evaluated over the past years in order to hit different melanoma subpopulations sequentially or concurrently in order to prevent the outgrowth of resistant clones.

As mentioned previously, patients treated with BRAF inhibitors relapse rapidly due to resistance, which is mostly caused by MAPK pathway reactivation (Rizos *et al.*, 2014; Shi, Hugo, *et al.*, 2014; Johnson *et al.*, 2015). Targeting the MAPK pathway simultaneously at multiple levels by combining BRAF and MEK inhibitors has been demonstrated to improve patient survival and to delay resistance onset (Larkin *et al.*, 2014; Long *et al.*, 2015). Currently, also an inhibitor targeting ERK1/2, another kinase involved in this pathway and acting downstream of MEK, is investigated in a clinical trial in combination with MEKi (Najem *et al.*, 2017). This approach seems to be a reasonable strategy against BRAF mutant, NRAS mutant and BRAF-wild type tumors (Hatzivassiliou *et al.*, 2012; Morris *et al.*, 2013). In addition, taking into account the aforementioned heterogeneity and the fact that BRAF-wild type melanoma cell populations can coexist with BRAF mutant counterparts within one tumor (Yancovitz *et al.*, 2012) the combination of MEK and ERK inhibitors could be beneficial in fighting both phenotypes concurrently. Other therapeutic strategies include the combination of MAPK pathway inhibitors with small molecules targeting the compensatory PI3K-AKT pathway, which is frequently activated in melanoma upon

MAPK inhibition and thus confers resistance to BRAFi and/or MEKi (Sanchez-Hernandez *et al.*, 2012). First clinical trials in patients with melanoma and other solid tumors investigating the concomitant inhibition of MAPK and PI3K-AKT pathways suggested clinically relevant activity though combined with considerable cytotoxicity, demonstrating the need for more specific drugs and precise scheduling of treatment protocols to minimize severe side effects (Shimizu *et al.*, 2012; Tolcher *et al.*, 2015).
Apart from combining different targeted approaches also the concomitant application of the immune checkpoint antibodies anti-CTLA-4 and anti-PD-1 has been successfully tested in the clinics and demonstrated superior effects achieving objective response rates in about 60 % of patients and providing almost four times longer progression-free survival when compared with anti-CTLA-4 mono-therapy (Larkin, Hodi and Wolchok, 2015; Postow *et al.*, 2015). However tumor cells manage to escape immune surveillance by especially decreasing their immunogenicity through various mechanisms, as demonstrated by us (Sucker *et al.*, 2017) and reviewed recently (Sharma *et al.*, 2017).
Thus, with regard to the immunogenic potential, therapeutic approaches increasing tumor cell immunogenicity could synergize with immune checkpoint blockade. One promising strategy is the combination of epigenetic drugs e.g. HDAC (*histone deacetylase*) inhibitors (HDACi) with immune modulating antibodies. Besides inducing cell-cycle arrest and apoptosis in melanoma, HDAC inhibitors have been shown to induce the expression of differentiation antigens and surface molecules including HLA class I and II as well as PD-L1 on tumor cells leading to augmented antitumor responses in mouse studies in the context of adoptive T cell transfer and anti PD-1 blockade (Vo *et al.*, 2009; Woods *et al.*, 2013, 2015). Interestingly, HDAC inhibitors have been also shown to have an impact on NK cell-mediated tumor recognition by upregulating activating ligands such as NKG2DL on cancer cells (Skov *et al.*, 2005). In our recent *in vitro* study we demonstrated that the BRAFi-mediated downregulation of NKG2DL on melanoma cells and therefore the impaired recognition of this tumor cells by NK cells could be efficiently overcome by the application of the HDAC inhibitor sodium butyrate (López-Cobo *et al.*, 2018). Importantly, the recovery of NK cell recognition by HDAC inhibition in the context of BRAFi treatment could only be achieved by simultaneous but not sequential application of sodium butyrate and BRAFi, indicating the relevance of precise scheduling of treatment protocols in combinatorial approaches to ensure the best

possible antitumor effects. Thus, we suggested that the co-administration of BRAFi and HDACi could be a reasonable strategy to avoid immune escape variants by improving NK cell-mediated tumor recognition combined with the direct pro-apoptotic effects of both drugs (López-Cobo *et al.*, 2018).

BRAF inhibition does not only impact on the immunogenicity of tumor cells with regard to NK cell recognition, as it has been demonstrated in short-term treatment by us and others (Frazao *et al.*, 2017; López-Cobo *et al.*, 2018). Over the past years data has been accumulated associating the MAPK pathway inhibition with many favorable and early effects in the tumor microenvironment including increased melanocytic antigen and HLA expression on tumor cells and increased T cell infiltration (Wilmott *et al.*, 2012; Frederick *et al.*, 2013; Bradley *et al.*, 2015) as well as decreased immunosuppression by myeloid derived suppressor cells and decreased levels of immunosuppressive cytokines (Frederick *et al.*, 2013; Schilling *et al.*, 2013).

Preclinical *in vivo* studies combining MAPK inhibition and immune checkpoint blockade (ICB) or adoptive T cell transfer have demonstrated very promising results with synergistic antitumor effects including durable responses and complete remissions in mouse models (Cooper *et al.*, 2014; Hu-Lieskovan *et al.*, 2015; Liu *et al.*, 2015; Ebert *et al.*, 2016) providing evidence for testing the combination of ICB and MAPKi in clinics. Table 2 summarizes a selection of clinical trials currently ongoing in the context of malignant melanoma and testing different combinations of BRAF inhibitors (vemurafenib, dabrafenib) and/or MEK inhibitors (trametinib, cobimetinib) with immune checkpoint antibodies against CTLA-4 (ipilimumab), PD-1 (nivolumab, pembrolizumab) or PD-L1 (atezolizumab, durvalumab) using different treatment protocols including various sequential and simultaneous combinations.

However it will take a considerable amount of time to complete these studies and to evaluate the results. Until then, the combinatorial treatment with ICB and MAPK inhibitors remains a subject of further investigations, as crucial insights on the optimal scheduling and sequencing of both therapies are largely missing.

Table 2: Selection of clinical trials combining targeted therapies and immune checkpoint inhibitors (www.clinicaltrials.gov, May 2018)

Compound	Checkpoint inhibitor	Phase	Trial number
trametinib and dabrafenib	ipilimumab and nivolumab	III	NCT02224781
trametinib and dabrafenib	pembrolizumab	II	NCT02858921
trametinib and dabrafenib	pembrolizumab	I/II	NCT02130466
trametinib and dabrafenib	durvalumab	I	NCT02027961
vemurafenib and cobimetinib	atezolizumab	III	NCT02908672
vemurafenib and cobimetinib	atezolizumab	II	NCT02902029
vemurafenib and cobimetinib	atezolizumab	I	NCT01656642
vemurafenib and cobimetinib	pembrolizumab	I	NCT02818023

To date, findings from melanoma patient-derived tissue samples and mouse studies revealed that the positive effects of MAPKi on tumor immunogenicity are transient and that the numbers of infiltrating lymphocytes return to levels present in pretreatment biopsies when tumors acquire resistance to MAPK pathway inhibition at the time of disease progression (Wilmott *et al.*, 2012; Frederick *et al.*, 2013; Kakavand *et al.*, 2015). Furthermore, Hugo and colleagues demonstrated highly recurrent immune-related alterations in transcriptomes from MAPKi-resistant tumor biopsies pointing to deficiency of cytotoxic T cells and loss of antigen presentation (Hugo *et al.*, 2015). Complementary to these findings, in our recent functional *in vitro* study (Pieper *et al.*, 2018), we demonstrated that BRAF inhibition alone or in combination with MEK inhibition strongly altered the antigen expression profile of melanoma cells over time, resulting in an impaired recognition of long-term treated tumor cells by antigen-specific $CD8^+$ T cells. In contrast, short-term BRAFi-treated melanoma cells displayed transiently increased expression of almost all analyzed melanocytic antigens and were therefore efficiently recognized by differentiation antigen-specific $CD8^+$ T cells, which is in line with previous findings (Boni *et al.*, 2010; Frederick *et al.*, 2013). With this study, we provided functional insights into the dynamic immunogenic changes on the level of tumor antigen expression, which occurred in the course of MAPKi in melanoma cells and were causative for the development of resistance to $CD8^+$ T cells in our autologous patient models. Thus, we suggest that a simultaneous or early sequential application of MAPKi and immunotherapy could be most effective in preventing the emergence of tumor clones

able to escape immune surveillance (Pieper *et al.*, 2018). Our conclusion and treatment suggestion is further supported by the findings of Ackermann and colleagues who have demonstrated in a retrospective analysis that the outcomes for patients treated with BRAF inhibitors prior to immune therapy with anti-CTLA-4 antibody were poor (Ackerman *et al.*, 2014).

However, larger-scale analyses on the repertoire of shared and tumor-specific antigens in melanoma are needed, to fully understand the kinetics and the impact of MAPKi-mediated alterations on the recognition of malignant cells by tumor-reactive cytotoxic $CD8^+$ T cells.

8.3. First-line treatment and cross-resistance

For patients with $BRAF^{V600E/K}$-mutated melanoma two main therapeutic options as first-line treatment are currently available – either the immune checkpoint inhibition or the dual treatment with BRAF and MEK targeting agents (Rozeman *et al.*, 2017). Currently, there is no clinical data available comparing these two therapeutic strategies directly with each other to guide the decision of which approach should be chosen first (Ugurel *et al.*, 2017). However, a lot of effort has been ventured to find biomarkers predicting response to each of the therapies, in order to optimize the selection of patients who might benefit from a certain treatment.

With regard to ICB, a combination of PD-L1/PD-1 expression on tumor/immune cells, high mutational load, and the infiltration of $CD8^+$ T cells into the tumor, has been recently proposed to be the best indicator of clinical response to anti-PD-1 treatment (Tumeh *et al.*, 2014; Topalian *et al.*, 2016). However, recent studies have demonstrated that progressing melanoma lesions from patients who have initially responded to anti-PD-1 or anti-CTL-4 therapy still might display a profound $CD8^+$ T cell infiltrate indicating that these immune cells, although present in the tumor side, are functionally impaired and not able to exert their cytotoxic activity anymore (Cooper *et al.*, 2016; Zaretsky *et al.*, 2016). Mechanistically, Zaretsky et al. could show that this acquired resistance to anti-PD-1 therapy was associated with genetic alterations in pathways involved in type II IFN signaling and antigen presentation (Zaretsky *et al.*, 2016). Moreover, multiple genomic defects in IFN-γ pathway genes have also been found in melanoma biopsies from non-responding and thus primary resistant patients to anti-PD-1 or anti-CTLA-4 treatment (Gao *et al.*, 2016; Shin *et al.*, 2017). In accordance with these findings, our recent study on melanoma patient

derived tissue samples and cell lines indicates that genetic aberrations in type II IFN signaling frequently evolve in the course of the disease and cause not only insensitivity towards the direct anti-tumorigenic effects of IFN-γ, but might also mediate cross-resistance to $CD8^+$ T cell effector functions. This is the case in tumor cells with very low or completely silenced HLA class I antigen presentation, which can no longer be restored by IFN-γ due to genetic defects in the signaling cascade (Sucker *et al.*, 2017).

These results together highlight the prognostic relevance of genomic information concerning IFN-γ-related genes for the successful treatment with immunotherapeutic approaches. Thus, screenings for genetic defects in IFN-γ-related genes should be considered a strategy for therapeutic decision-making with respect to first-line treatment as IFN-γ signaling aberrations have been shown to play a role in primary resistance to immune checkpoint blockade (Gao *et al.*, 2016; Shin *et al.*, 2017). Treatment-naïve patients displaying such alterations would most likely benefit more from targeted therapy as first-line treatment.

Moreover, tumor lesions that relapse after an initial response to one of the immune modulators and show genetic alterations in components of the IFN-γ signaling pathway might be prone to develop cross-resistance to the second immune checkpoint inhibitor. Recently, Chen and colleagues performed a study supporting this hypothesis, in which they analyzed longitudinal melanoma biopsies from patients who initially received an anti-CTLA-4 antibody followed by the treatment with an anti-PD-1 immune blockade at progression. In this process, potential mechanism of resistance including, among others, decreased expression of HLA molecules and IFN-γ pathway effectors have been identified early on treatment with anti-PD-1 immune checkpoint inhibition in non-responding patients (Chen *et al.*, 2016). In such cases a direct or early switch to targeted therapy might be reasonable to spare the patient unnecessary immune therapy-related cytotoxicity. This approach could eliminate T cell-resistant subpopulations and re-sensitize patients to immune-based therapies.

As already mentioned, the dual inhibition of the MAPK pathway with BRAF and MEK inhibitors is the alternative first-line treatment in BRAF mutant melanoma patients. The main advantages of targeted therapy as frontline treatment are the high and rapid response rates and the massive shrinkage of the tumor mass (Luke *et al.*, 2017). Beyond this, MAPKi has been shown to increase tumor immunogenicity by

various mechanisms, as discussed in the prior chapter of the thesis and reviewed recently by Mandala et al. (Mandala *et al.*, 2017), suggesting that preluding the anti-melanoma therapy with targeted approaches might enhance the anti-tumor responses during subsequent immunotherapy. However, there is a growing evidence indicating that the duration of the MAPK inhibitor treatment plays a crucial role in this process, since the tumor-immunogenicity-increasing-effects of targeted therapy, as the influx of TILs and the induction of melanoma associated antigens, have been demonstrated in *in vitro* and *in vivo* models as well as in patient-derived biopsies to be very short-lived and to disappear in progressing lesions (Wilmott *et al.*, 2012; Frederick *et al.*, 2013; Kakavand *et al.*, 2015; Deken *et al.*, 2016). In accordance with these findings, in our recent *in vitro* study we demonstrated the development of a cross-resistance mechanism of long-term MAPKi treated human melanoma cell lines to autologous TILs and multiple antigen-specific T cell clones present within the TIL repertoire. The cross-resistance mechanism was based on an altered antigen expression profile (including shared and tumor-specific antigens), which was caused by prolonged MAPK inhibition and protected drug-tolerant cancer cells from the recognition by antigen-specific $CD8^+$ T cells (Pieper *et al.*, 2018). Our results are in line with previous findings demonstrating that resistance to MAPK inhibition frequently coevolves with fatal changes in the immune landscape of lesions resistant to targeted therapy including decreased $CD8^+$ T cell numbers and function as well as decrease in antigen presentation (Hugo *et al.*, 2015). Additionally, the same group demonstrated in a subsequent work the presence of an innate anti-PD-1 resistance signature (IPRES) in tumors resistant to anti-PD-1 immune checkpoint therapy, which could be also found to a similar extend in MAPKi-resistant lesions, indicating that cross-resistance to immunotherapy in melanomas resistant to MAPK inhibition might be a common issue (Hugo *et al.*, 2016).

In summary, first-line MAPKi treatment is known to induce a profound tumor control in a very short period of time in BRAF mutated melanomas, but responses are generally transient. Thus, to induce long-lasting benefit and to avoid cross-resistance, as described above, it seems reasonable to replace or to complement MAPK inhibition by an early sequential immune checkpoint therapy, but only if no genetic defects in IFN-γ signaling pathway and/or antigen presentation components are present.

8.4. NK cell-based immunotherapeutic approaches

Although the importance of NK cells in immune surveillance of primary solid tumors, such as melanoma, remains to date a matter of investigation, as NK cell infiltrates are rarely found in the tumor tissue, there is an accumulating evidence for the role of these cells in controlling tumor metastasis (López-Soto *et al.*, 2017). Accordingly, increased numbers of highly cytotoxic NK cells have been found in tumor-cell-infiltrated lymph nodes from melanoma patients (Ali *et al.*, 2014) and the significant involvement of NK cells in repressing melanoma metastases in the context of BRAFi treatment has been demonstrated in mice (De Andrade *et al.*, 2014). Increased NK cell presence has been also noted in regressing melanoma lesions compared with non-regressing melanomas (McKay *et al.*, 2011) as well as a positive correlation between the expression of the activating NK cell receptor NKp46 and the prolonged survival of stage IV melanoma patients (Fregni *et al.*, 2013). Moreover, the high killing capacity of NK cells towards human and murine melanoma cells has been demonstrated *in vitro* as well as in mouse models (Lakshmikanth *et al.*, 2009). In addition, it has been reported that NK cells in patients with metastatic melanoma often display an exhausted phenotype with increased expression of the inhibitory checkpoint receptor TIM-3 and decreased levels of NK cell activating receptors (da Silva *et al.*, 2014; Gallois *et al.*, 2014).

These findings argue for an important and direct role of NK cells in the immune surveillance of melanoma. Accordingly, escape mechanisms from NK cell recognition have been described for melanoma cells as the increased proteolytic cleavage of cell surface-bound ligands (e.g. NKG2D ligands) for activating NK cell receptors, which has been associated with poor prognosis and unfavorable clinical outcome to immunotherapy in melanoma patients (Paschen *et al.*, 2009; Raneros *et al.*, 2015; Maccalli *et al.*, 2017). Thus, multiple NK cell-based immunotherapeutic approaches are currently under investigation in order to enhance NK cell activation and to exploit their cytotoxic potential towards tumor cells.

In this process, one important approach is the increase of tumor immunogenicity. This can be achieved by a pharmacological blockade of ADAMs (*a disintegrin and metalloproteinases*) and MMPs (*matrix metalloproteinases*), which are involved in the shedding of immunostimulatory stress ligands from the surface of malignant cells (Waldhauer and Steinle, 2006; Waldhauer *et al.*, 2008; Boutet *et al.*, 2009; Chitadze *et al.*, 2013). In addition, MMP inhibition has also been demonstrated to prevent the

cleavage of the CD16 (FcγR-III) surface receptor on activated NK cells, which is involved in ADCC (*antibody-dependent cell cytotoxicity*), a process by which NK cells recognize and kill antibody-coated tumor cells. The combination of therapeutic tumor-specific monoclonal antibodies, which use ADCC as a mechanism of action, and MMP inhibitors, has been therefore proposed for cancer treatment in order to sustain the CD16 binding to the constant Fc chain of antibodies and to enhance cytotoxic functions of NK cells (Shuptrine, Surana and Weiner, 2012; Zhou *et al.*, 2013). In regard to therapeutic tumor-specific mAbs considerable progress has been achieved in engineering bi- and tri-specific antibodies consisting of one or two variable single-chain fragments against tumor-specific epitopes, respectively. These so called “killer engagers” crosslink tumor cells and NK cells trough CD16 engagement, and thus mediate tumor-specific ADCC (Gleason *et al.*, 2012; Tay, Carol and Biro, 2016).

Besides the usage of small-molecules inhibiting MMPs and/or ADAMs, as outlined above, specific antibodies targeting the cleavage site of the NKG2D ligand MICA have been recently developed and demonstrated to prevent the shedding of the MICA and MICB proteins and thus to increase NK cell-mediated anti-tumor activity in a mouse model (Ferrari de Andrade *et al.*, 2018). Another possibility to increase the density of activating ligands on the surface of cancer cells is the application of epigenetic drugs e.g. HDAC inhibitors or demethylating substances which have been shown to increase the expression of NKG2D ligands and to reduce their shedding from the surface of tumor cells (Chavez-Blanco *et al.*, 2011; Zhu *et al.*, 2015). Recently, we have also demonstrated that the application of the HDAC inhibitor sodium butyrate induced the expression of NKG2D ligands on the surface of melanoma cells after BRAFi-mediated downregulation of these molecules and thus restored the NK cell recognition of the analyzed tumor cells (López-Cobo *et al.*, 2018).

Further approaches to improve anti-tumor NK cell effector functions comprise the blockade of inhibitory receptors on NK cells such as the KIR family of receptors or the NKG2A receptor, which bind to HLA class I molecules on tumor cells and thus prohibit NK cell activation, as outlined in the introductory section. Currently, the anti-KIR mAb lirilumab is tested in a phase I/II clinical trial in combination with anti-PD-1 and as a triple combination with anti-PD-1 and anti-CTLA-4 immune checkpoint blockade in patients with advanced solid tumors (clinicaltrial.gov; trial number: NCT01714739). This study may open up new supporting combination strategies for

the immunotherapy of malignant melanoma. However, increased toxicity and autoimmune reactions have to be considered as inhibitory signals are removed from both $CD8^+$ T cells and NK cells.

Another strategy to enhance NK cell recognition of tumor cells and to circumvent escape mechanism, such as NKG2DL downregulation or HLA class I upregulation, is the use of genetically engineered CAR-expressing NK cells (*chimeric antigen receptor*-expressing NK cells), which like the bi-specific or tri-specific monoclonal antibodies, recognize given tumor-associated antigens and induce NK cell-mediated cytotoxicity upon binding to a specific tumor epitope (Hermanson and Kaufman, 2015). In melanoma, the killing efficiency of the CAR-transduced human immortalized NK cell line NK-92 recognizing the melanoma antigen gp-100 in the context of HLA-A2 was demonstrated *in vitro* and in a human melanoma xenograft model using adoptive NK cell transfer in mice (Zhang *et al.*, 2013). Indeed, genetically modified NK cell lines such as NK-92 are thought to be a promising strategy to improve the efficacy of adoptively transferred NK cells in cancer immunotherapy as they are very well characterized, relatively easy to expand *in vitro*, and offer a robust base for genetic engineering (Suck *et al.*, 2016).

8.5. Liquid biopsy as an approach in personalized treatment of malignant melanoma

As discussed previously, malignant melanoma is a highly heterogeneous and versatile tumor that has to be fought with a multitude of likewise manifold therapeutic approaches (Hachey and Boiko, 2016). In this process, selection of patients who might benefit from a particular treatment strategy is crucial. Currently, therapeutic decision-making relies largely on the molecular profile of melanoma with respect to oncogenic drivers, which mutational status is determined by sequencing of tumor DNA isolated from a surgically obtained tumor biopsy.

However, the genomic and molecular profile of cancer is highly dynamic and subject to constant changes that may arise during the course of the disease due to selective pressure (Burrell *et al.*, 2013). Accordingly, we demonstrated that genomic heterogeneity evolved within different patient models in the course of melanoma progression under the selective pressure of the immune system and resulted in individual IFN-γ-resistant and to some extend also in $CD8^+$ T cell-resistant lesions

(Sucker *et al.*, 2017). Further, we were able to show that under the pressure of BRAFi, next to the drug resistance also cross-resistance to tumor-reactive antigen-specific $CD8^+$ T cells evolved over time, which was caused by remarkable changes in the antigen expression profile of melanoma cells (Pieper *et al.*, 2018). In addition, we demonstrated that already very early during BRAFi treatment melanoma cells may arise that escape from NK cell recognition by downregulating the expression of stimulatory ligands but can be re-sensitized to NK cells through the application of epigenetic drugs (López-Cobo *et al.*, 2018). These and other previously discussed studies indicate, that a longitudinal and close mashed monitoring of melanoma development is indispensable for early detection of molecular and phenotypic alterations occurring over time. Only this strategy can ensure that appropriate and individual therapeutic strategies and treatment combinations can be applied at the right time to counteract the development of escape mechanisms leading to disease progression.

However, in addition to the invasive nature of surgeries there is also the problem of random sampling while taking tissue biopsies. This method facilitates only a very limited monitoring of tumor alterations restricted to a small sample size, which do not represent the whole molecular and phenotypic variety present within a tumor (Marusyk and Polyak, 2010; Gerlinger *et al.*, 2012).

Currently, the development of ‘liquid biopsies’ of blood, which is a non-invasive technique that can be applied repeatedly prior to therapy, during treatment and for long-term surveillance of cancer patients, is a very promising approach to closely monitor therapy responses and the evolution of cancer in real-time. Blood-based liquid biopsies allow a very comprehensive molecular profiling of tumors as they contain intact circulating tumor cells (CTC), cell-free circulating tumor DNA (ctDNA) and RNA (ctRNA) as well as tumor cell-derived exosomes containing diverse nucleic acids, proteins and lipids (Haber and Velculescu, 2014; Siravegna *et al.*, 2017; Cui *et al.*, 2018; Halvaei *et al.*, 2018). Thus, genetic alterations that are present within tumor tissue e.g. somatic mutations, copy number alterations or chromosomal anomalies, can also be found in ctDNA, CTCs and exosomes isolated from blood of cancer patients (Siravegna *et al.*, 2017).

Accordingly, recent studies have demonstrated that the analysis of patient-derived ctDNA with respect to genetic alterations, has the potential to reflect the spatial and temporal heterogeneity found in tissue biopsies from tumors and to track different

responses to therapies across metastases (Murtaza *et al.*, 2015; Gremel *et al.*, 2016). Moreover, isolated CTCs can be used in *in vitro* assays for testing of drug sensibility, allowing for monitoring of subclones with differential drug susceptibility, which may evolve during the course of the disease. This approach may enable a rapid therapeutic intervention upon detection of a possible drug tolerance (Yu *et al.*, 2014; Khoo *et al.*, 2016). The multitude of studies exploring different sources of liquid biopsies (e.g. blood, saliva, urine) and their clinical feasibility have been reviewed recently by Siravegna et al., (Siravegna *et al.*, 2017), while the manifold clinical application possibilities of blood-based liquid biopsies are depicted in Figure 4.

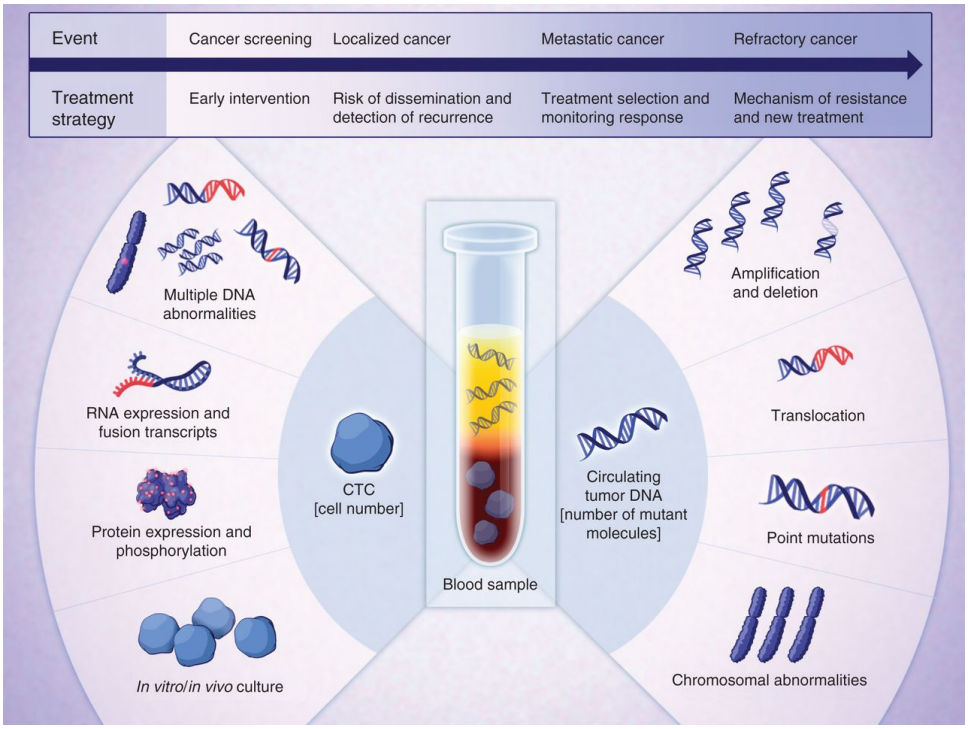

Figure 4: Molecular analyses of CTCs and ctDNA derived from blood-based liquid biopsy and their clinical applications, (Haber and Velculescu, 2014).

For malignant melanoma, it was demonstrated that low or not detectable levels of ctDNA in blood of patients before treatment positively correlated with response to targeted therapy and to immunotherapy (Gray *et al.*, 2015; Lee *et al.*, 2017). Further, decreased amounts of ctDNA were detected in blood biopsies in patients displaying tumor regression during targeted therapy (Gray *et al.*, 2015; Haselmann *et al.*, 2018). Moreover, the analysis of ctDNA have been shown to uncover arising therapy resistance, prior to detection of progressive disease on radiological scans, by revealing the presence of resistance-conferring mutations (e.g. *NRAS* mutation) or

increased levels of $BRAF^{V600E/K}$ mutant ctDNA (Gray *et al.*, 2015; Haselmann *et al.*, 2018). These studies demonstrate the suitability of ctDNA-based monitoring of melanoma progression and therapy responses in patients. However, the long-term clinical benefit of liquid-biopsy driven diagnostics and treatment-decisions remains to be proven in further patient-based studies.

9. References

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10. Abbreviations

ACT	adoptive cell transfer
ADAM	a disintegrin and metalloproteinase
ADCC	antibody-dependent cell cytotoxicity
AKT	v-akt murine thymoma viral oncogene homolog
APM	antigen processing machinery
ARF	alternate reading frame
BAGE	B melanoma antigen
BRAF	v-Raf murine sarcoma viral oncogene homolog B1
BRAFi	BRAF inhibition
c-MET	tyrosine-protein kinase Met or hepatocyte growth factor receptor
CAR	chimeric antigen receptor
CD	cluster of differentiation
$CD8^+$	CD8 positive
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2A
CNV	copy number variation
CSPG4	chondroitin sulfate proteoglycan 4
CTC	circulating tumor cells
ctDNA	circulating tumor DNA
CTLA-4	T-lymphocyte-associated protein-4
ctRNA	circulating tumor RNA
DNA	deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
EGFR	epidermal growth factor receptor
ER	endoplasmatic reticulum
ERAP1/2	endoplasmatic reticulum aminopeptidase 1 and 2
ERK	extracellular signal-regulated kinase 1 and 2
FADD	Fas-associated death domain protein
FasL	FS7-associated cell surface antigen Ligand
FDA	Food and Drug Administration
GAGE	G antigen 1
gp-100	glycoprotein 100

HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
ICB	immune checkpoint blockade
IFN-γ	interferon-gamma
IFNGR1/2	interferon-gamma receptor 1 and 2
IGF-1R	insulin-like growth factor 1 receptor
IPRES	innate anti-PD-1 resistance signature
IRF1	interferon regulatory factor 1
JAK1/2	janus kinase 1 and 2
KIR	killer-cell immunoglobulin-like receptors
LEF1	lymphoid enhancer-binding factor 1
LOH	loss of heterozygosity
mAb	monoclonal antibody
MAGE	melanoma-associated antigen
MAPK	mitogen-activated protein kinase
MAPKi	mitogen-activated protein kinase pathway inhibition
MART-1	melanoma-associated antigen recognized by T cells
MEK1/2	mitogen-activated protein kinase kinase 1 and 2
MEKi	mitogen-activated protein kinase kinase inhibition
MHC	major histocompatibility complex
MICA/B	MHC class I chain related proteins A and B
MITF	microphthalmia-associated transcription factor
MMP	matrix metalloproteinase
NCR	natural cytotoxicity receptor
NK cell	natural killer cell
NKAR	NK cell activating receptor
NKG2D	Natural Killer Group 2, member D
NKG2DL	NKG2D ligand
NKIR	NK cell inhibitory receptor
NKT cell	natural killer T cell
NRAS	neuroblastoma RAS viral oncogene homolog
NY-ESO-1	New York esophageal squamous cell carcinoma-1

ORR	overall response rate
OS	overall survival
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PDGFRβ	platelet-derived growth factor receptor beta
PFS	progression free survival
PI3K	phosphatidyl-inositol-3-kinase
PRAME	preferentially expressed antigen in melanoma
PTEN	phosphatase and tensin homolog
RAF	rapidly accelerated fibrosarcoma protein
RAS	rat sarcoma viral oncogene homolog
RGP	radial-growth phase
RNA	ribonucleic acid
RTK	receptor tyrosine kinase
sNKG2DL	soluble NKG2D ligand
SNV	single nucleotide variation
STAT1	signal transducer and activator of transcription 1
sULBP2	soluble ULBP2
TAP1/2	antigen peptide transporter 1 and 2
TCGA	The Cancer Genome Atlas
TCR	T cell receptor
TIL	tumor-infiltrating lymphocytes
TNF-α	tumor necrosis factor alpha
TNFR1	tumor necrosis factor receptor 1
TRP1/2	tyrosinase-related protein 1 and 2
ULBP1-16	UL-16 binding proteins 1-16
UV	ultraviolet
VGP	vertical-growth phase
YAP1	yes-associated protein 1
β-2m	beta-2-microglobulin
γδT cell	gamma-delta-T cell

11. List of figures

12. List of tables

13. Author contributions

Article I

Acquired IFNγ resistance impairs anti-tumor immunity and gives rise to T cell-resistant melanoma lesions.

Journal: Nat Commun. 2017 May 31; 8:15440.

Authors: Antje Sucker, Fang Zhao, **Natalia Pieper**, Christina Heeke, Raffaela Maltaner, Nadine Stadtler, Birgit Real, Nicola Bielefeld, Sebastian Howe, Benjamin Weide, Ralf Gutzmer, Jochen Utikal, Carmen Loquai, Helen Gogas, Ludger Klein-Hitpass, Michael Zeschnigk, Astrid M. Westendorf, Mirko Trilling, Susanne Horn, Bastian Schilling, Dirk Schadendorf, Klaus G. Griewank, Annette Paschen

I hereby certify that Natalia Pieper contributed to the concept and experimental design of this article. She performed experiments depicted in figures 1 i, 2 g-k, 4 e-f, as well as in supplemental figures S1 c, S2 d-e and S3 c, and was responsible for planning, execution, analysis and illustration of depicted results and corresponding figures.

Place, Date	Annette Paschen
Place, Date	Natalia Pieper

Article II

Impaired NK cell recognition of vemurafenib treated melanoma cells is overcome by simultaneous application of histone deacetylase inhibitors.

Journal: Oncoimmunology 2017 Nov 6; 7(2): e1392426.

Authors: Sheila López-Cobo, **Natalia Pieper**, Carmen Campos-Silva, Eva M. García-Cuesta, Hugh T. Reyburn, Annette Paschen & Mar Valés-Gómez

I hereby certify that Natalia Pieper contributed to the concept and experimental design of this article. She performed experiments depicted in figures 2 D and 5 D, and was responsible for execution, analysis and illustration of depicted results. Additionally, she performed replicates of data depicted in figure 2 B and supplemental figure 1 B. Moreover, she supervised and advised the first-author's work in the lab during an internship, during which a considerable part of the data presented in the above mentioned article was generated. In this process she provided her methodological expertise and help during the entire placement.

_____	_____
Place, Date	Annette Paschen
_____	_____
Place, Date	Natalia Pieper

Article III

Evolution of melanoma cross-resistance to CD8$^+$ T cells and MAPK inhibition in the course of BRAFi treatment.

Journal: Oncoimmunology 2018 April 18; doi.org/10.1080/2162402X.2018.1450127

Authors: **Natalia Pieper**, Anne Zaremba, Sonia Leonardelli, Franziska Noelle Harbers, Marion Schwamborn, Silke Lübcke, Barbara Schrörs, Jolanthe Baingo, Alexander Schramm, Sebastian Haferkamp, Ulrike Seifert, Antje Sucker, Volker Lennerz, Thomas Wölfel, Dirk Schadendorf, Bastian Schilling, Annette Paschen, Fang Zhao

I hereby certify that Natalia Pieper 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 contributed to the writing, review and revision of the manuscript. She performed all experiments shown, except for figures 1 A-B, 2 B, 4 C, 4 E, 6 A-B. In Addition, she contributed to the execution, analysis and illustration of data depicted in figures 1 D, 4 F, 6 C, 6 D as well as in the supplementary figure S1 A-B.

_____ _____

Place, Date Annette Paschen

_____ _____

Place, Date Natalia Pieper

14. Acknowledgments

An dieser Stelle möchte ich mich bei allen Personen bedanken, ohne die diese Arbeit nicht möglich gewesen wäre:

Bei Prof. Dr. Dirk Schadendorf, für die Möglichkeit meine Arbeit in seiner Klinik anfertigen zu können sowie für die interessanten wissenschaftlichen Retreats.
Bei Prof. Dr. Annette Paschen für die hervorragende Betreuung und Unterstützung während meiner Doktorarbeit, für die zahlreichen Ideen und Anregungen, die Weiterbildungsangebote, die sie mir ermöglichte, sowie für die Möglichkeit an verschiedenen sehr interessanten Projekten mitwirken zu können.
Bei Prof. Dr. Wiebke Hansen und Prof. Dr. Christine Falk für die Begutachtung meiner Dissertation.
Bei Dr. Fang Zhao für ihre Fachkompetenz, stetige Hilfe, ihre gute Laune und positive Einstellung, mit der sie mich immer angesteckt hat.
Bei der gesamten Arbeitsgruppe „Molekulare Tumorimmunologie": Annette, Antje, Fang, Bea, Birgit, Inga, Jana, Julia, Marion, Nadine, Song, Sonia und Svenja für die tolle und entspannte Arbeitsatmosphäre, für die gute Organisation und die schönen und lustigen Momente in unserer Freizeit.

Bei Nadine, Sonia und Julia für die schweißtreibenden Stunden beim Sport.
Bei Sonia und Reni für TGIT und eure ungebremste Frauenpower.
Bei Bea für ihre Formatierungs- und Backkünste.
Bei Franzi für ihren Pragmatismus, ihren Humor und ihre große Hilfsbereitschaft.
Bei Raffaela und Nadine für eure Freundschaft und unsere Frühstückseskapaden.

Bei Bärbel und Bruno dafür, dass sie immer für mich da waren, besonders dann, wenn es darauf ankam, für ihr offenes Ohr und die guten Gespräche, aber auch für die unzähligen schönen Erinnerungen, die wir uns geschaffen haben und die mich heute immer wieder zum Lachen bringen.
Bei meinen Eltern für die schöne und unbeschwerte Kindheit und ihren Mut für ihre Kinder ins Unbekannte zu ziehen, ohne den ich heute nicht da wäre, wo ich bin.
Bei Morten, für die liebevolle Unterstützung, das Verständnis und die Opferbereitschaft, die er aufgebracht hat sowie für seine Liebe und das ungebrochene Vertrauen in mich.

15. Curriculum Vitae

Aus datenschutzrechtlichen Gründen ist der Lebenslauf in der Online-Version nicht enthalten

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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.

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