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Cell-state dynamics and therapeutic resistance in melanoma from the perspective of MITF and IFN γ pathways

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Abstract

Targeted therapy and immunotherapy have greatly improved the prognosis of patients with metastatic melanoma, but resistance to these therapeutic modalities limits the percentage of patients with long-lasting responses. Accumulating evidence indicates that a persisting subpopulation of melanoma cells contributes to resistance to targeted therapy or immunotherapy, even in patients who initially have a therapeutic response; however, the root mechanism of resistance remains elusive. To address this problem, we propose a new model, in which dynamic fluctuations of protein expression at the single-cell level and longitudinal reshaping of the cellular state at the cell-population level explain the whole process of therapeutic resistance development. Conceptually, we focused on two different pivotal signalling pathways (mediated by microphthalmia-associated transcription factor (MITF) and IFN γ) to construct the evolving trajectories of melanoma and described each of the cell states. Accordingly, the development of

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Author contributions

All authors made substantial contributions to researching data for the article, discussions of content and writing and reviewing and/or editing of the manuscript before submission.

Competing interests

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therapeutic resistance could be divided into three main phases: early survival of cell populations, reversal of senescence, and the establishment of new homeostatic states and development of irreversible resistance. On the basis of existing data, we propose future directions in both translational research and the design of therapeutic strategies that incorporate this emerging understanding of resistance.

Introduction

Melanoma, the most deadly malignancy of the skin, has been associated with steadily decreasing mortality despite the rising incidence of this disease over the past few years¹⁻⁴. The reason behind this encouraging phenomenon is the greatly improved prognosis of patients with stage IV (metastatic) melanoma owing to the two major therapeutic breakthroughs made within the past 10 years: targeted therapy⁵⁻⁷ and immunotherapy⁸⁻¹⁰. These two treatment options, however, are not curative for most patients with metastatic melanoma owing to the rapid development of acquired resistance to targeted therapy¹¹ and the predominant innate resistance to immunotherapy^{8,12}.

Accumulating evidence indicates the existence of two subpopulations of melanoma cells that contribute to resistance to targeted therapy and immunotherapy¹³⁻¹⁵, which are both characterized by slow cell cycle activity, a de-differentiated state and invasiveness¹⁶⁻¹⁹. The first insights into the presence of these cells precede the current era^{20,21}. Accordingly, two different models have been developed to explain the biology of these subpopulations, namely, the cancer stem cell (CSC) model and the microphthalmia-associated transcription factor (MITF)-rheostat phenotype switching model.

In the CSC model, melanoma cells are hierarchically organized and can differentiate from CSCs to progenitor cells and then to terminally differentiated melanoma cells without the option of dedifferentiating in the opposite direction^{22,23}. CSCs contribute to multidrug resistance, to cell survival under various stress conditions and to the establishment of a new drug-resistant heterogeneous melanoma cell population^{24,25}. Thus, unique features of CSCs could be targeted to eradicate these cells and overcome resistance to therapies that successfully eliminate the more differentiated cell population. To date, early attempts to target one such marker, CD20, were terminated owing to lack of efficacy ([NCT01032122](#) and [NCT01376713](#)).

In the MITF-rheostat model, melanoma cells are horizontally organized and their phenotypes, namely, proliferative with high levels of MITF expression (MITF^{hi}) or invasive with low levels of MITF (MITF^{low}), are interchangeable²⁶⁻²⁸. In this model, senescent subclones with extremely high or low levels of MITF expression contribute to therapeutic resistance²⁹. This MITF-centric model does not, however, intrinsically provide an obvious target for drug development.

To reconcile these two models and to direct the development of future therapies, we propose a new model that combines the dynamic oscillation of cell states at the single-cell level with constant reshaping of the cell ensemble at the population level. In this model, the dynamic

fluctuation of cell states provides a reservoir of cells for tumour restructuring, which contributes to the development of therapeutic resistance.

Evidence obtained over the past few years supports a crucial role for IFN γ and its related pro-inflammatory cytokines (for example, TNF)^{30,31} in the therapeutic efficacy but also in the development of resistance to T cell-activating immunotherapies^{19,32,33}. Indeed, cumulative data suggest that both IFN γ and TNF can induce therapy resistance via a persist cell state characterized by dedifferentiation^{34,35}. Thus, we focus on IFN γ -JAK1/2-signal transducer and activator of transcription (STAT)1/STAT3 pathway (referred to herein as the IFN γ pathway) as the master regulator of the whole cytokine network³⁶ in order to illustrate the dynamic fluctuating cell-state model in the development of resistance to immunotherapy. Furthermore, we integrate the modulation of melanoma biology by both MITF and IFN γ in a model that explains resistance to the major therapeutic modalities used to treat patients.

Dynamic fluctuation of protein expression

At the single-cell level, the expression of a given protein fluctuates dynamically around a predetermined homeostatic level³⁷ (FIG. 1a), which is probably pre-set according to intrinsic epigenetic mechanisms, gene regulatory networks (a multiplicity of binary regulatory events) and extrinsic factors³⁸. Protein expression levels vary substantially both longitudinally within the same cell (temporal variation) (FIG. 1a) and between different cells at a given time point (spatial variation)³⁹ (FIG. 1b), contributing to the heterogeneity of the entire cell population.

The reasons contributing to this highly dynamic variation in protein expression could be either stochastic (owing to noise) or deterministic⁴⁰. Stochastic variability in protein expression can arise from random patterns of gene expression, gene location and randomness in transcription and translation, all resulting from differences in chromatin remodelling, the number of transcription factor binding sites and the availability of cofactors, as well as from intracellular biochemical fluctuations, such as cytoskeleton rearrangements, metabolic state, protein localization and interactions, natural variation in the concentrations of key reactants and post-translational modifications⁴¹⁻⁴⁴. Variation in protein expression owing to noise alone is thought to be generally small and transient in non-malignant cells^{45,46} but is higher in malignant cells, especially at metastatic stages^{37,42,47}, indicating that heterogeneity in protein expression is higher in tumour cells than in non-malignant cells. Deterministic protein variation occurs in response to signals from other cellular components of the microenvironment that are mediated by cytokines and cell signalling pathways^{38,45}. The joint contribution of stochastic variation and deterministic variation can result in substantially increased variability in protein levels.

At the cell-population level, the expression of a given protein fits a log-normal distribution (FIG. 1b) and can be influenced by external factors, such as drug exposure⁴³. This perturbed protein state can be transmitted from mother to daughter cells transiently, giving rise to temporally stable non-genetic, phenotypic switching^{41,44,48}; however, stochastic single-cell variation promotes rapid phenotypic divergence between sister cells after several cell

divisions^{41,44,48}, leading to either the previous or a new heterogeneous homeostatic state, in an extracellular-dependent way⁴⁹. The mechanisms driving the tendency towards a particular homeostatic state can include, but are not limited to, cell subclonal cooperativity, evolutionary advantage, cell–cell physical communication, paracrine effects and microenvironment remodelling⁴⁰.

The state of a cell is mostly determined by the expression levels of different proteins⁵⁰, which is a continuous quantitative variable. At any given time point, the cellular state is a continuous spectrum as opposed to fitting into artificially defined categories. This concept can be well illustrated by reversing the distribution of variation in protein expression at the cell-population level (FIG. 1b) along the horizontal axis, such that the cell state (determined by the levels of protein expression) is shown as a continuous variable along the *x* axis, whereas the likelihood of cells to fluctuate between a given temporary cell state and other cell states is plotted along the *y* axis (FIG. 1c). In this chart, differences in the state of an individual cell are directly related to instability: outlier cells are the most unstable and thus are most likely to revert back to the bulk-population cell state⁵¹ (FIG. 1d). Presumably, somatic genetic variances lead to the existence of a different spectrum of expression of given proteins, contributing to the massive heterogeneity of tumour cells.

MITF and resistance to targeted therapy

MITF plays a pivotal role in melanoma

MITF, which has been described to regulate the fluctuation between an invasive and a proliferative phenotype in melanoma cells^{26–28}, illustrates how a single protein can serve as a marker of a particular cell state. Melanomas are derived from transformed melanocytes⁵², whose primary role is to produce melanin, a pigment that shields adjacent keratinocytes from ultraviolet radiation⁵³. MITF is the master regulator of differentiation of neural crest cells into melanocytes and of the expression of pigmentation-related genes (encoding pigment-producing enzymes and other factors involved in the maturation and export of melanin), such as *TYR*, *TYRP1* and *DCT*⁵⁴ (Supplementary Fig. 1). Relative to melanocytes, *MITF* expression levels are maintained in most melanomas⁵⁵, amplified in 5–20% and mutated in a subset of familial melanomas⁵⁶. The results of an in vitro study suggest that *MITF* can be considered as a lineage-specific oncogene in most melanomas^{57,58}. Pigmentation-related genes have been documented to be recognized by T cells⁵⁹ and thus contribute, at least in part, to the highly immunogenic nature of melanoma. Accordingly, vitiligo has been observed as a sequela of effective immunotherapy in patients with melanoma^{60–62}.

MITF also maintains the homeostasis of melanoma cells, in which it is a pivotal regulator of cell growth through mechanisms including upregulation of cell cycle-related genes (such as *CDK2*, *CDKN1A* and *CDKN2A*) and cell survival via upregulation of anti-apoptotic genes (such as *BCL2* and *BCL2A1*) (Supplementary Fig. 1). MITF can also inhibit angiogenesis and a proliferative-to-invasive phenotypic switch, both of which are correlated with resistance to targeted therapy^{63–65}. In general, the MITF^{hi} subgroup of melanoma cells is considered to have a proliferative or differentiated phenotype, whereas MITF^{low} melanoma cells are invasive, dedifferentiated or senescent. Of note, melanoma cells with extremely

high levels of MITF expression can lose their proliferative properties and revert to a senescent, paradoxically differentiated, status^{29,66}.

MITF regulation in melanoma progression

Owing to its pivotal roles in melanoma, the expression of MITF is tightly regulated. A detailed description of the MITF-regulating network is beyond the scope of this article but is depicted in Supplementary Fig. 2 and has been described elsewhere^{29,67,68}. Under the influence of extracellular signalling pathways (including those commonly activated by mutations in *BRAF*, the most commonly activated oncogene in melanoma^{29,69}), natural selection and other factors, this elegant gene regulatory network maintains a predetermined homeostatic level of MITF expression^{70,71} during melanoma progression; this pre-set level ensures that the phenotype of most melanoma cells is optimally balanced between invasiveness and proliferation, and thus the cells thrive (FIGS 2,3). In this context, exposure to a BRAF-targeted agent can lead to rewiring of the MITF gene regulatory network, affecting its downstream network and leading to a new homeostasis (FIG. 3).

Development of resistance to targeted therapy

The conflicting CSC^{22,23} and MITF-rheostat phenotype-switching²⁹ models were developed on the basis of bulk-cell analyses that reflected the ensemble cell phenotype and behaviour or averaged results of whole melanoma cell populations⁷², which differ from those of any individual cell⁷³ (FIG. 1d). If we instead consider resistance from the single-cell perspective, these two theories can be reconciled.

Single-cell RNA sequencing data indicate that MITF expression levels vary continuously from cell to cell (spatial continuum)¹³, indicating the existence of a continuous spectrum of cells with different states at a given time point. In the absence of therapy, some melanomas have a skewed cell population in terms of MITF expression levels, generally with a predominance of MITF^{hi} cells. The expression of MITF within a given single cell also follows the dynamic fluctuation model and varies longitudinally owing to either stochastic⁷⁴ or deterministic mechanisms. In the entire melanoma cell population, owing to the exquisite gene regulatory network (Supplementary Fig. 2), MITF expression levels vary across a spectrum¹³ but are maintained within a narrow dynamic range in the vast majority of melanoma cells (FIG. 2a). Meanwhile, even before the initiation of therapy, stochastic or deterministic factors (extracellular signals or intracellular genetic alterations) enable melanoma cells to establish outlier phenotypes with extremely high or low expression of MITF, which are associated with cellular behaviours that differ from those of the bulk melanoma cell population and serve as reservoirs of fitness-enhanced cells that can resist eventual exogenous stress or adverse impacts, such as chemotherapy or targeted therapy^{14,29,45,46,75–79} (FIG. 2a,b). MITF levels have to be maintained at a certain level in order for melanoma cells to proliferate and, thus, the outlier cells are marked by senescence. Noticeably, owing to its complex regulating network, MITF can be autoregulated by counteracting extrinsic noise and return to homeostasis after induced stress^{46,80,81}.

Heterogeneity in MITF expression exists even within outlier cells^{13,76}, the most clear example being MITF^{low} cells, which generally have an invasive phenotype (persister cells).

Historically, some of these persister cells were qualified as CSCs owing to high expression levels of certain CSC markers^{82–84}. The expression of CSC markers tends to fit a bimodal log-normal distribution reflecting a population of stem-like cells with high expression of CSC markers and a different population of highly dedifferentiated cells that tend to express barely any known CSC or mature-melanocyte lineage markers^{34,66,85}. Similar to MITF, the expression of CSC markers dynamically fluctuates, and thus CSCs do not constitute a static clone with hard-wired differences determined by unique somatic genetic alterations⁴⁸ but rather reflect transcriptional states combined with underlying genetic heterogeneity that persist over time — that is, CSCs comprise a dynamic population of different individual cells at different time points⁸⁶. This heterogeneity provides an explanation for the failure to prevent resistance in preclinical studies in which known CSC biomarkers (such as *NGFR* or *KDM5B*) were silenced¹⁷, along with other fruitless efforts to target CSCs in clinical studies in patients with melanoma (NCT01032122 and NCT01376713).

In general, spatial heterogeneity provides cell diversity that facilitates Darwinian selection during melanoma development and enables different cells to react differently to Lamarckian induction. Owing to longitudinal oscillation in the levels of different proteins, which contributes to the constant temporal fluctuation of cell states at the single-cell level³⁹, this process is highly dynamic. As a result, even within the same cell subgroup, individual cells will have different states at different time points. Treatment with a targeted agent, for example, a MAPK inhibitor, will reset the MITF homeostatic point, leading to a MITF expression-defined cell distribution that shifts either towards lower levels, higher levels or both directions in a context-dependent manner⁸⁷ (FIGS 2,3). The mechanisms driving this shift in MITF expression include Lamarckian induction (owing to BRAF being an upstream regulator of MITF; Supplementary Fig. 2) and/or Darwinian selection^{88,89}. At the level of the whole-cell population, a phenotype switch takes place (FIG. 2c,d).

At the single-cell level, the level of MITF expression is an indicator of MAPK pathway dependency. Median levels of MITF expression indicate pathway dependency, whereas either extremely high or low levels are associated with resistance to MAPK inhibitors^{29,90,91}. The MITF^{extremelyhi} subset of melanoma cells is the most differentiated and least addicted to MAPK signalling and is, therefore, associated with intrinsic resistance to MAPK inhibitors, whereas the MITF^{low} population comprises dedifferentiated, invasive and apoptosis-resistant cells, programmed to be persisters capable of surviving harsh environmental conditions, such as hypoxia, a lack of nutrients, targeted therapy and/or immunotherapy^{17,75,92}. A shift in the distribution of MITF expression levels results in an expansion of the therapy-resistant outlier populations (FIG. 2a,b versus FIG. 2c,d). During treatment with targeted therapy, those surviving outliers serve as a reservoir cell population in which de novo epigenetic and genetic aberrations lead to the development of irreversible resistance, ultimately giving rise to a new melanoma cell population resistant to targeted therapy^{92,93} (FIG. 3j,h). The results of preclinical studies suggest that even the persistence of only one single cell⁹⁴ after different types of therapy can eventually give rise to a heterogeneous population and establish a new homeostasis⁹³.

IFN γ and resistance to immunotherapy

IFN γ network in melanoma

The crucial components of IFN γ signalling are context-dependent, varying greatly between different cancer types and between different mutational contexts even within the same tumour type⁹⁵. Herein, we focus solely on melanoma cells and on a few elements of the JAK–STAT signalling network (IFN γ –JAK1/JAK2–STAT1/STAT3 in this article), which are essentially activated by IFN γ and TNF, both of which have been shown to be simultaneously released by immunotherapy-reinvigorated T cells^{31,96}. A detailed description of IFN γ pathway components in melanoma is beyond the scope of this article but has been depicted in FIG. 4 (REFS^{97,98}).

IFN γ and immune response of melanoma

The IFN γ pathway is centrally involved in the regulation of diverse biological functions of melanoma cells, which could be roughly divided into immune-pertinent aspects (such as antigen processing and presentation, cytokine production and immune-checkpoint molecule expression) and those concerning the cell-autonomous functions of melanoma cells (for example, cell proliferation, growth, apoptosis and differentiation). IFN γ has long been considered as the primary mediator of the immune response to melanoma cells owing to its capacity to increase the expression of proteins involved in the antigen-processing machinery (such as components of the immunoproteasome⁹⁹, MHC class Ia molecules, antigen peptide transporter 1 (TAP1), TAP2, several IFN γ -inducible proteasome subunits, tapasin and β 2-microglobulin^{100–104}), as well as pro-inflammatory cytokines that attract T cells, such as CXCL9, CXCL10 and CXCL11 (REF.¹⁰⁰).

Counteracting the immune response

As the key regulator of immune response in melanoma, an important intrinsic function of IFN γ is to prevent severe immune overactivation by exerting immune inhibitory functions⁹⁹. One of the inhibitory mechanisms regulated by the IFN γ pathway is the upregulation of non-classical MHC class Ib molecules^{105,106}, most notably HLA-E and HLA-G, which inhibit both natural killer (NK) cells and T cells¹⁰⁷. The expression of MHC class II molecules^{106,108–110}, which are negatively correlated with melanoma cell differentiation¹¹¹ and can protect melanoma cells from apoptosis through a lymphocyte-activation gene 3 (LAG3)-dependent mechanism¹¹², is also upregulated upon IFN γ signalling. Very high levels of MHC class Ia molecules could also impair immune responses. In the B16 mouse model, the elimination of melanoma cells by cytotoxic T lymphocytes (CTLs) targeting tyrosinase-related protein 2 is impaired in response to IFN γ -dependent induction of high levels of noncognate MHC class I molecules, which probably compete with cognate MHC molecules³¹. The IFN γ pathway also has an immunosuppressive role through the upregulation of immune-checkpoint molecules including cytotoxic T lymphocyte antigen 4 (CTLA-4)^{105,113}, programmed cell death 1 ligand 1 (PD-L1)^{114–119}, PD-L2 (REF.¹⁰⁴), indoleamine 2,3-dioxygenase (IDO)^{114,120}, carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)¹²¹ and possibly TIM3 (also known as HAVCR2) and LAG3 (REF.¹¹³). A third inhibitory mechanism involves STAT3-dependent inhibition of the production

and sensing of inflammatory signals in melanoma cells, leading to the impairment of dendritic cell maturation¹²².

Cell-autonomous functions in melanoma

In most contexts, IFN γ exerts an antiproliferative effect on melanoma cells^{100,101,110,111,123–126} by inducing G1 cell cycle arrest through mechanisms related to the regulation of p27^{Kip1} (also known as CDKN1B), CDK6, cyclin A, cyclin E and miR-29 family members^{127–129}. IFN γ -induced cell cycle arrest has long been known to be associated with increased invasiveness of melanoma cells. Early data showed that IFN γ successfully impairs the proliferation and increases the metastatic ability of B16 mouse melanoma cells¹²⁴; this process was positively correlated with in vitro invasiveness^{27,130} and with downregulation of MITF and several downstream melanocyte differentiation antigens¹³¹ and leads to impaired recognition of melanoma cells by CTLs^{110,111,132,133}. The mechanisms underlying this process include the inhibition of canonical WNT signalling and the activation of the JUN and p38 pathways¹³² (probably through activation of STAT1)¹³⁴. These changes resemble those observed during the generation of persister cells in response to targeted therapy¹³⁵.

Clinical evidence confirming the effect of IFN γ on the metastatic potential of melanoma cells came from a randomized phase III clinical trial of IFN γ as the adjuvant treatment of patients with high-risk melanoma, who had less favourable disease-free survival (DFS) and overall survival outcomes than patients in the observation arm¹³⁶. Correlative evidence was reported years later, indicating that the presence of detectable IFN γ levels in plasma was an independent prognostic factor of shorter DFS¹³⁷.

IFN γ pathway reshaping in melanoma

IFN γ has important roles in melanomagenesis¹³⁸, especially with regard to pro-survival and immune-evasion effects involved in ultraviolet radiation-induced melanomagenesis^{105,139}. Thus, IFN γ pathway signalling needs to be maintained at a certain level in the vast majority of initially activated melanocytes and newly transformed melanoma cells (FIG. 3). Subsequently, a nascent melanoma undergoes constant interaction with the host immune system, which promotes multiple processes that favour immune escape during evolution to a clinically evident melanoma^{140,141}.

As discussed, IFN γ acts as a double-edged sword in melanoma, and thus maximal melanoma fitness would favour the presence of either low or high levels of activity in order for melanoma cells to evade recognition and elimination by T cells. In any particular melanoma, the presence of one or both subgroups (hypoactivated and hyperactivated IFN γ pathway) and the dominance of one of them are context-dependent. Very low or no activation of the IFN γ pathway leads to a lack of MHC molecules and pro-inflammatory cytokines, favouring NK cell recognition while providing melanoma cells opportunities to evade T cell surveillance. Conversely, very high activation levels of this pathway lead to overexpression of both classical and non-classical MHC molecules, upregulation of immune checkpoints and melanoma cell dedifferentiation. Both situations (FIG. 3c,d) result in a lack of immune response, which provides survival fitness in the face of host immune

surveillance^{99,126,142–147}. This immune selection process leads to the bifurcation of the distribution curve of IFN γ activation status in melanoma cells¹⁴⁸ (FIG. 3c,d).

Development of resistance to immunotherapy

Similar to the correlation between the bifurcation of MITF expression and resistance to targeted therapy, melanoma cells resistant to immunotherapy can be divided into two subcategories. The first one is associated with hypoactivation of the IFN γ pathway^{149,150}, which thereby has a lack of immunogenicity that makes it innately resistant to immunotherapy. The second one has hyperactivation of the IFN γ pathway, which overlaps with a dedifferentiated and invasive phenotype; this population reflects a stress-induced persister status^{15,35}. As with targeted therapy, resistance to immunotherapy is caused by both Darwinian selection and Lamarckian induction^{19,151–153}. Overall, Darwinian selection primarily contributes to the enrichment of the IFN γ hypoactivation subgroup, whereas Lamarckian induction favours the hyperactivation state.

The cell subgroup with IFN γ hypoactivation can be divided into two subcategories on the basis of responsiveness to IFN γ released by T cells reinvigorated by immune-checkpoint inhibition (ICI). The IFN γ -responsive subpopulation, in which ICI-induced activation of IFN γ increases immunogenicity, is recognized by T cells and then eliminated. The IFN γ -non-responsive subpopulation, which has previously been reported as being characterized by homozygous loss-of-function mutations in *JAK1* and *JAK2*, genomic loss of *IFNGR1* and/or *IFNGR2* and amplification of *SOCS1*, is present before exposure to immunotherapy^{33,154–156}, survives and is, therefore, enriched after initiation of therapy. In cells with genetic loss-of-function of IFN γ resulting in hypoactivation, the lack of reactive PD-L1 expression prevents ICI from activating T cells at all¹⁰⁰. In addition, the lack of expression of MHC class I further enables melanoma cells to evade recognition by T cells^{33,101} and impairs T cell infiltration into melanomas, which is a prerequisite for a response to the currently available immunotherapies¹⁰⁰. Importantly, the IFN γ pathway can also be silenced through epigenetic modifications, providing a rationale to explore combinations of epigenetic modulators with immunotherapy^{155,157}. During the enrichment of melanoma cells with non-responsive hypoactive IFN γ signalling, both genetic and epigenetic aberrations continue to accumulate owing to chromosomal instability in melanoma cells, leading to a new homeostatic cell state (FIG. 3g). Of note, the mechanism underlying hypoactivation of IFN γ pathway that enables melanoma cells to escape immunotherapy aligns with the immunological roles of the IFN γ pathway; whether this subgroup of melanoma cells is proliferative and MITF^{hi} (as anticipated) remains to be determined.

The mechanism underlying effective ICI involves the use of immune checkpoints against immune clearance in melanomas with a hyperactivated IFN γ pathway; the blockade of these checkpoints reinvigorates certain groups of T cells to initiate an immune response against melanoma. As described, treatment with anti-programmed cell death 1 (PD-1) antibodies induces the expansion of CD8⁺ T cell populations with an exhausted phenotype, whereas anti-CTLA-4 treatment induces an additional expansion of an ICOS⁺ T helper 1 (T_H1) cell-like CD4⁺ (activated) T cell population¹⁵⁸. Meanwhile, immunosuppressive regulatory T

(T_{reg}) cells are also inhibited primarily by CTLA-4 blockade^{159–161}. The activated effector T cells release large amounts of IFN γ and TNF^{96,156,162}, inducing apoptosis, antigen processing and presentation, secretion of various chemokines and upregulation of PD-L1 and/or PD-L2 (REF.¹⁰⁴), which can then be neutralized by ICI¹⁵⁵, further facilitating melanoma cell clearance. Accumulating preclinical evidence has shown that, in some scenarios, this additional release of IFN γ and TNF (which has stress-related effects¹⁰¹) will push the already IFN γ -hyperactivated cells into more extreme states, thus leading to the induction of a persister status in melanoma cells^{152,155,163}.

Early evidence suggested that recombinant IFN γ could stimulate shedding and suppress expression of nevus-associated antigens¹⁶⁴. Further evidence came from adoptive cell transfer strategies targeting melanocytic differentiation antigens (MART-1 or PMEL (commonly referred to as gp100)), which predominantly inhibited tumour growth via cytostatic effects induced by increased secretion of IFN γ and TNF (REFS^{35,127}). An inflammation-associated reversible, invasive, senescence-like dedifferentiation phenotype was induced by in vitro treatment with IFN γ and TNF (REF.³⁴), together with the upregulation of *NGFR* and genes associated with an invasive phenotype, such as *AXL*, *JUN*, *WNT5A* and *EZH2* (REFS^{127,165–170}). Similar phenomena have been reported in the context of resistance to ICI^{160,168,171}. Data from studies reported over the past few years reinforce this phenomenon by showing that inhibition of JAK1/JAK2 by ruxolitinib overcomes resistance of immunotherapy in a mouse model of melanoma¹⁵⁵.

Three phases of therapeutic resistance

We propose a model in which therapeutic resistance to both targeted agents and ICI comprises three different phases: early survival of cells with innate resistance or induced persister cells, reversal of senescence and, finally, establishment of irreversible resistance. These three phases overlap with the dynamics of the bulk-cell population, yet the single-cell characteristics are enriched — and will become dominant — over time. On the basis of current knowledge, drawing a clear boundary between different resistance phases is difficult, in part owing to the very limited amount of tumour samples obtained throughout the course of therapy and the paucity of studies in animal models using single-cell fate mapping technologies. A deeper characterization of these resistance phases would facilitate in vivo monitoring in animals and patients, an understanding of the influence of different cell populations on one another and mechanistic studies aimed at identifying novel vulnerabilities.

Phase 1: early survival

Two subgroups of melanoma cells resistant to targeted therapy and immunotherapy, respectively, exist: innate resistant ones, namely, MITF^{extremelyhi} cells that are resistant to targeted therapy and cells with IFN γ hypoactivation that are resistant to immunotherapy (FIG. 3i,j); in addition, two groups of induced persisters that express senescence markers^{172–175} exist: MITF^{low} cells that are resistant to targeted therapy and cells with IFN γ hyperactivation that are resistant to immunotherapy^{34,77} (FIG. 3f).

During the early survival phase, which lasts from days to weeks in vitro, no new epigenetic or genetic aberrations occur^{17,176}. Instead, this phase is mainly driven by dynamically fluctuating gene and protein expression in both persisters and cells with innate resistance. Accordingly, drug resistance in persisters has been shown to be completely reversible. Indeed, a transiently pre-resistant cell state matching our definition of persister was proved to exist in studies that used Luria–Delbrück fluctuation analysis¹⁷⁶, but whether innate resistant cells can be shifted towards drug-sensitive states remains to be determined.

To target cell subpopulations with innate resistance to either targeted therapy or immunotherapy, switching to another therapeutic modality is a reasonable choice. For persisters, however, accumulating data suggest that this cellular state is a pre-programmed senescent state that enables cells to survive multiple stresses including hypoxia¹⁷⁷, oxidative stress¹⁷⁸, nutrient deprivation¹⁷⁹, inflammation¹⁸⁰, radiotherapy¹⁸¹, chemotherapy^{182,183}, targeted therapy^{173,184} and T cell-activating immunotherapy¹⁵², thus contributing to cross-resistance to different therapies. A detailed description of the rewiring of key signal transduction pathways in persisters is beyond the scope of this article; briefly, the main characteristics of this senescent persister subpopulation are the activation of MAPK bypass pathways³⁴ as well as induction of an inflammatory network. More specifically, the activation of JAK1–STAT3 is commonly observed in persister cells and leads to the further upregulation of multiple receptor tyrosine kinases (RTKs) and other bypass signalling pathways^{34,64}, as well as the activation of multiple pro-inflammatory molecules (including TNF (REF.¹⁸⁵), NF- κ B, TGF β and IFN γ) with MITF-inhibitory effects (Supplementary Fig. 2) — thus, this population has a dedifferentiated, MITF^{low}, invasive phenotype.

During the early survival phase, the persister cell population does not remain constant but expands over time through induced subpopulation-specific secretome changes, which stimulate cell outgrowth and dissemination¹⁸⁶ by turning adjacent melanoma cells into persisters¹⁸⁷. The mutual positive feedback between melanoma cells, particularly mediated by the secretome of persister cells, and other cells in the tumour microenvironment (TME)^{188,189} is also important. The paradoxical MAPK inhibitor-induced activation of tumour-promoting cellular components within the TME^{190–192} reshapes the TME into a resistance-promoting niche; in turn, this niche induces drug resistance by activating MAPK bypass pathways in melanoma cells (such as PI3K–AKT¹⁹³ or STAT3 (REF.¹⁸⁷)) or by releasing supportive cytokines (such as IL-1 β , IL-6 and CCL2)^{194–197}. This process facilitates the existence of spatially clustered resistant persister cell populations, which serve as a cell reservoir for the future development of clones with irreversible therapeutic resistance. Although less dominant in the resistant subpopulation of melanoma cells in patient samples obtained early after initiation of targeted therapy¹⁹⁸, persisters become more prevalent in samples obtained during clinical disease progression⁷⁹.

To summarize, after the initial hit of targeted therapy or immunotherapy, three different subgroups of melanoma cells can remain: MITF^{extremelyhi} cells with innate resistance to targeted therapy, cells with IFN γ hypoactivation and innate resistance to immunotherapy and MITF^{low} persisters with IFN γ hyperactivation (FIG. 3), although only one or two subgroups might exist within a given tumour. As the external stress of therapy persists, these three different subgroups gradually enter the second phase of resistance development.

Phase 2: reversal of senescence

In this section, we focus on MITF to depict the changes that persister cells undergo during the second phase of therapy resistance because MITF-related epigenetic and genetic rewiring has been better described than IFN γ -related mechanisms. Similar phenomena could be depicted by describing the activation of the IFN γ pathway in response to immunotherapy but are beyond the scope of this article.

The MITF^{extremelyhi} subgroup, which remains senescent during phase 1 (REF.¹⁹⁹), will similarly develop reactivation of the MAPK pathway to restore proliferation during phase 2 (REF.³⁴). As discussed, MITF levels have a tendency to revert back to their heterogeneous homeostatic range. In this phase, after a persistent exposure to MAPK inhibitors, de novo epigenetic and genetic aberrations start to occur and accumulate in a time-dependent manner^{14,79}, enabled by a drug tolerance-facilitating TME¹⁹¹ and leading to a rewiring of the MITF-regulating system, which in turn gradually leads to a new MITF homeostatic status. As MITF expression shifts from the extremes back towards a new homeostatic point, melanoma cells with a senescent phenotype gradually subside and are substituted by a new subgroup with a proliferative phenotype — these changes are associated with the phenomenon of clinical progression⁷⁹. Of note, senescent persisters and/or MITF^{extremelyhi} cells do not disappear but rather remain present in small numbers.

This phase is the transitory period of time that leads to the establishment of a new homeostasis (phase 3). In terms of targeted therapy, in vitro, this phase generally starts after several weeks of exposure to MAPK inhibitors and probably after a longer time in the setting of immunotherapy, but no clear end time boundary exists. In general, phase 2 is highly dynamic, and persister-related resistance is mostly reversible in the early stage²⁰⁰ and mostly irreversible at later stages.

Phase 3: new homeostasis

In phase 3 of our model of drug resistance, after months to years of exposure to MAPK inhibitors or T cell-activating immunotherapy, the accumulation of both epigenetic and genetic aberrations and the modulation of the TME and melanoma cell secretome¹⁸⁶ finally result in the establishment of a new homeostasis and of irreversible resistance, which has been shown in vitro to be associated with a high degree of tumour heterogeneity²⁰¹. One of the most conspicuous features of heterogeneity is that persisters induced by targeted therapy and immunotherapy seem to follow different trajectories (FIG. 3g,h). Specifically, patients with disease progression on targeted therapies tend to have less durable remissions and more prompt disease progression than those receiving immunotherapy, some of whom gain durable benefit after reaching a maximal response²⁰². This difference might result from the persistent immune-activating effect of immunotherapy or, rather, from the reshaping of the TME, which probably reduced the fitness of persisters cells, limiting their ability to switch towards a proliferative phenotype by accumulating both genetic and epigenetic aberrations. Thus, although the persister subgroups induced by targeted therapy and immunotherapy initially overlap (as indicated by dedifferentiation, a mesenchymal phenotype, angiogenic features, interferon and inflammatory features and increased expression of CSC biomarkers, immune checkpoints and multiple RTKs)^{15,79,88,166,203–207}, the trajectories of development

of further resistance eventually separate. In the clinic, different progression patterns can be observed when comparing the Kaplan–Meier curves of both progression-free survival and overall survival of patients treated with targeted therapy²⁰⁸ or immunotherapy²⁰⁹, implying that the persister stage might be more vulnerable to therapeutic intervention than that of cells attaining irreversible resistance, which have higher heterogeneity.

In phase 3, some melanoma cells become addicted to MAPK inhibitors and, thus, drug withdrawal provides a new stress^{204,210,211}, which can lead to the inhibition of cell growth and proliferation. This rationale supports the discontinuation of and rechallenge with targeted therapies in patients with melanoma after weeks or months of therapy; however, a concern that remains with this approach is that the increased stress of treatment discontinuation could enrich for persistent cells with higher levels of heterogeneity²¹², diminishing the responsiveness of the remaining cell population to drug rechallenge. Indeed, therapeutic rechallenge following the development of acquired resistance and a treatment break has been shown to lead to responses of lower magnitude and shorter duration than those reported for the initial treatment²¹³, and thus this strategy should be generally reserved for patients with disease progression on targeted therapy in which irreversible cell states are likely to predominate.

Whether some melanoma cells become addicted to immunotherapy-enhanced expression of both IFN γ and TNF remains to be determined. Preliminary clinical evidence indicates that a substantial group of patients remain free from disease progression after immunotherapy discontinuation and for long periods of time, which might result from the near complete elimination of melanoma cells by immunotherapy. Other potential explanations for this observation include the known long half-life of immunotherapy agents, the persistent pharmacodynamic effects long after clearance of these agents (for example, mediated by memory T cells) and remodelling of the TME that limits the proliferation of melanoma cells, among others.

Future directions

The model we have proposed was developed on the basis of observations made in vitro, although attempts have been made to find analogy between the distinct three phases in mouse models and patient samples⁷⁹. These attempts have been limited, however, by the overlapping epigenomic and genomic signatures observed during early survival, reversal of senescence and irreversible resistance, as well as the challenge of obtaining patient samples at time points in relation to the start of therapy when tumour volumes are at a minimum.

Translational research

Correlation between MITF and IFN γ pathway.—One of the most intriguing questions for which answers remain elusive is the direct correlation between the MITF network, the most clearly depicted network in development of resistance to targeted therapy, and IFN γ , its counterpart in resistance to immunotherapy. To date, most of the correlative evidence has been generated indirectly by analysing the properties of persister cells. Whether mechanistic intersections exist between the MITF network and immunotherapy resistance, beyond the involvement of pigmentation-related antigens, remains to be determined. The effect of the

IFN γ pathway on the MITF network (excluding regulation of the persister state) is also elusive. Understanding these interactions will be key to addressing the overlap between cells with innate resistance to targeted therapy and immunotherapy. Ongoing single-cell RNA sequencing analyses of patients with ICI-treated melanoma, in particular, of tumour cells, should provide the required resolution to establish more direct links between IFN γ and MITF.

Description of different phases of development of therapeutic resistance.—

One of the conundrums regarding longitudinal surveillance during therapy with targeted agents or immunotherapy is the failure to correlate different observations from preclinical studies using in vitro assays, animals models and patient samples. The tumour specimens described in the vast majority of published on-therapy analyses are collected in the first few weeks of treatment or at the time of clinical progression. This approach relates to the fact that patients with a clinical response have tumours that have regressed compared with baseline measurements and are less feasibly and safely biopsied for research purposes. To circumvent these limitations, some researchers have tried to describe the evolving phenotypes over longer periods of drug exposure using both in vitro and mouse models^{66,88}. The characteristics and potential therapeutic vulnerabilities of melanomas for which early on-treatment samples have been obtained (when patients are in the midst of a clinical response) cannot be established. Similarly, we do not know what impedes infiltrating CD8⁺ cells (that have effectively contributed to a response in the case of targeted therapy or directly mediated this response in the case of immunotherapies) from being able to eliminate persister cells. Additional challenges face the use of peripheral blood samples to gain these insights. In the near future, the development of more human-relevant, immune-competent mouse models might provide new insights on the dynamics of persisters and the possibility of tracking their resistant biology in peripheral blood. Studies should be carried out with the aim of monitoring cell state switching as well as addressing uncertainties regarding the optimal time points of assessment (for example, 1–2 days, 1–2 weeks, 1–2 months or later time points after initiation of therapy with MAPK inhibitors). Samples from different time points might reflect very different disease biology: simultaneous elimination of sensitive cells and enrichment of resistant populations.

Epigenetic and genetic aberrations are accumulated during long-term exposure to immunotherapy, leading to irreversible resistance and, thus, whether cell subgroups with different IFN γ activation states have a tendency to acquire particular aberrations needs to be understood. Additionally, establishing the relationship between MITF, IFN γ and markers of the MITF and IFN γ -defined states in tumour cells with TME-related factors (such as shed RTKs or exosomes) would facilitate monitoring of patients and guide therapeutic interventions as relevant changes in cell state emerge. Lastly, further characterization of cells with innate resistance to both targeted therapy and immunotherapy is needed to elucidate novel therapeutic strategies.

Therapeutic strategy

Implications from a dynamic fluctuation model.—According to our dynamic fluctuation model, two properties of melanoma cells are important: heterogeneity and the

existence of distinct homeostatic states. Certain therapeutic strategies need to be considered with caution owing to the potential shortcoming of increasing particular resistance mechanisms. A continuous oscillation in drug exposure with standard doses of agents such as MAPK inhibitors might increase genomic complexity during ‘drug holidays’ (interruptions of treatment lasting days or weeks for reasons other than toxicity management). This strategy is distinct from pulsatile dosing strategies, in which doses much higher than those that can be tolerated continuously are administered. If pulsatile dosing results in more tumour cell elimination, an inherent benefit will be the restriction of the survivor cell subpopulation. In addition, strategies deployed against minority cell populations are unlikely to generate clinical, radiographic evidence of single-agent efficacy; for this reason, therapies targeting CSC biomarkers are not efficacious as monotherapies. Melanoma CSCs, however, must not be considered a static subpopulation and might not be amenable to elimination; instead, prevention of the transition from tumour cell to CSC might be more advantageous.

We consider several strategies to be supported by the emerging understanding of the dynamics of therapy resistance. First, to counteract heterogeneity, the combination of targeted agents or immunotherapies with epigenetic modulators, stress response inhibitors or metabolic agents (for example, inhibitors of peroxisome proliferator-activated receptor- γ co-activator 1 α (PGC-1 α), steroid hormone receptor- α (ERR α) or oxidative phosphorylation) warrants investigation^{38,214,215}. This higher-order combination therapy might enable a thorough eradication of both subpopulations with innate resistance and persists at very early stages of therapy, limiting the potential of these cells to serve as reservoirs for future epigenetic and genetic aberrations.

A second potential strategy is predicated on exploiting tumour heterogeneity using a dosing schedule for targeted agents that maintains therapy-sensitive cells within the melanoma cell population, with the rationale that these cells will compete with and prevent the outgrowth of certain resistant subpopulations^{216–218}. On the other hand, the possibility that cooperation might occur between different subgroups of cells^{40,186,219} is a concern and needs to be further investigated. These competing hypotheses highlight the dire need for better monitoring of the resistant cell states and their evolution.

Third, the ability of cells to shift between or remain in certain homeostatic states might be amenable to disruption. A key element of this strategy is to prevent MITF and IFN γ expression from reverting back to the predefined homeostatic or activation status set points, such that persists and/or innate resistant cells remain in cell cycle arrest. To achieve this goal, novel drug combinations are needed; agents that target cell cycle would seem to be leading candidates²²⁰ and should be explored on the backbone of targeted therapies and immunotherapies. Of note, CDK4 and/or CDK6 inhibitors are available, whereas CDK2 inhibitors remain a less well-developed drug class.

Implications of melanoma evolution.—Different tumour cell states arise during melanoma progression and the subsequent development of therapeutic resistance (FIG. 3). Those cell states remain heterogeneous but can be distinguished on the basis of certain

properties. By depicting cell states and their properties more clearly, new therapeutic strategies can be devised.

In terms of therapeutic resistance, the longer the duration of therapeutic exposure, the greater the degree of tumour heterogeneity (FIG. 3f–j). This observation implies that combination therapy regimens, applied after a short initial period of single-agent targeted therapy or immunotherapy, might be able to eradicate cells with innate resistance or persisters cells. The collection of serial biopsy samples at an early on-treatment time point will help discriminate which cell subpopulations dominate early during therapeutic resistance and which combination regimens should be prioritized.

The currently available data indicate that one of the most promising strategies is to target persister cells^{221,222}; owing to the heterogeneous epigenetic and genetic aberrations accumulated during both the reversal of senescence and irreversible resistance phases, the persister cell phase seems to be more vulnerable to intervention¹⁹⁴. Among direct targeting strategies that should be considered, JAK inhibitors, metabolic modulators, multiple RTK inhibitors and epigenetic modulators²²² (to block phenotype switching) have promise; these strategies aim to prevent or reverse the epithelial–mesenchymal transition²²¹. Nevertheless, caution should be taken regarding combination therapies, especially those involving candidates that might have a negative impact on immune system. Indeed, a pilot clinical trial of combination therapy with a JAK inhibitor and an anti-PD-1 antibody was closed early owing to a lack of differences in intratumoural T cell subsets (CD8⁺ T cells and T_{reg} cells) and no effect on tumour cells despite a statistically significant reduction in peripheral T cell activation²²³.

Reshaping of the tumour microenvironment.—Persisters cells that survive targeted therapy or immunotherapy follow different evolutionary trajectories, with a more rapid and widespread outbreak described for targeted therapy than for immunotherapy. This behaviour might relate to the different TME-modulating effects of targeted therapy and immunotherapy. Thus, when considering the addition of an agent to a targeted therapy or immunotherapy backbone, we need to consider and assess its effects on the cellular components of the TME — both tumour-related and immune-related.

A second TME-related aspect worth considering is treatment with agents that remodel the either non-cellular or cellular components of the TME. These agents include those that can limit the invasive behaviour of the persister cells by inhibiting extracellular proteolytic proteins or that can make the TME more hostile to melanoma cells by inhibiting angiogenesis or reducing the accessibility of essential nutrients to melanoma cells. Another category includes agents that eliminate immune inhibitory cells. Examples include the depletion of melanoma-promoting B cells using an anti-CD20 monoclonal antibody^{188,224} (NCT01307267) and the further release T cells or other immune stimulatory cellular components from immune inhibitory signals by combining anti-PD-1 antibodies with other immune-checkpoint inhibitors (such as anti-TIM3 antibodies), antagonists of other immune inhibitory signals (such as TGFβ inhibitors) or T cell agonists.

Conclusions

The advances in melanoma therapy have resulted in the current combinations of two agents from the same therapeutic modality. Ongoing investigations seek to build higher-order combination strategies on a backbone of targeted therapy or immunotherapy. Beyond clinical end points, the mechanisms driving resistance in each individual patient need to be ascertained with great precision in order to understand whether they will be addressed by the novel therapeutic approaches and thus develop a precision medicine-based principle driving the subsequent development and application of novel regimens. Our improving understanding of melanoma cells, at both the single-cell level and the cell-subpopulation level, is crucial to achieving this goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key points

- In any particular cell, the expression of a given protein fluctuates dynamically around a pre-set homeostatic level, contributing to temporal heterogeneity. At the cell-population level, the expression of a given protein fits a log-normal distribution, contributing to spatial heterogeneity.
- Cell state is mostly determined by the expression levels of different proteins, which is a continuous quantitative variable and can be perturbed by extrinsic stress, such as drug exposure.
- The development of resistance to targeted therapy and immunotherapy can be divided into three phases, namely, early survival (including persister cells and innate resistant cells), reversal of senescence and new homeostasis; along these phases, resistance gradually changes from reversible to irreversible.
- The persister cell subpopulation is programmed to tolerate cell death and capable of surviving harsh environmental conditions, such as hypoxia, lack of nutrients and exposure to targeted therapy and/or immunotherapy.
- Future therapeutic developments should take into account the highly dynamic heterogeneity and the existence of distinct homeostatic states of tumour cells.

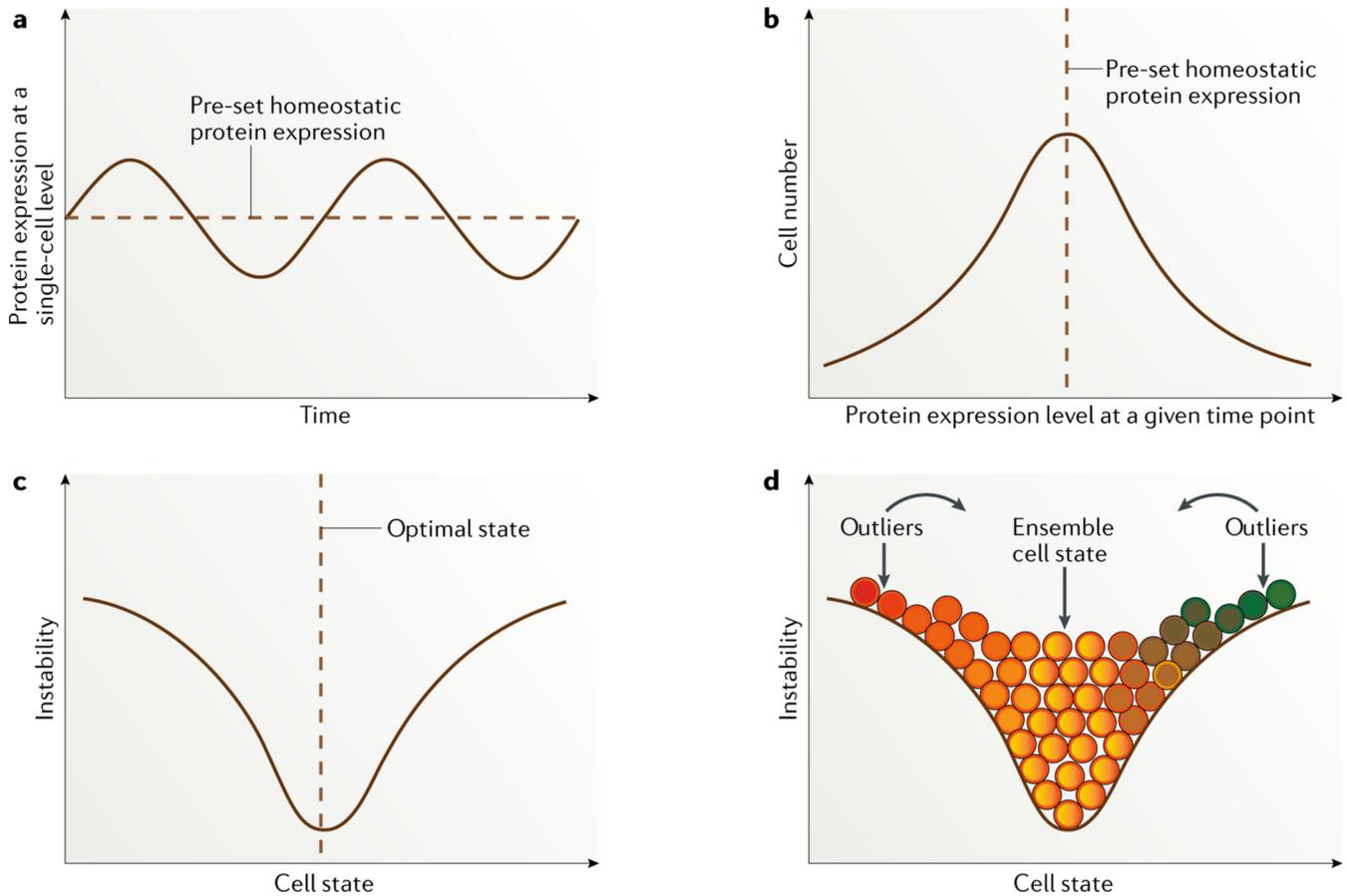


Fig. 1 | Dynamic fluctuation model.

a | Dynamically fluctuating expression pattern of a given protein longitudinally at the single-cell level. **b** | Cell distribution based on expression levels of a particular protein, with the vast majority of cells located around the predefined homeostatic protein expression level. **c** | Cell state defined by a particular protein or a group of correlated proteins entangled within a regulatory pathway, with the majority of cells located at the optimal ensemble state. The more distant from this pre-set state a cell is, the more unstable it is likely to be, with a strong tendency to switch back to the point of homeostatic balance. **d** | Distribution of cells of different states (depicted in different colours). Continuous colour changes indicate that cell-state transitions occur across a spectrum. Unlike the perfectly symmetric distribution shown, the cell-state curve varies for proteins that define different cell states. Immortalized melanoma cells are probably skewed towards one end of the spectrum in a context-dependent way. Importantly, unstable outliers are in a cell state that differs substantially from the majority of the cell population and are therefore expected to behave differently in a given situation, such as stress.

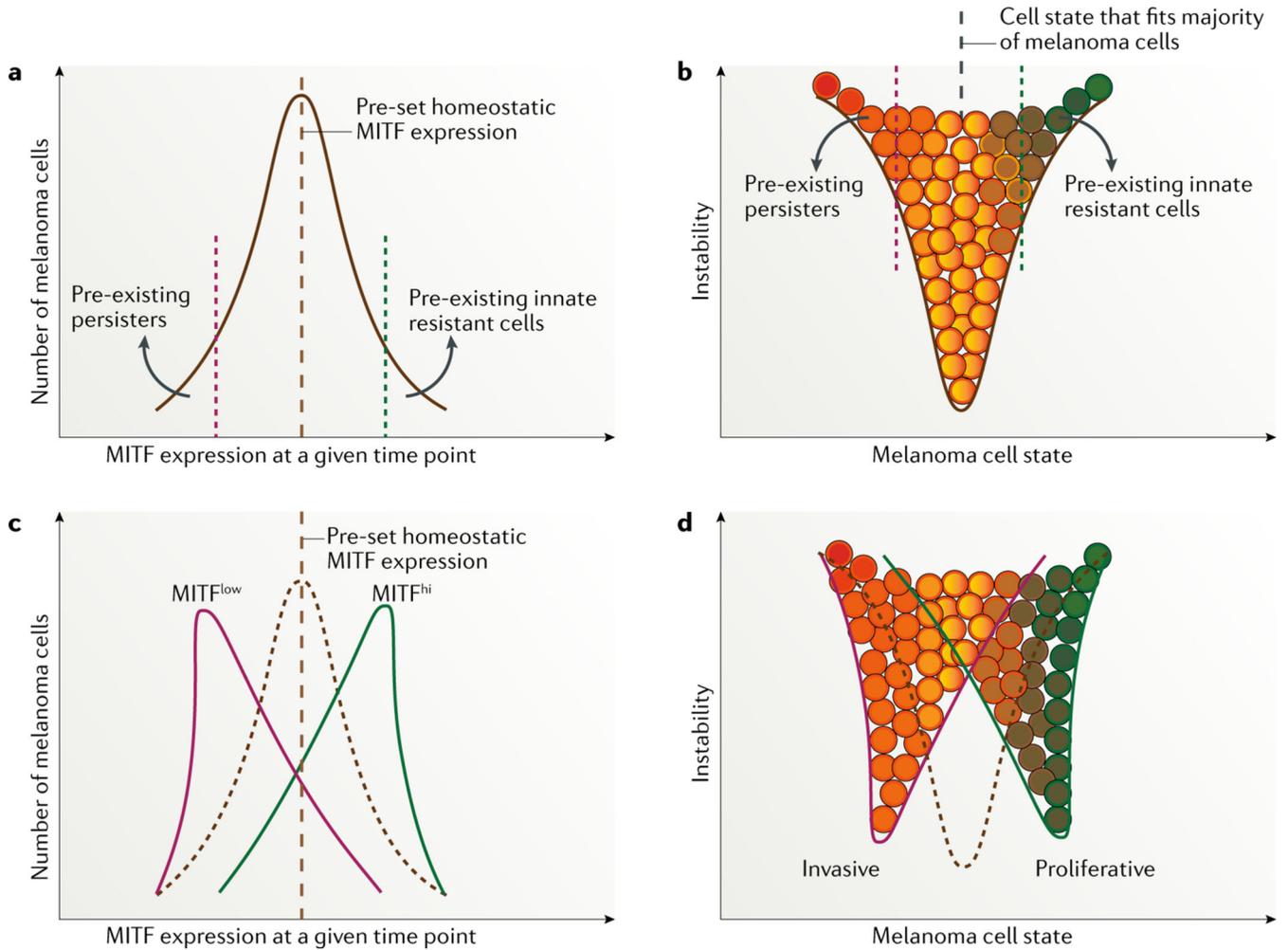


Fig. 2 | MITF expression and phenotype switching in melanoma before and after treatment with targeted agents.

a,b | Microphthalmia-associated transcription factor (MITF) expression and melanoma cell-state pattern before exposure to targeted therapy, representing a unimodal distribution of MITF expression in the same population of melanoma cells. Of note, variations in predefined points exist between melanoma cell lines, mouse models and patient samples^{72,225} owing to differences in melanoma initiation and in microenvironment selective pressure. **c,d** | Alterations in MITF expression and variation in cell state after starting therapy.

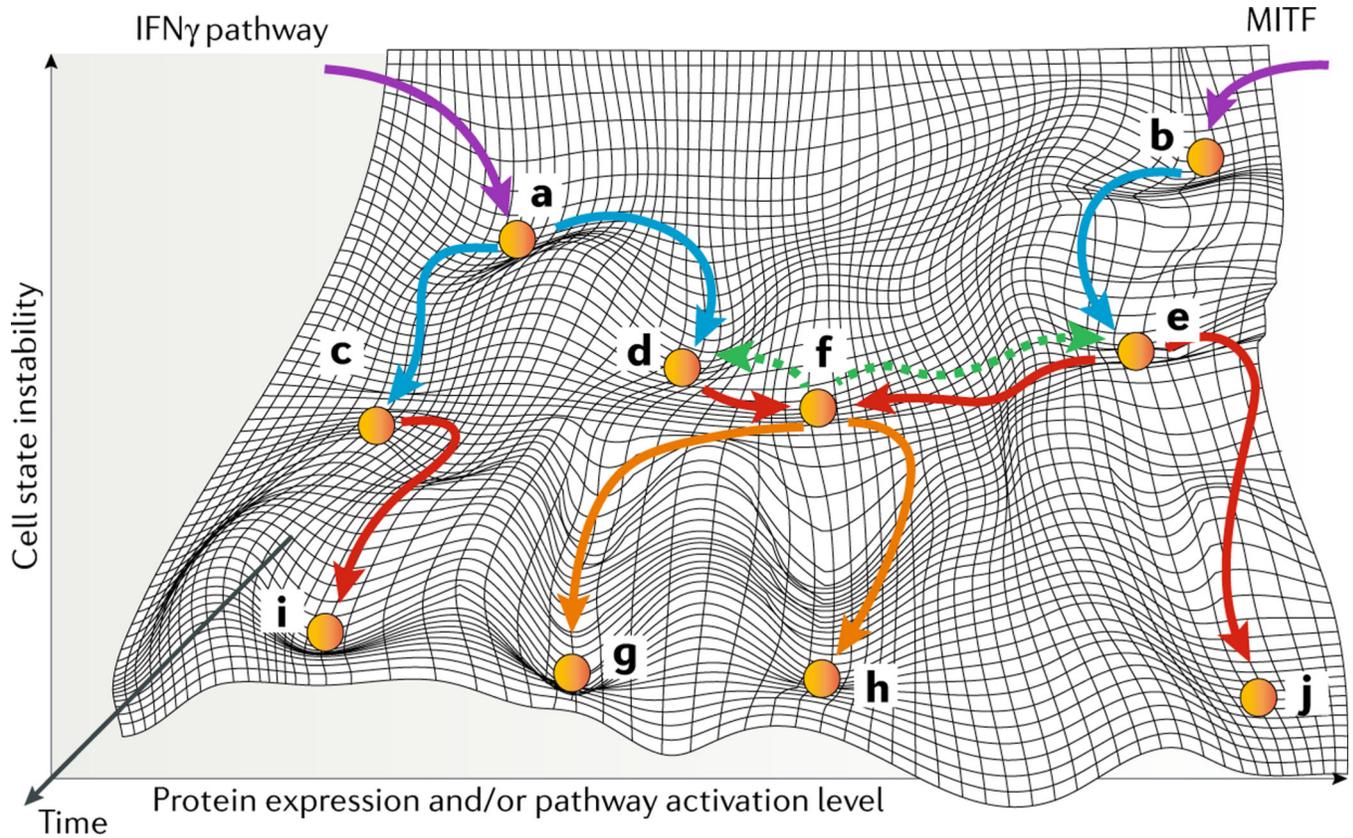


Fig. 3 | Reshaping of cell states at different stages in melanoma.

The initiation, progression and development of initial and secondary therapy resistance are designated with purple, blue, red and orange arrows, respectively. Green dashed arrows demonstrate the complete reversibility of a cellular state soon after therapy initiation. Melanoma initiation can occur through an IFN γ -dependent and immunogenicity-dependent mechanism (part **a**) or a microphthalmia-associated transcription factor (MITF)-related mechanism (part **b**). Through immune editing, melanoma cells bifurcate into different states characterized by IFN γ hypoactivation and low immunogenicity (part **c**) and IFN γ hyperactivation and high immunogenicity (part **d**). MITF-related cell state is maintained, with inter-individual variations, during disease progression (part **e**). A persister cell state is characterized by IFN γ hyperactivation, high immunogenicity, low expression of MITF and an invasive phenotype that contribute to resistance to both targeted agents and immunotherapy (part **f**). This persister state is not stable and will reverse back to that seen in part **d** or part **e** if therapy is withdrawn or, otherwise, through the accumulation of both epigenetic and genetic aberrations, will evolve into more stable states (parts **g,h**). Epigenetic and genetic alterations can lead to a cell state of IFN γ hypoactivation and low immunogenicity (part **i**) that contributes to resistance to immunotherapy and to a state with extremely high MITF expression and a senescent phenotype (part **j**) that contributes to resistance to targeted agents.

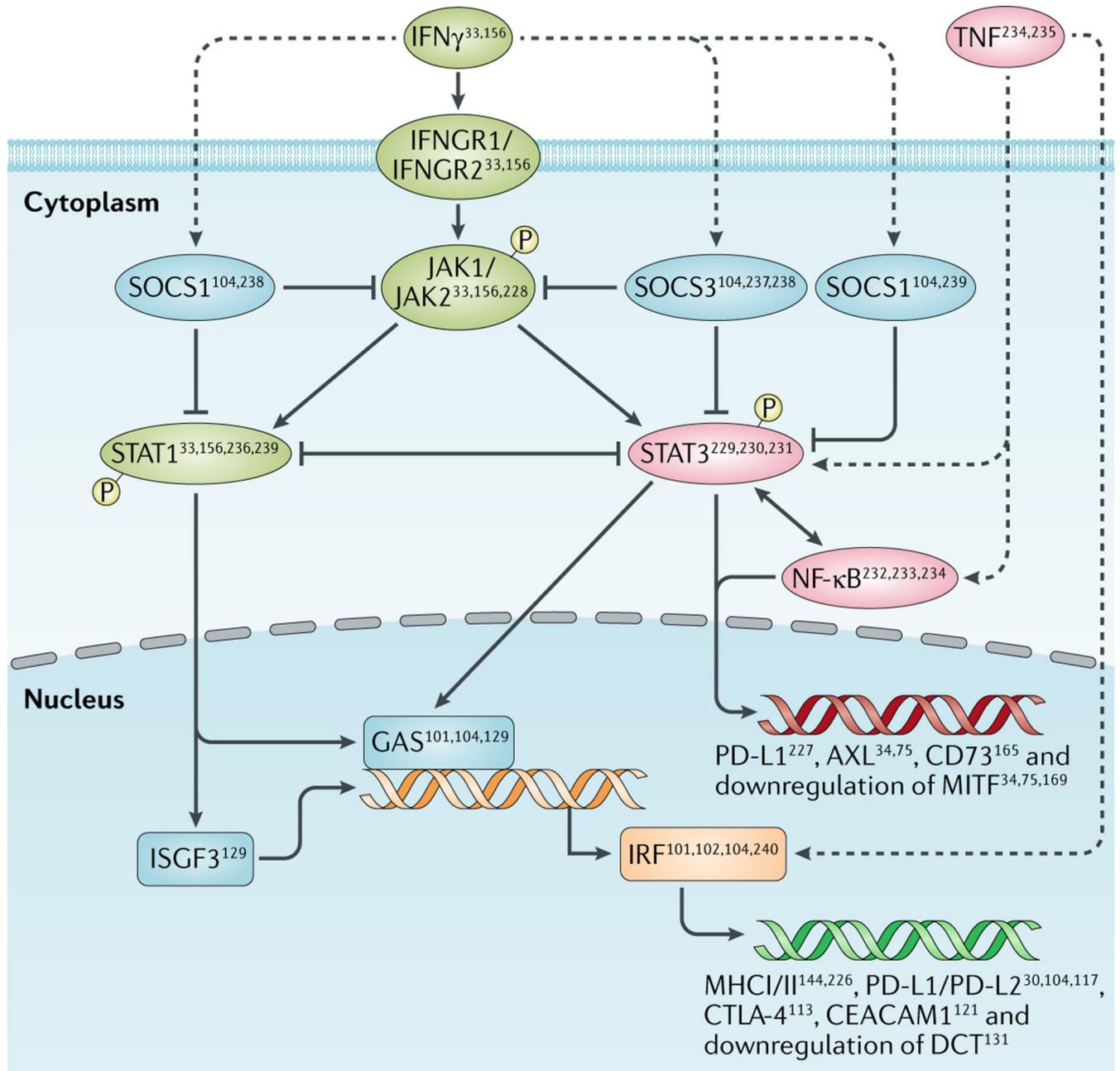


Fig. 4 | IFN γ -JAK1/JAK2-STAT1/STAT3 pathway in melanoma.

Dashed lines represent positive regulation with no specific regulatory mechanism elucidated yet. Examples of regulated downstream molecules are provided. CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CTLA-4, cytotoxic T lymphocyte antigen 4; GAS, growth arrest-specific protein; IFNGR, IFN γ receptor; IRF, interferon regulatory factor; ISGF, interferon-stimulated transcription factor; MITF, microphthalmia-associated transcription factor; P, phosphorylated molecule; PD-L, programmed cell death 1 ligand; SOCS, suppressor of cytokine signalling; STAT, signal transducer and activator of transcription.