Mechanisms of Drug Resistance in Melanoma

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Abstract

Metastatic melanoma is associated with poor outcome and is largely refractory to the historic standard of care. In recent years, the development of targeted small-molecule inhibitors and immunotherapy has revolutionised the care and improved the overall survival of these patients. Therapies targeting BRAF and MEK to block the mitogen-activated protein kinase (MAPK) pathway were the

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first to show unprecedented clinical responses. Following these encouraging results, antibodies targeting immune checkpoint inhibition molecules cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), programmed cell death (PD)-1, and PD-ligand1(PD-L1) demonstrated sustained tumour regression in a significant subset of patients by enabling an anti-tumour immunologic response. Despite these landmark changes in practice, the majority of patients are either intrinsically resistant or rapidly acquire resistance to MAPK pathway inhibitors and immune checkpoint blockade treatment. The lack of response can be driven by mutations and non-mutational events in tumour cells, as well as by changes in the surrounding tumour microenvironment. Common resistance mechanisms bypass the dependence of tumour cells on initial MAPK pathway driver mutations during targeted therapy, and permit evasion of the host immune system to allow melanoma growth and survival following immunotherapy. This highlights the requirement for personalised treatment regimens that take into account patient-specific genetic and immunologic characteristics. Here we review the mechanisms by which melanomas display intrinsic resistance or acquire resistance to targeted therapy and immunotherapy.

Keywords

Acquired resistance • BRAF inhibitor • Checkpoint inhibitor • Immunotherapy • Intrinsic resistance • MAPK pathway • MEK inhibitor • Melanoma • Targeted therapy

1 Melanoma Incidence and Clinical Subtypes

Malignant melanoma accounts for >80% of skin cancer-related deaths despite representing <1% of cases (NICE 2015). The current incidence and mortality of melanoma in Europe is over 100,000 new cases and over 22,000 deaths each year (http://www.IARC.fr). In most European countries rates are doubling every decade and in many countries melanoma will soon be the fourth most common cancer (Ferlay et al. 2013). The yearly incidence in the UK is 12,000 cases, which is rising by ~2% per year, and approximately 2,000 patients die from advanced disease. Approximately one third of melanoma patients are under 50 years of age, so this cancer disproportionately affects the elderly population (NICE 2015).

Melanoma stems from the malignant transformation of melanocytes, which are neural crest-derived, and migrate during development to colonise the skin, eye, and in scarce numbers, other tissues of the body. The most common type of melanoma arises from melanocytes in the skin and predominantly affects the population of European descent (Whiteman and Green 1999). The only known environmental risk factor for cutaneous melanoma is ultraviolet radiation (UVR) and informing the population at risk remains a public health challenge (Gilchrest et al. 1999). Previous efforts to use environmental factors to classify cutaneous melanoma include proposals for a divergent pathway model (Whiteman and Green 1999), where UVR exposure pattern, host susceptibility, and the site of the primary lesion are

used as criteria to identify epidemiologically uniform subsets of patients. Indeed, cutaneous melanoma arising at sites chronically exposed to UVR (head and neck) is more likely to occur in adults with a high lifetime accumulation of UVR exposure, whereas melanomas arising at sites that are only intermittently exposed to UVR (trunk) appear in younger patients who received a lower lifetime exposure. This group of patients were more likely to report intermittent UVR exposure on the trunk through recreational activities (Whiteman et al. 2003; Curtin et al. 2005; Chang et al. 2009).

The most common founder mutation in melanomas arising in patients younger than 55 years of age is ^{V600E}BRAF (Davies et al. 2002; Cancer Genome Atlas Network 2015). In contrast, melanomas arising in the elderly are more likely to be driven by mutations in *NRAS*, *NF1* and *BRAF* mutations that do not affect the codon V600 (Cancer Genome Atlas Network 2015; Krauthammer et al. 2015; Shain and Bastian 2016). Thus, the protein kinase BRAF is mutated in almost half of all cutaneous melanomas, and *BRAF* mutations are more frequently found to drive melanomas arising over skin that has been only intermittently exposed to sun such as the trunk and upper limbs (Cancer Genome Atlas Network 2015; Shain and Bastian 2016; Shain et al. 2015).

2 Melanoma Treatment Overview

Until the last decade, the standard of care for advanced disease included surgical resection, chemotherapy with the alkylating agent dacarbazine (DTIC), and high-dose interleukin 2 (IL-2), which only led to durable responses in a few patients with metastatic disease. The only approved adjuvant treatment for patients at high risk of progression was interferon-α-2b. Despite these treatments, metastatic melanoma remained associated with extremely poor prognosis and only curable if detected at an early, pre-metastatic stage (Balch et al. 2009). More recently, insights into the molecular mechanisms driving melanomagenesis have led to the development of targeted therapies, and the advances in immune checkpoint inhibitor therapies have also contributed to a revolution in melanoma care.

3 The Rationale for Targeted Therapies in Melanoma

One of the principal signalling pathways in melanoma is the MAPK pathway, which signals via RAS (Young et al. 2009; Stephen et al. 2014) and RAF (Davies et al. 2002; Wan et al. 2004; Marais et al. 1997). The signalling cascade is triggered by stimulatory input from extracellular growth factors, cytokines and hormones that bind to the receptor tyrosine kinases (RTK) on the cell membrane (Schlessinger 2000). Once activated, the RTKs form stable dimers that undergo phosphorylation at multiple tyrosine residues that are in the cytosolic component of the protein. This chemical change drives activation of RAS proteins, and downstream proteins BRAF and CRAF, leading to activation and phosphorylation of the dual-specificity

kinases MEK1 and MEK2; which in turn phosphorylate ERK1 and ERK2 (Holderfield et al. 2014). ERK proteins then phosphorylate approximately 50 cytoplasmic and nuclear proteins to control cell proliferation, differentiation, adhesion and migration (Roberts and Der 2007). This powerful signalling pathway presents negative regulators that limit ERK effects supplying feedback mechanism systems (Lito et al. 2013). In addition to the MAPK pathway, however, extracellular stimuli may affect multiple other pathways. Of particular significance to melanoma is the PI3K/AKT/mTOR pathway, which is upregulated across many cancer types (Fruman and Rommel 2014). Critically, these signalling pathways not only drive signals in a single direction from the extracellular space to the nucleus, but also interconnect at different levels creating a complex network (Fey et al. 2012). Thus, targeted therapies aim to not only interfere with a single signalling axis but also aim to target secondary signalling streams that the tumour may use to circumvent the target of a single node within the network.

Nearly half of all cutaneous melanomas present a gain-of-function (GOF) mutation in the *BRAF* gene at the codon V600, which constitutively drives MAPK pathway activation via MEK1/2 and ERK1/2 (Davies et al. 2002; Wan et al. 2004). A single activating mutation in the V600 codon of *BRAF*, which usually leads to substitution of a valine residue to glutamic acid residue (V600E) (Dhomen et al. 2009), renders BRAF constitutively active but is not enough to drive tumour initiation (Pollock et al. 2003). Additional hits in tumour suppressors *PTEN* (Dankort et al. 2009), *TP53* (Goel et al. 2009) or *CDKN2A* (Sharpless et al. 2003) are required to progress melanomagenesis. Critically, in physiological conditions where BRAF is wild type, RAF proteins dimerise upon activation (Wan et al. 2004; Luo et al. 1996; Farrar et al. 1996). By contrast, ^{V600E}BRAF is able to dimerise with CRAF (Wan et al. 2004), a RAF family protein, and function as a monomer vulnerable to inhibition (Poulikakos et al. 2011).

The second most common mutation is a GOF at codons G12/13 (approximately 5% of melanomas) or Q61 (17% of melanomas) of NRAS (Sekiya et al. 1984; Albino et al. 1989; van't Veer et al. 1989). BRAF and NRAS mutations occur in a mutually exclusive pattern in pre-treatment samples (Cancer Genome Atlas Network 2015). NRAS presents GTPase activity and when wild type, following the phosphorylation of RTKs, is activated by a guanine nucleotide exchange factor (GEF), which permits the exchange of GDP for GTP (Young et al. 2009). When RAS is mutated, it remains in GTP-bound state (Katz and McCormick 1997). RAS signals downstream by a variety of pathways, including both the MAPK and PI3K/Akt/mTOR pathways, and in human melanoma and mouse models of NRAS melanoma, like in BRAF, there is synergy to drive tumour progression when RAS alterations are combined with additional genetic hits in *CDKN2A*, *TP53* and *PTEN* (Feng et al. 2013; Conde-Perez and Larue 2014). Critically, there are currently no successful drugs in clinic targeting oncogenic RAS.

In the tumours that are wild type for both BRAF and NRAS mutations, the more frequent identified drivers are *NF1* loss-of-function (LOF) mutations, as well as alterations in *KIT*, *TERT* and CCND1 (Cancer Genome Atlas Network 2015; Krauthammer et al. 2015; Shain et al. 2015; Hodis et al. 2012).

4 RAF Inhibition in Melanoma

The discovery of the BRAF oncogene and MAPK pathway signalling led to the development of targeted BRAF inhibition therapy (vemurafenib (Chapman et al. 2011; Sosman et al. 2012; McArthur et al. 2014) and dabrafenib (Hauschild et al. 2012)) and targeted MEK kinase inhibition (trametinib (Flaherty et al. 2012)), although MEK inhibitor trials have shown less impressive responses than BRAF inhibitors. More recently, combination therapies have improved initial monotherapy results (Long et al. 2014; Ascierto et al. 2016). These new drugs achieve improved progression-free and overall survival, and about 90% of patients show some improvement and tumour regression, with up to approximately 50% achieving partial or complete responses. Although these responses are encouraging, the response to these drugs is limited, with only a small subset of patients developing long-term, durable responses. Unfortunately, approximately 50% of patients that present an initial improvement generally relapse within 7 months of treatment (acquired resistance) (Chapman et al. 2011; Hauschild et al. 2012). Additionally, there are about 10% of patients who do not respond to the targeted inhibition at all, and these are termed intrinsic or primary resistant to targeted therapy. Importantly, BRAF inhibitors lead to characteristic side effects, including photosensitivity, which can limit treatment, and the rapid development of cutaneous squamous cell carcinoma (cuSCC) (Su et al. 2012). Keratinocytic secondary neoplasia are thought to arise due to the paradoxical activation of the MAPK pathway in keratinocytes that are wild type for BRAF but present upstream RAS activation in chronically damaged skin (Su et al. 2012). In this cellular context, the BRAF inhibitor leads to increased downstream ERK signalling in BRAF wild type keratinocytes that give rise to cuSCC (Heidorn et al. 2010). Thus, combining BRAF and ERK inhibitors was predicted to decrease this side effect, and indeed, this combination treatment not only was demonstrably linked to improved progression free and overall survival compared to BRAF inhibitor in monotherapy (Larkin et al. 2014; Robert et al. 2015) but also decreased development of cuSCC. However, as in BRAF inhibitor monotherapy, resistance develops in most melanoma patients, and great efforts have been invested to understand how and when tumours resist or stop responding.

5 Tumour Cell Intrinsic or Autonomous Resistance to RAF Inhibition in Melanoma due to Mutational or Genetic Events

Resistance to BRAF inhibitors is predominantly linked to reactivation of the MAPK pathway (Lito et al. 2013; Nazarian et al. 2010; Maertens et al. 2013; Whittaker et al. 2013). As monomeric ^{V600E}BRAF is the relevant target of RAF inhibitors, all changes that increase the proportion of RAF dimerisation likely decrease sensitivity to BRAF inhibitors (Poulikakos et al. 2011). Following the rationale that increasing RAS signalling to drive RAF heterodimer formation should decrease therapy

response, we find that upstream, acquired GOF mutations in *RAS* (Nazarian et al. 2010), LOF mutations in *NF1* (Maertens et al. 2013) and several GOF mutations in RTKs (Whittaker et al. 2013) have all shown to drive tumour resistance. Apart from these mechanisms that reactivate RAS, there are structural changes in oncogenic BRAF due to aberrant splicing that can lead to resistance (Poulikakos et al. 2011; Solit and Rosen 2011). For example, p61-^{V600E}BRAF splice variants retain an active kinase activity but are unable to bind RAS. They dimerise regardless of RAS status and drive constitutive signalling to ERK, uncoupled from upstream regulation (Poulikakos et al. 2011). More recently, a screen for resistance mechanisms using patient derived xenografts (PDX) found that duplications of the BRAF kinase domain led to resistance in approximately 10% of PDX (Kemper et al. 2016). This was found to hold true in a validation cohort of resistant patient samples. Additional drivers of resistance are the increased expression of CRAF or copy number increase of BRAF, which possibly also drive dimer formation and increase signalling throughput.

There are also mechanisms of resistance that arise downstream of the inhibition site BRAF within the MAPK cascade, that bypass the effect of the drug. Importantly, the specific mutations in *MEK1* (*MAP2K1*) coding amino acid changes P124L and Q56P are able to decrease response to BRAF inhibition (Wagle et al. 2011), but not all MEK1 somatic mutations lead to equivalent reactivation of the pathway downstream of MEK1 (Shi et al. 2012). Additionally, the increased expression of the MAPK kinase kinase COT (*MAP3K8*) is thought to lead to direct activation of MEK1, circumventing RAF inhibition (Johannessen et al. 2010). More recently, the role of ERK reactivation driving resistance has been further highlighted by massively parallel sequencing efforts revealing mutations in genes encoding for proteins in the cohesion complex that participate in the organisation of chromatids, *STAG2* and *STAG3* (Shen et al. 2016). Mutant proteins are shown to drive the reactivation of ERK signalling to drive resistance by inhibiting the expression of the phosphatase DUSP6, an inhibitor and regulator of ERK activity in melanoma. Other implicated negative regulators include the LOF of DUSP4 (Shen et al. 2016).

Further molecular alternatives driving resistance are the activation of parallel signalling, such as the activation of PI3K/AKT/mTOR pathway by deletion or inactivating mutations of the negative pathway regulator and tumour suppressor *PTEN* (Xing et al. 2012), and the inactivation of the tumour suppressor *RB1* (Xing et al. 2012) to decrease the requirement for BRAF/MEK signalling. The discovery of these contributing signalling pathways provides new rationales of second-line therapies, as demonstrated by the success of combinations of MAPK and PI3K/AKT/mTOR pathway inhibitors to overcome acquired resistance to monotherapy targeting BRAF alone (Greger et al. 2012).

To add further complexity, patients who present disease progression following initial therapeutic response to BRAF inhibition are more likely to progress presenting metastasis at previously uninvolved sites, and there is a selection for more aggressive clones (Paraiso et al. 2015). Importantly, tumour heterogeneity and heterogeneous mechanisms of resistance are present in tumours and at different

metastatic sites simultaneously (Shi et al. 2014). Recent efforts to dissect the degree of heterogeneity and its clinical implications have led to transcriptional studies targeting thousands of single tumour cells, and show that at the cellular level in all tumours, there are distinct transcriptional patterns within each tumour that display varying degrees of predicted responsiveness to BRAF inhibition (Tirosh et al. 2016). Thus, there are subclones of cells, in varying proportions within each tumour, expressing molecular programmes that make them less likely to respond to therapy and vulnerable to selection during disease progression (Tirosh et al. 2016).

6 Tumour Cell Intrinsic or Autonomous Resistance to RAF Inhibition in Melanoma due to Non-mutational Events

As described above, additional genetic damage to *PTEN* or *RB1* leads to activation of alternative signalling pathways that will lead to therapeutic failure (Xing et al. 2012). However, we already observe the activation of parallel signalling pathways, leading to progressive "dampening" (adaptation) of response to BRAF inhibitors during therapy in the absence of additional genetic hits. This occurs because there is a progressive switch of cells to rely on other signalling pathways such as PI3K/AKT as a natural consequence of the new pressures exerted by BRAF inhibition (Lito et al. 2012). This rewiring or switch happens because BRAF inhibitors will initially shut down transcription of key targets of proliferation, leading to response, via suppression of ERK function, but will also decrease the expression of negative pathway regulators downstream of ERK such as DUSP6 (negative regulator of ERK activity) and Sprouty (*SPRY* (Tsavachidou et al. 2004); inhibitor of GRB2, an adaptor protein transducing signals between RTKs and RAS) (Lito et al. 2013; Lito et al. 2012). Thus, inherent tumour cell adaptation to targeted therapy, in the absence of additional genetic events, per se provides an explanation for progressive decline in therapeutic response.

Another way tumour cells co-opt established biological cellular signalling pathways to advance tumour progression in the absence of genetic mutations is by up-regulation or activation of RTKs (Whittaker et al. 2013). Tumour cells express multiple RTKs that integrate extracellular signals to modulate intracellular events feeding multiple cascades that will affect the fundamental MAPK and PI3K/AKT pathways, and influence proliferation and survival. Therefore, up-regulation of RTKs and increases in soluble RTK ligands and growth factors are all described mechanisms to reduce innate drug sensitivity and drive acquired resistance. Critical RTKs that are implicated in reducing response to treatment due to activation or up-regulation include MET, IFG-1R (Villanueva et al. 2010), EGFR and PDGFRbeta (Wilson et al. 2012). In melanoma, the expression of the RTK ligand HGF is a well-known driver of poor response (Straussman et al. 2012).

Both the expression and loss of MITF, the critical melanoma lineage survival factor, are additional mechanisms of resistance to BRAF pathway inhibition. MITF

plays a central role in ensuring melanocyte survival, and regulates multiple critical survival and antiapoptotic genes (Levy et al. 2006).

Different MITF expression levels have been linked to distinct melanoma tumour and cell behaviour, where high levels mediate differentiation, moderate levels promote proliferation, and low/absent levels drive a more invasive phenotype (Levy et al. 2006). Therefore, the multifaceted role of MITF as a consequence of varying expression levels, with seemingly opposing effects, is further mirrored during the development of resistance. Specifically, overexpression of MITF reduces the therapeutic effect of BRAF inhibitors (Johannessen et al. 2013; Smith et al. 2016; Haq et al. 2013; Van Allen et al. 2014), MEK inhibitors (Smith et al. 2013) and combination treatments (Shi et al. 2014) via increased cAMP pathway signalling (Johannessen et al. 2013).

However, at the other end of the expression spectrum, loss of MITF is also a common occurrence in acquired resistance (Muller et al. 2014). Critically, in cell clones down-regulating MITF, there is an inversely correlated up-regulation of the RTK AXL that enhances tumour resistance. The expression programme in MITF low/AXL high tumours is implicated in driving the resistant phenotype and has now been found to exist in a continuum in subsets of tumour cells that may otherwise express a predominating "AXL low" expression programme that would predict response. A recent study has found the expression programme observed in "MITF low/AXL high" resistant samples can already be detected at the single cell level in treatment-naïve samples, and these transcriptomic features are subsequently increased in resistant, progressive disease (Muller et al. 2014; Konieczkowski et al. 2014). Additionally, the neighbouring stromal cells also influence the expression programme at the single cell level (Tirosh et al. 2016).

A landmark 2015 study performed transcriptome and methylome analysis of matched melanoma samples taken before treatment and during disease progression. One of the critical findings in this paper is that resistant samples displayed a more homogeneous pattern of expression that was in contrast to heterogeneity shown at the DNA level. The acquired resistance transcriptome correlated with YAP1 enrichment, MET high and LEF1 low expression levels. Moreover, MAPK pathway inhibition with targeted therapies led to direct methylation changes that were therapy time-dependent, affecting key regulatory genes and the transcriptome across the resistant samples. One of the other striking findings in the paper is that in resistant samples, there is a predominance of NF-Kβ inflammation that correlates with monocyte expression and M2 macrophages (tumour associated macrophages), suggesting MAPK pathway inhibition affects the inflammatory context of the tumour. Indeed, as a consequence of the M2 switch in macrophage phenotype, the researchers observed that as MAPK pathway inhibitor resistance arose, there was a parallel increase in $CD8^+$ T cell deficiency, exhaustion of the cell population and down-regulation of the antigen presentation molecules in about half the resistant samples (Hugo et al. 2015). Thus, this shows that the study of resistance must extend beyond DNA analysis to incorporate non-genomic approaches with a focus on the consequences on the tumour as well as on the immune tumour ecosystem. Unravelling these changes impacts the therapeutic options of patients, as this work

shows that when patients progress on targeted therapies they likely respond less to immunotherapy. Providing further evidence of how a deep understanding of the immune context underpins therapeutic choice, there are data to support the up-regulation of PD-L1 and the tumour-infiltrating immune populations in metastatic melanoma before MAPK inhibition are associated with progression and survival (Hugo et al. 2017; Massi et al. 2015).

7 Tumour Cell Extrinsic or Microenvironmental Resistance to RAF Inhibition in Melanoma

Research focus into the mechanisms of drug resistance in melanoma has predominantly centred on properties intrinsic to tumour cells, but disease progression and resistance to targeted therapies is no longer considered an exclusive function of genomic and non-genomic modifications of tumour cells. The importance of the tumour microenvironment in supporting resistance to MAPK inhibition is slowly unravelling, and a more complex, comprehensive interpretation must incorporate knowledge of cross talk between discrete cellular compartments including the tumour and supporting stroma (Tape et al. 2016). As we have already alluded to, recent studies delineate a clear contribution of macrophages and fibroblast-derived factors that are well known to confer resistance to MAPK pathway inhibitors.

Fibroblasts are well-known facilitators of melanoma progression, and there is a bidirectional communication through direct cell-to-cell contact and via the secretion of soluble factors to promote melanoma invasion, survival and growth (Li et al. 2003). In melanoma, an extrinsic, non-cell autonomous signal leading to HGF secretion by the stromal cells may lead to resistance (Straussman et al. 2012). HGF can bind to RTKs that will increase intracellular signalling to drive up-regulation of RAS, and ultimately, reactivation of MAPK pathway (Straussman et al. 2012). Furthermore, HGF is also known to contribute to resistance in human cell lines treated with a BRAF inhibitor by down-regulating the pro-apoptotic response. A more recent paper investigated how genetically unharmed stroma evolves under treatment with targeted therapy. Importantly, they demonstrated that in areas of high stromal density, fibroblasts present a hyperactivation of MAPK pathway that elicits a qualitative change in the tumour matrix via integrin β1 and FAK to induce ERK, providing an early subset of melanoma cells with the capacity to rapidly tolerate treatment (Hirata et al. 2015). Moreover, the effect of fibroblasts may vary in patients who are elderly, as melanoma cells in contact with aged fibroblasts are more invasive (Kaur et al. 2016). A recent study has shown that aged fibroblasts increase the secretion of sFRP2, a β-catenin inhibitor that decreases MITF expression, leading to downregulation of the redox regulator APE1, thus rendering melanoma cells more sensitive to oxidative stress and secondarily driving resistance to BRAF inhibition (Kaur et al. 2016). Together, all these studies demonstrate that melanoma cells and fibroblasts are both under pressure to adapt to targeted inhibitors. Thus, fibroblasts change their matrix providing melanoma

cells with a new microenvironment that will enable adjacent tumour cells to evade therapy at a very early stage.

Additional stromal cells that play a demonstrated role in the development of resistance are macrophages (Ruffell and Coussens 2015). Tumours from patients treated with MAPK pathway inhibitors present an increased density of tumour-associated macrophages. These tumour macrophages secrete the melanoma growth factor TNFα that drives, in an NF-kβ-dependent manner, the expression of MITF, leading to resistance (Smith et al. 2014). Moreover, studies show that combination of MAPK and NF-kβ inhibitors delay the appearance of resistance (Smith et al. 2014). TNFα is known to block apoptosis in cells where BRAF is inhibited, and additionally contributes to melanoma invasion and vascularisation of tumours (Gray-Schopfer et al. 2007). As TNFα and NF-kβ play context-specific roles within cancer progression, it will be critical to further investigate whether the signalling pathways described to promote therapy failure secondary to macrophage infiltration hold true across different cancer types and other targeted therapies. These studies, together with the recent discovery that MAPK pathway inhibition leads to $CD8^+$ T cell depletion, highlight how the pressure exerted by novel MAPK inhibitor therapies not only impact tumour evolution by genomic and non-genomic events, but also shape the behaviour of the supporting connective tissue and immune cell populations and function.

8 Immunotherapies in Melanoma

Another landmark change in melanoma therapy has been the development of antibodies to elicit an antitumor immunologic response (Brahmer et al. 2012; Hodi et al. 2010; Topalian et al. 2012; Kaufman et al. 2013; Mellman et al. 2011; Topalian et al. 2014; Wolchok et al. 2013). These novel therapies target the immune checkpoints that exist in physiological conditions to counterbalance immune activation. In cancer, these critical signalling barriers inhibit the activation of antitumoural immune responses. The inhibition of these checkpoints allows the immune system to target cancer cells and leads to long-term disease control. In melanoma, there is improved survival by targeting the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) checkpoint molecule, the inhibitory T-cell receptor programmed death 1 (PD-1) receptor and the PD-1 ligand PD-L1. The mechanisms of action have been extensively reviewed. Other novel immunotherapies include adoptive T cell therapy, which is based on the isolation and ex vivo expansion of T cells that are tumour-specific (Restifo et al. 2012). This therapy allows the vast expansion of T cells, which are then infused into patients to target tumour cells. However, there is great variability of response to immunotherapies across different cancer types, and even within cancer types where immunotherapy is successful, such as melanoma, lung cancer and renal cell cancer, there is a proportion of patients who never respond. Similarly to targeted therapy patients, these are termed intrinsically resistant patients. There is also a subset of patients who initially respond but later progress, indicating the development of acquired resistance to

immunotherapy. Thus, there are tumours that cannot be detected by the immune system (intrinsically resistant), and tumours where the cells adapt to immunotherapy and progressively outgrow the inhibition (adaptive immune resistance).

9 Intrinsic and Adaptive Resistance to Immunotherapies in Melanoma

Intrinsic resistance arises in cells that present genetic or non-genetic changes that afford the tumour natural resistance, as seen, for example, in tumours that express few molecular changes that are recognised as foreign by the immune system (Snyder et al. 2014; McGranahan et al. 2016). This affords one explanation as to why tumours with fewer mutation loads are less likely to respond to immunotherapy. During tumour evolution, there is a tendency for tumours to lose a proportion of their non-silent mutations, which can potentially lead to a lower ratio of antigenic epitopes, leading to immunoadaptation of tumours (Rooney et al. 2015). One of the necessary elements linked to response is the presence of pre-existing T cells within the tumour. The absence of effector T cells within a melanoma has been linked in humans and mice to melanoma-cell-intrinsic oncogenic activation of the WNT/β-catenin pathway (Spranger et al. 2015; Spranger and Gajewski 2016). Importantly, analysis of pre-anti-PD-1 treatment and post-treatment samples has shown a greater proportion of mutations in the DNA repair gene *BRCA2* in responding patients (Hugo et al. 2017). By contrast, the samples that are intrinsically resistant to anti-PD-1 therapy have upregulation of genes involved in a non-melanocytic, more mesenchymal behaviour (*AXL*, *ROR2*, *WNT5A*, *LOXL2*, *TWIST2*, *TAGLN*, *FAP*), as well as genes involved in angiogenesis and wound healing (Hugo et al. 2017). Curiously, this expression profile significantly overlaps with the expression profile observed in samples that have developed resistance to MAPK pathway inhibition (Hugo et al. 2017), implying convergence of resistance-expression programmes.

Cooperating with the loss of antigenicity and the intrinsic characteristics in the tumour that render them more or less vulnerable to immunotherapy are additional mechanisms arising during immune response that directly inhibit tumour-targeting T cells. For example, resistances can be acquired by new resistance-driving mutations in genes involved in interferon-receptor signalling and in antigen presentation (Zaretsky et al. 2016). Additionally, during the initial phase of the interaction between a tumour cell and an antigen-specific T cell, the T cell produces interferon-γ (Ribas 2015; Peng et al. 2012). Interferon-γ signalling has a dual anti-tumour and immune inhibitor role in this interaction (Shankaran et al. 2001). It first serves as a mediator and amplifier of the immune effect, exerting a chemoattraction role to recruit further leukocytes, macrophages and natural killer T cells, but also has a second effect dampening the immune response to cancer cells by expressing factors aimed to reduce the immune anti-tumour effect (Rooney et al. 2015; Bald et al. 2014), such as indolamin 2.2 dioxygenase (IDO) (Peng et al. 2016). IDO is a critical enzyme that when expressed, interferes with

appropriate T cell function. Most critically, following an interferon-γ stimulus, T cells express the ligand PD-L1 that binds to the PD1 receptor leading to inactivation of T cells (Spranger et al. 2013; Tumeh et al. 2014; Pardoll 2012). Other interferon-γ-dependent checkpoints that have also been described to mediate inhibitory immune loops are most prominently the carcinoembryonic antigen cell adhesion molecule-1 (CEACAM1) that forms heterodimers with TIM-3, an activation-induced inhibition molecule involved in immune tolerance and T-cell exhaustion, to inhibit T cell function (Huang et al. 2015). This interaction is an attractive target for additional immune checkpoint blockade in cancers expressing CEACAM1 and TIM-3 (Huang et al. 2015).

There are additional interferon-independent mechanisms of adaptation to immunotherapy. For example, in some tumours, mutations in cancer cells affecting major signalling pathways per se can also lead to expression of PD-L1 in tumours (Parsa et al. 2007; Mittendorf et al. 2014; Marzec et al. 2008; Atefi et al. 2014; Akbay et al. 2013; Shin et al. 2016). There is also a $CD8^+$ T cell-dependent accumulation of regulatory T cells expressing $FOXP3^+$ that exert an inhibitory influence over the immune response, which is mediated by chemokine release (Spranger et al. 2013). Regulatory T cells are immunosuppressive, downregulate the proliferation and induction of effector T cells and are critical to control autoimmunity or excess immunity. Additionally, tumour specific T cells mounting an immune response during the killing of cancer cells produce the major modulating inflammatory cytokine TNFα, which leads to a decrease in the expression of melanoma-specific genes, involved in melanocyte lineage differentiation, and an increase in the expression of genes that signal dedifferentiation and a biological profile more in keeping with neural crest-derived cells or immature melanocytes (Ribas and Tumeh 2012). In adoptive T cell transfer therapy, melanoma evolves to a less differentiated state that mediates resistance due to TNFα secretion (Landsberg et al. 2012). Thus, the loss of melanoma-specific antigens provides another mechanism of immunoevasion. Furthermore, a seminal recent study shows progressive selection of non-immunogenic clones, a process termed immunoediting, occurs during cancer progression via a T cell-dependent immunoselection process (Matsushita et al. 2012).

Taken together, research shows the fine regulatory relationships aimed to deliver accurate immune responses to infection and limit excess cytotoxicity in homeostasis are co-opted to the tumours' advantage in cancer.

10 Conclusions and Future Avenues

There is a rapid changing landscape of therapies for advanced melanoma patients. Current efforts aim to enhance the efficacy of existing targeted and immunotherapies, by understanding the biology driving response and resistance to guide therapeutic care. One of the challenges is identifying the first line therapies or combinations of therapies that will lead to more prolonged clinical responses, taking into consideration the genetic and immunological differences in each patient.

Moreover, in this rapidly changing landscape of novel therapies, new strategies to overcome resistances are currently underway. For example, promising new drugs block the reactivation of MAPK pathway in both BRAF and NRAS tumours, without driving the paradoxical activation of the MAPK pathway (Girotti et al. 2015). Another novel approach to overcome resistance includes the disruption of mitochondrial biogenesis using the small-molecule HSP90 inhibitor gamitrinib (Zhang et al. 2016). New drug discovery efforts have also found encouraging results using compounds triggering ER Stress in MAPK inhibitor resistant cells (Cerezo et al. 2016). For novel immunotherapies, understanding the activation levels of significant checkpoint molecules and the immunosuppressive context within each tumour sample will aid physicians select appropriate therapies or combinations of therapies to improve survival.

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