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UNIL | Université de Lausanne
Faculté de biologie et de médecine

University Hospital of Lausanne
Department of Oncology

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Altered responsiveness and potential implication for the efficiency of immunotherapy

Doctoral Thesis in Life Sciences (PhD)

Presented to the Faculty of Biology and Medicine
of the University of Lausanne

By

Christophe MARTIGNIER

Master of Sciences of the University of Lausanne

Jury

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PhD. Karin Schäuble, Thesis co-director
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PhD. Mauro Delorenzi

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pour le Doyen
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Abstract

Characterization of melanoma cells' responsiveness to T cell derived cytokines and its potential impact on the efficiency of immunotherapy

Today we are living in the novel era of immunotherapy thanks to immune checkpoint blockade with great results in the fight against cancer. However, some patients do not benefit from these therapies because of various mechanisms of resistance. Recent researches have shown that resistance can be due to loss of responsiveness of the tumor cells to T cell-derived cytokines. In contrary, other researchers concluded that the response of the tumor cells to T cell-derived cytokines allows them to express chemokines that lead to the recruitment of immunosuppressive cells. These observations are calling for a better understanding of the immunological phenotype of the tumor cells. In this study, we characterized the responsiveness of patient-derived melanoma cell lines to T cell-derived cytokines. The initial screening showed that melanoma cells were not responding to the majority of cytokines. We continued the screening with IFNγ and TNFα as they were the cytokines that induced most changes. In addition, the combination of both these cytokines showed synergistic effects. We identified two melanoma cell lines that were not responding and others were only poorly responding. Grouping of the patients based on the ability of the corresponding cell lines to respond to the cytokines showed some trends indicating that the responsiveness could be associated with a better patient survival. The lack of responsiveness of one of the non-responding cell lines was explained by a mutation in the JAK1 gene. Reconstitution of JAK1 restored the responsiveness. The second cell lines did not upregulate IRF1 upon IFNγ treatment. Forcing the IRF1 did change its phenotype while treatment with HDAC inhibitor restored its responsiveness to IFNγ.

In conclusion, our results show that the response of melanoma cells to T cell-derived cytokines is heterogeneous and that some cell lines are poorly or non-responding which likely dampens the efficiency of immunotherapy. Our data call for a better characterization of the cause and the consequence of this lack of responsiveness and find ways to overcome these mechanisms. Recent studies suggest that patients treated with TLR stimulators acting directly on melanoma cells in vivo may indeed be able to overcome this type of resistance, an approach that is currently being tested in large clinical trials. Other trials will determine whether drugs that modify epigenetic imprinting may become useful.

Résumé

Caractérisation de la réponse des cellules du mélanome aux cytokines produites par les lymphocytes T et de ses effets potentiels sur l'efficacité de l'immunothérapie

Aujourd'hui, le succès des inhibiteurs de points de contrôle immunitaire dans le traitement du cancer nous a fait rentrer dans l'ère de l'immunothérapie. Cependant, de nombreux patients ne répondent pas à ces traitements suggérant l'existence de mécanismes de résistance. Des études ont montré que l'altération de la réponse des cellules tumorales à certaines cytokines produites par les cellules T mais aussi la capacité d'autres cellules tumorales à recruter des cellules immunosuppressives en réponse à ces mêmes cytokines participent à cette résistance.

Ce travail étudie la réponse de lignées cellulaires de mélanomes de patients à des cytokines produites par des lymphocytes T. Les premiers résultats ont indiqué que la majorité des cytokines n'avaient pas d'effet sur les cellules tumorales à l'exception de l'IFNγ et du TNFα. Parmi les 21 lignées de mélanomes, 2 lignées ne répondaient pas tandis que plusieurs autres montraient une faible réponse à ces cytokines. En comparant la survie des patients et la capacité des cellules tumorales correspondantes à répondre aux cytokines, nous avons établi plusieurs associations. Nous avons identifié qu'une des lignées qui ne répondait pas avait une mutation dans le gène JAK1 et que la restauration de ce gène permettait aux cellules de répondre à nouveau. La seconde lignée ne montrait pas d'augmentation d'expression d'IRF1 et forcer son expression n'a eu aucun effet. Cependant, un traitement avec des inhibiteurs d'HDAC a restauré la réponse à l'IFNγ.

Ensemble, ces observations montrent que différents mécanismes peuvent conduire à une absence de réponse à l'IFNγ et qu'il est donc important de caractériser ces altérations afin de développer des solutions pour contourner ces résistances. Des recherches récentes ont montré que le traitement de patients avec des activateurs de TLR permettait de contrecarrer ces résistances. D'autres études détermineront si des traitements épigénétiques peuvent améliorer la situation.

Abbreviations

PRM	Pattern Recognition Molecules
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
DAMP	Damage Associated Molecular Pattern
TLR	Toll-Like Receptor
MHC	Major Histocompatibility Complex
APC	Antigen Presenting Cell
BCR	B Cell Receptor
TCR	T Cell Receptor
ER	Endoplasmic Reticulum
TAP	Transporter Associated with Antigen Processing
Th	T Helper
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
IFNγ	Interferon gamma
TNFα	Tumor Necrosis Factor alpha
IL2	Interleukin 2
CTL	Cytotoxic T Lymphocytes
CXCL	Chemokine C-X-C motif Ligand 9
CXCL10	Chemokine C-X-C motif Ligand 10
NK	Natural Killer
TNFR1	Tumor Necrosis Factor Receptor 1
TNFR2	Tumor Necrosis Factor Receptor 2
Treg	Regulatory T cells
IL10	Interleukin 10
PD1	Programmed cell Death 1
PD-L1	Programmed Death Ligand 1
PD-L2	Programmed Death Ligand 2
UV	Ultraviolet
BRAF	B-Rapidly Accelerated Fibrosarcoma
NRAS	Neuroblastoma Rat Sarcoma Virus
KRAS	Kirsten Rat Sarcoma Virus

HRAS	Harvey Rat Sarcoma Virus
NF1	Neurofibromin 1
MEK	Mitogen-Activated Protein Kinase Kinase
CTLA4	Cytotoxic T-Lymphocytes-Associated Protein 4
MDSC	Myeloid-Derived Suppressor cells
TNFβ	Tumor Necrosis Factor Beta
LTα	Lymphotoxin Alpha
IL6	Interleukin 6
IL4	Interleukin 4
IL5	Interleukin 5
IL13	Interleukin 13
GM-CSF	Granolocyte Macrophage-Colony-Stimulating Factor
CCL3	Chemokine (C-C motif) Ligand 3
CCL4	Chemokine (C-C motif) Ligand 4
CCL2	Chemokine (C-C motif) Ligand 2
TME	Tumor Micro Environment
RNA	Ribonucleic Acid
mRNA	Messenger Ribonucleic Acid
DNA	Deoxyribonucleic Acid
CAR	Chimeric Antigen Receptor
CD19	Cluster of Differentiation 19
CD20	Cluster of Differentiation 20
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
TGFβ	Tumor Growth Factor Beta
IDO	Indoleamine 2,3-Dioxygenase 1
CSF1	Colony-Stimulating Factor 1
PTPN2	Protein Tyrosine Phosphatase Non-Receptor Type 2
JAK1	Janus Kinase 1
RPMI	Roswell Park Memorial Institute Medium
NHM	Normal Human Melanocyte
HEMA-LP	Human Epidermal Melanocytes, Lightly Pigmented
FCS	Fetal Calf Serum
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction

FACS	Fluorescence Assisted Cell Sorting
GMFI	Geometric Mean Fluorescence Intensity
HLA	Human Leukocyte Antigen
ICAM-1	Intercellular Adhesion Molecule 1
CTV	Cell Trace Violet
EDTA	Ethylenediaminetetraacetic Acid
qPCR	quantitative Polymerase Chain Reaction
cDNA	complementary Deoxyribonucleic Acid
PBMC	Peripheral Blood Mononuclear Cells
GAPDH	Glyceraldehyde 3-Phosphate dehydrogenase
S16	Ribosomal Protein Subunit 16
Ct	Cycle Threshold
RIPA	RadioImmunoPrecipitation Assay
DNase	Deoxyribonuclease
TEMED	Tetramethylenediamine
BSA	Bovine Serum Albumin
STAT1	Signal Transducer and Activator of Transcription 1
pSTAT1	phosphorylated Signal Transducer and Activator of Transcription 1
IRF1	Interferon Regulatory Factor 1
IgG	Immunoglobulin G
hIRF1	human Interferon Regulatory Factor 1
hJAK1	human Janus Kinase 1
EGFP	Enhanced Green Fluorescent Protein
LB	Lysogeny Broth
DMEM	Dulbecco's Modified Eagle Medium
ANOVA	Analysis of variance
ns	non significative
Ctrl	Control
SNP	single-nucleotide polymorphism
SNV	single-nucleotide Variant
TCGA	Tumor Cell Genome Atlas
CNA	Copy Number Alteration
KEGG	Kyoto Encyclopedia of Genes and Genomes
rtPCR	realtime Polymerase Chain Reaction
vJAK1	vector with Janus Kinase 1 and Enhanced Green Fluorescent Protein genes

vEGFP	vector with Enhanced Green Fluorescent Protein gene
vIRF1	vector with Interferon Regulatory Factor 1 and mCherry genes
vmCherry	vector with mCherry gene
ATAC	Assay for Transposase-Accessible Chromatin
B2M	Beta 2 Microglobulin
HDAC	Histone Deacetylase

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Introduction

The immune system

A complex system such as the human body faces many challenges that can arise from the environment (pathogens) but also from alterations in the tissues. In order to protect the body from the damages these events can produce, the organism has developed a system called the immune system consisting of a multitude of cells responding in a coordinated manner. As the challenges are diverse, the immune system can use different mechanisms depending on the type of alterations. The immune response is divided in two categories which are the innate and the adaptive response.

The innate immune response

The innate immune system is responsible for the first line of defense against alterations of any type. It can be further divided in three main categories which are the physical barriers, the innate immune cells and the non-cellular innate immune components (Figure 1)[1].

The mucosa and the skin are part of the physical barriers protecting the organism from the entry of pathogens, based on their epithelial cell layers and their functions[2]. We can also include chemical barriers such as pH regulation and the presence of antimicrobial molecules (e.g. mucins that aggregate bacteria in the saliva, or anti-microbial cathelicidins and defensins in the skin)[3].

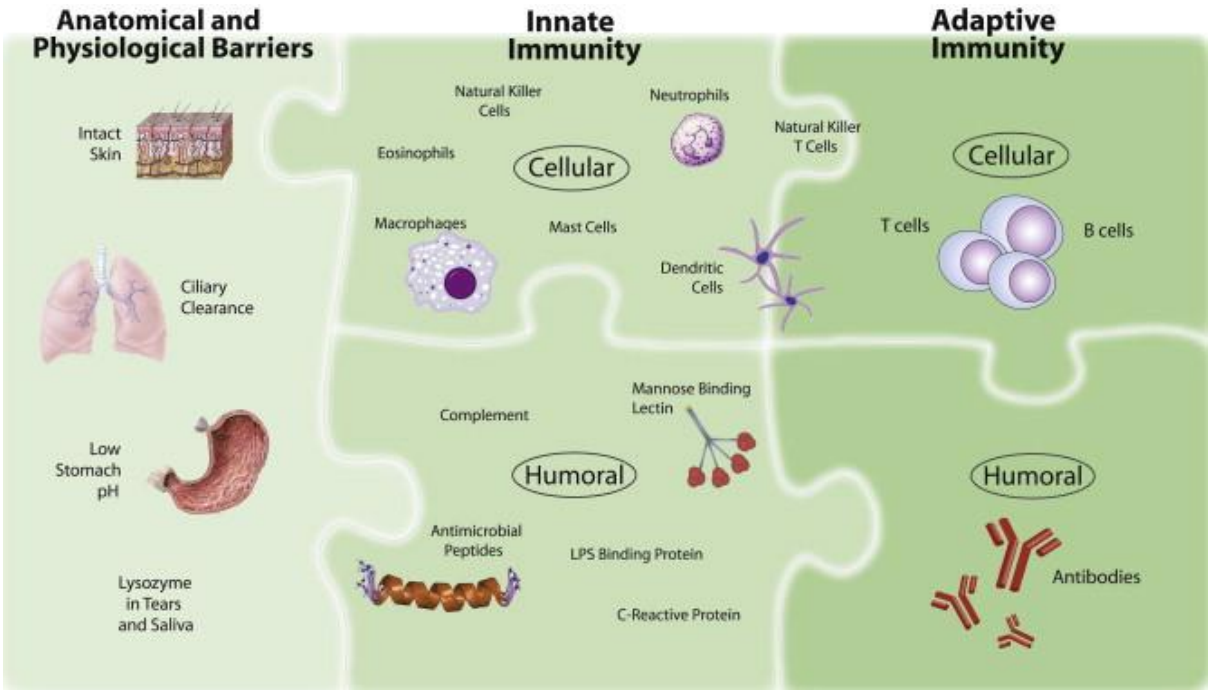


Figure 1. Components of the immune system. As the role of the dendritic cells is to make the link between the innate and adaptive immune system, they are positioned at the interphase of the two (Figure from Turvey et al., 2010)[2]

The innate immune response is also composed of circulating molecules that can recognize signals associated with danger. In fact, these molecules are recognizing pattern that are shared between different pathogens and are thus called Pattern Recognition Molecules (PRMs)[4]. In addition, they can also recognize the constant regions of antibodies that are bound to pathogens. In cooperation with other complement proteins in the plasma, they opsonize invaders by binding to their surface directly or via antibodies. This opsonization leads to the recruitment of phagocytic cells that eliminate the invaders or it can also directly induce the lysis of the pathogen by recruiting additional complement proteins with protease activities[5].

The innate immune cells are very diverse as they need to have a wide range of activities to defend the organism against a multitude of pathogens. It includes natural killer cells, dendritic cells and cells with phagocytic properties such as macrophages and neutrophils. The innate immune cells are working together with the non-cellular innate immune response which includes the complement system. Both are based on a similar mechanism consisting of recognition of patterns that are shared between different types of related pathogens. These patterns are called pathogen associated molecular patterns (PAMPs). These specific structures are evolutionary conserved as they are usually crucial for the microbe's survival and thus hard to change without altering the fitness of the microbes. This limits the chances of evading the recognition by the innate immune system. The recognition of these PAMPs is mediated by pattern recognition receptors (PRRs) that are encoded directly in the human genome. The innate immune system can also detect the presence of an alteration by recognizing molecules that are only produced in the case where damages are inflicted to cells. These molecules are called Damage Associated Molecular Patterns (DAMPs) and are released by damaged or dying cells.

The recognition of PAMPs is mediated by sentinel and phagocytic cells such as mast cells, macrophages, dendritic cells, neutrophils and other immune cells that express PRRs. Toll-Like receptors (TLRs) belong to the PRR family and are expressed by many different innate immune cells. TLRs expressed by dendritic cells will recognize PAMPs present in the environment and initiate the phagocytic process[4]. Once ingested, the phagocytosed material will be processed which will lead to the initiation of an adaptive immune response through the presentation of the antigens on Major Histocompatibility Complex (MHC) molecules at the cell surface, as dendritic cells are an important part of the professional Antigen-Presenting Cells (APCs). PAMPs are necessary to activate the APC as in absence of PAMPs, the captured antigens will be presented without additional costimulatory signals which are required for the activation of the adaptive immunity[2].

The adaptive immune system

Similarly, to the innate immune system, the adaptive immune system recognizes molecules that can elicit an immune response and are thus called immunogenic. These molecules are called antigens. However, the receptors involved in the recognition of these antigens are not germline encoded which allows them to recognize a much wider range of molecules. In addition to this broad range of specificity, the adaptive immune system keeps track of previous immune responses in a process that is called memory formation and thus allows a quicker and more effective response when a similar event should occur again (e.g. infection by the same virus). It is composed of a humoral component that involves B lymphocytes (B cells) and their production of antibodies, and a cellular component involving T lymphocytes (T cells)[6].

B lymphocytes

B cells arise from precursors located in the bone marrow. They express B cell receptors (BCR) on their cell surface. Each B cell expresses only one type of BCR which is explained by the mechanisms involved in the generation of the BCR. The BCR is composed of two parts. The first one is constant and called Fc while the second part is a variable region that is acquired by the VDJ recombination which is a mechanism of somatic recombination occurring during the development of the B cells and leading to a very diverse BCR repertoire[7].

The recognition of a specific antigen by the BCR on the cell surface leads to the activation of the B cell, its proliferation and the subsequent secretion of antibodies as well as the formation of long-lived memory B cells. They have the capacity to be rapidly activated upon encounter of the corresponding antigen[7].

T lymphocytes

As for B cells, T cells also find their roots in the bone marrow. However, their development requires further maturation steps in the thymus. The generation of their T cell receptor (TCR) also relies on somatic recombination. While each T cell only has one type of TCR, there exists a high diversity of TCRs in the T cell repertoire. There are at least two downsides of this mechanism. The first one is that the recombination may lead to the production of TCRs that are not functionally expressed at the cell surface but such cells are eliminated during the development in the thymus.[8] This process is called positive selection where only T cells presenting a functional TCR at the cell surface can mature in the thymus. In contrast, the recombination can also lead to the production of TCRs that present a high affinity for self-antigens which could lead to an immune response targeting healthy tissue and thus auto-immune disease. To prevent such events, T cells have to

undergo a “test phase” where specific APCs are presenting self-antigens in the thymus, during which the T cells that are able to recognize these antigens are eliminated. This process is called negative selection[8].

Antigen presentation

In contrast to B cells that can directly recognize antigens in their environment, T cells require the processing of the antigens and their presentation by a specific molecule called Major Histocompatibility Complex (MHC)[10]. In the case of an infection by microbes such as viruses or bacteria, professional APCs like dendritic cells will recognize PAMPs which will lead to their activation. Activated APCs process the antigens taken up from their environment and migrate to the lymph nodes where they will present the antigens on their MHC, together with the expression of co-stimulatory molecules. The recognition of the antigen by a T cell and the costimulatory signals will lead to the activation and the proliferation of antigen-specific T cells.

As the tools necessary to fight an intracellular or an extracellular pathogen are not the same, two different classes of MHC molecules exist. MHC-I presents antigens that are derived from proteins present within the cell. It is expressed by almost all types of cells and indicates the “health status” of the cell. One exception is that MHC-I on APCs can present antigens derived from the extracellular compartment through a mechanism called cross-presentation. In order to present intracellular antigens to the cell surface, the cell needs to transfer the antigen from the intracellular compartment to the Endoplasmic Reticulum (ER) using a transporter called TAP. In the ER, the antigen will be coupled to the MHC-I and transported to the cell surface[9].

In contrast to MHC-I, MHC-II is mostly expressed by APCs and presents antigens that are derived from molecules present in the extracellular environment through their uptake by phagocytosis, their processing and coupling to MHC-II[10]. In the case of an infection by an extracellular pathogen, APCs will be activated through their PRRs and migrate to the lymph nodes where they will present the MHC-II-bound antigens from the environment to the T cells[9].

MHC-I and MHC-II are not recognized by the same kind of T cells and this specificity derives from coreceptors expressed by the T cells. There are two coreceptors called CD4 and CD8. CD4 binds to MHC-II and the T cells expressing CD4 are called CD4+ T cells while CD8 binds to MHC-I and the T cells expressing CD8 are called CD8+ T cells[11].

CD4+ T cells

CD4+ T cells recognize MHC-II bound antigens and thus antigens that are present in the extracellular environment. CD4+T cells can be divided into different types and we will mostly describe T helper 1 (Th1), T helper 2 (Th2) and T regulatory (Treg) cells but other types also exist

depending on the pathogen faced[12]. Th1 are mostly involved in the elimination of intracellular pathogens such as viruses. The name Helper comes from the fact that they activate and support the function of other immune cells and thus also shape the type of response established based on the cytokines they release. In order to fight intracellular pathogens, Th1 will activate cells such as CD8+ T cells as they have the ability to eliminate infected cells and maintain their activity by producing IFNγ, TNFα and IL2[13]. In contrast, Th2 is involved in the response against extracellular pathogens such as some bacteria. To achieve this goal, they can support the function of B cells and thus increase the production of antibodies which are efficient at neutralizing extracellular pathogens[13]. Finally, Treg has a different role than the previous two types described. Their role is to limit the activation of the immune system in order to prevent the damages that could be done to healthy tissues. In this way, they avoid auto-immunity reaction by blocking the activation of T cells that would be able to recognize self-antigens[13].

However, an immune response is usually a mix of the different CD4+ T cell responses.

CD8+ T cells

CD8+ T cells have a cytolytic function and are thus also called cytotoxic T lymphocytes (CTLs). This cytolytic activity is initiated by the recognition of a MHC-I-bound antigen by the TCR and the signal transmitted by the coreceptor CD8[14]. This initial recognition step leads to the formation of an immunological synapse between the CD8+ T cell and the target cell. The second step involves the release of vesicles containing perforin that will form holes in the membrane of the target cell. The disruption of the membrane allows the entry of granzymes previously present in the T cells. The granzymes will activate the caspase signaling which results in the apoptosis of the target cell. Another mechanism used by CTLs is the expression of FAS-Ligand (FAS-L) on their surface. FAS-L interacts with FAS on the target cells and also leads to the activation of the caspase cascade[15].

In addition to their cytolytic activity, CD8+ T cells also produce several cytokines such as IFNγ, TNFα and IL2[15]. These cytokines have different roles. One of them is to maintain and shape the immune response. In an autocrine manner, they can stimulate the activation and the proliferation of the T cells but they can also regulate the activities of other cell types such as APCs and macrophages. This mechanism is interesting in the way that APC presenting the antigen corresponding to the T cell will activate the T cell and the activated T cell will then produce the cytokines that will further activate APC presenting the same antigen. In conclusion, this system may strengthen an immune response targeting a specific antigen.

IFNγ

IFNγ is one of the key cytokines of the adaptive immune system. It can be produced by Natural Killer (NK) cells, Natural Killer T (NKT) cells as well as by CD4+ Th1 cells and CD8+ T cells[16]. It has antiproliferative and pro-apoptotic function. It plays a major role in the context of viral infection as it decreases viral replication and, by increasing antigen presentation on MHC-I and MHC-II, favors the recognition of viral antigens on infected cells by CD8+ T cells and thus the elimination of these cells to prevent further spreading of the virus. IFNγ also helps at maintaining and strengthening the immune response by inducing the expression of chemokines such as CXCL9 and CXCL10 which then recruit T cells[16]. However, as an uncontrolled immune reaction can be detrimental for the organisms, it also induces the expression of molecules that can dampen immune cell activation[17].

TNFα

TNFα is a very pleiotropic cytokine. It has first been identified as a cytotoxic molecule for cancer cells hence its name. It can be produced by a wide panel of cells from the innate and adaptive immune system such as T cells, macrophages and NK cells. Its primary role is to induce inflammation and apoptosis through its binding with TNFR1[18] but it can also be involved in wound healing and cell proliferation through its binding with TNFR2[19]. This underlines the fact that the role of TNFα is highly contextual.

Immune tolerance

As described previously, the generation of the TCR involves somatic recombination that may produce TCRs able to recognize self-antigens presented by MHC molecules. As the immune system is a very powerful system at eliminating its target, such events should be avoided. One way to prevent recognition of self-antigens by the TCR is to eliminate directly in the thymus self-reactive T cells. This goal is achieved by “testing” the self-reactivity of T cells to a wide range of self-antigens that are presented by specific cells. If the T cell is self-reactive, it will undergo apoptosis. The elimination of self-reactive T cells during their maturation in the thymus protects the body from auto-immune disease thanks to central tolerance[20]. Although this mechanism is efficient, some self-reactive T cells may still be able to mature and thus be found in the organism, potentially leading to auto-immune events. Fortunately, the presence of the antigen is not necessarily sufficient to induce an immune response. An antigen presented in absence of a stimulatory signal will not elicit an activation of the T cell and in contrast could lead to silencing or elimination of the self-reactive cell[21]. This process is called peripheral tolerance.

In the case where an immune response against self is nevertheless initiated, several mechanisms can still play a role. Some self-reactive Tregs are involved in the recognition of self-reactive effector T cells and can stop their activation by producing anti-inflammatory cytokines such as IL-10[22]. In addition, T cells express inhibitory receptors such as PD1 and these receptors can prevent the activation of the cell in the presence of its ligands PD-L1 / PD-L2 by interfering with the TCR signalling[23].

Cancer

Cancer is a group of diseases that is characterized by an abnormal cell growth that leads to the ability to invade healthy parts of the organism. More than 100 types of cancer affect humans, and the incidence is increasing with the general aging of the population[24]. Many risk factors have been associated with different types of cancer development. It includes smoking, obesity, poor diet and alcohol consumption but also environmental factors such as UV exposure and carcinogens in the environment[25]. Some viruses are also involved in the development of cancer such as papillomavirus[26]. In addition to these factors, it is now also clear that some people harbor some pre-existing genetic alterations that may favor cancer occurrence[27]. Diagnosis usually relies on symptoms, imaging and biopsy. Several treatments may be used depending on the type and status of the cancer. It usually includes surgery, radiotherapy, chemotherapy and targeted therapy when possible. The survival rates vary a lot depending on the type of cancer but also the stage in which the cancer was diagnosed. This last observation emphasizes that screening is an efficient tool to increase the rate of success of treatments but it is still debatable for some types of cancers.

Melanoma

Melanoma is a type of cancer that develops from melanocytes. These cells are found in the basal layer of the epidermis and are responsible for the production of the dark pigment known as melanin. Melanoma represents 5% of cases of cutaneous malignancies which makes it the third most common cutaneous malignancy[28]. Despite its low incidence, melanoma accounts for 65% of skin-cancer related deaths due to the high mortality rate of metastatic melanoma[28]. This is explained by the fact that melanoma is an aggressive cancer as it presents a high metastatic potential.

Risk factors of melanoma

Many risk factors have been associated with melanoma occurrence[29]. They can be divided into two categories: environmental and hereditary[30]. An excessive UV exposure is hugely associated with skin melanoma development and this is due to the DNA damages that UV radiation causes to skin

cells which leads to dysplastic changes. Other damages to the skin caused by chemicals or burns are also associated with melanoma although it is not as clear as for UV induced damages.

Hereditary factors are also influencing the risk of developing melanoma[30]. A lighter skin color favors the appearance of melanoma as the pigments present in the skin are responsible for the protection against UV induced damages. This is further confirmed by the high incidence of melanoma in patients with xeroderma pigmentosum (increased sensibility to UV) [31]. In addition, a deficient immune system may not be as efficient at clearing the cells that give rise to melanoma and thus we see an increased incidence of melanoma in patients with immunosuppression which is however only marginal and only found in some studies but not in others[32]. We also see correlations between the number and the size of the naevi and the apparition of melanoma[30]. With further investigations, specific germ line genes have been highlighted for the role of their mutations in the increased risk of developing melanoma. This gene list includes Cyclin dependent Kinase inhibitor 2A as well as the corresponding Cyclin Dependent Kinase 4 and the telomerase reverse transcriptase[30].

It is interesting to note that these risk factors may work in a synergistic manner to increase the incidence of melanoma in the population as an excessive UV exposure combined with a lighter skin strongly increases the risk of developing melanoma.

Diagnosis of melanoma

As for many other cancers, an early diagnosis of melanoma increases the survival of the patients. This is partially explained by the fact that thin melanoma (<0.76mm) can be rapidly excised before any melanoma cell started to spread in other parts of the body[33]. This observation prompted for the establishment of more general screening of potential patients (people with big and numerous nevi) by physicians but also directly by patients based on different criteria such as the size, the shape and the color of the potential malignancies (Figure 2) [33, 34]. Naevi with irregularities are excised and analyzed by histology to establish a precise diagnosis. This also allows to confirm that the whole mass has been removed. When facing advanced melanoma, a sequencing may be performed in order to identify mutations that could be used for targeted therapies[35].

Melanoma is classified in stages ranging from 0 to IV depending on the advancement of the disease. The staging is based on the melanoma thickness, the number of metastatic nodes, and the presence and the location of distant metastases (Figure 3)[36].

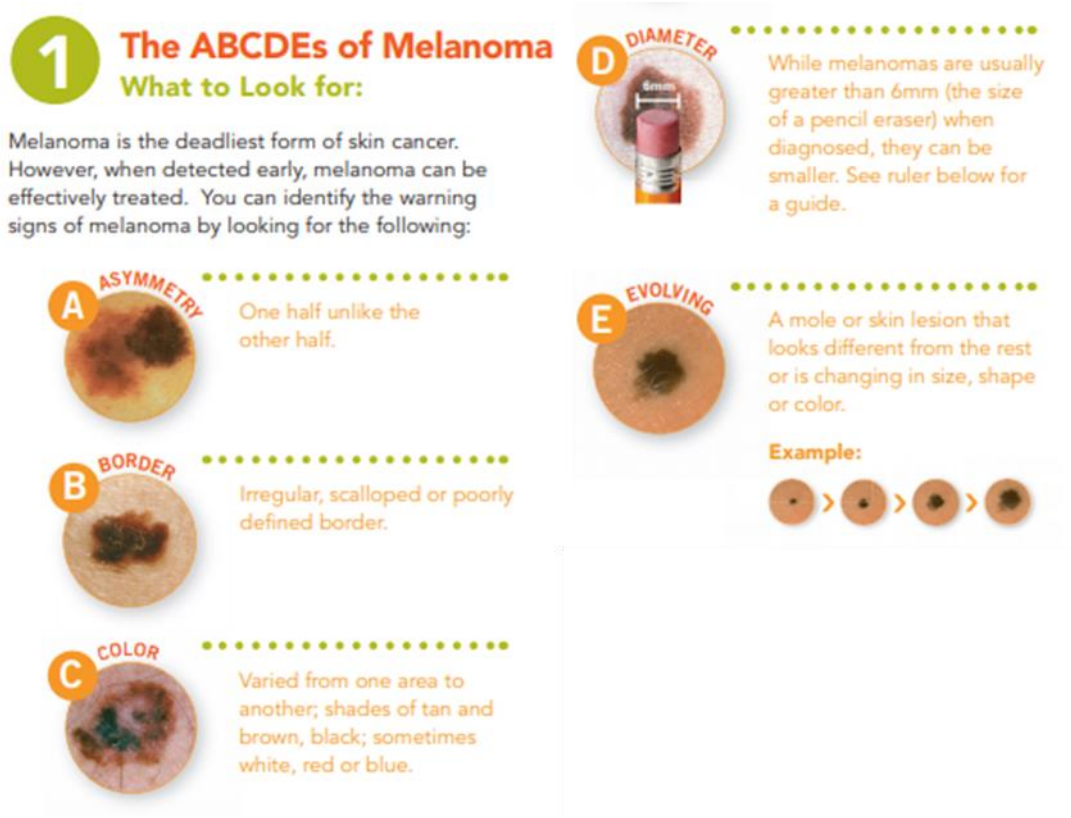


Figure 2. The ABDCEs of melanoma depicts the guidelines to recognize potential melanoma at early stages (American Academy of Dermatology, 2019, Infographic: Skin Cancer Body Mole Map, https://www.aad.org/public/diseases/skin-cancer/find/at-risk/mole-map, accessed in October 2021, .)

Oncogenic driver mutations in melanoma

As it is the case for other cancers, genetic alterations leading to tumor growth and malignancy have been identified in melanoma. The two most frequent oncogenic driver mutations found in melanoma are affecting the BRAF and the NRAS genes[37]. Presence or absence of these mutations can be used to classify melanoma cases in different subtypes, with the RAS subtype also including alterations occurring in the KRAS and HRAS genes. Besides BRAF and NRAS, NF1 is also frequently found altered[37]. The last subtype includes melanomas that do not harbor any of the previous driver mutations. This classification is relevant when discussing treatment options as some of these mutations can be directly targeted by specific drugs (Figure 4)[38].

Treatment of melanoma

The choice of treatment for Melanoma is influenced by the subtype and the stage. Early diagnosis of localized melanoma allows the excision of the primary tumor with margin to ensure complete removal. Stage III melanoma requires surgical removal of the tumor as well as the distant metastases. This can be achieved by conventional surgery or stereotactic irradiation which has the advantage of limiting damages on the healthy tissues[33].

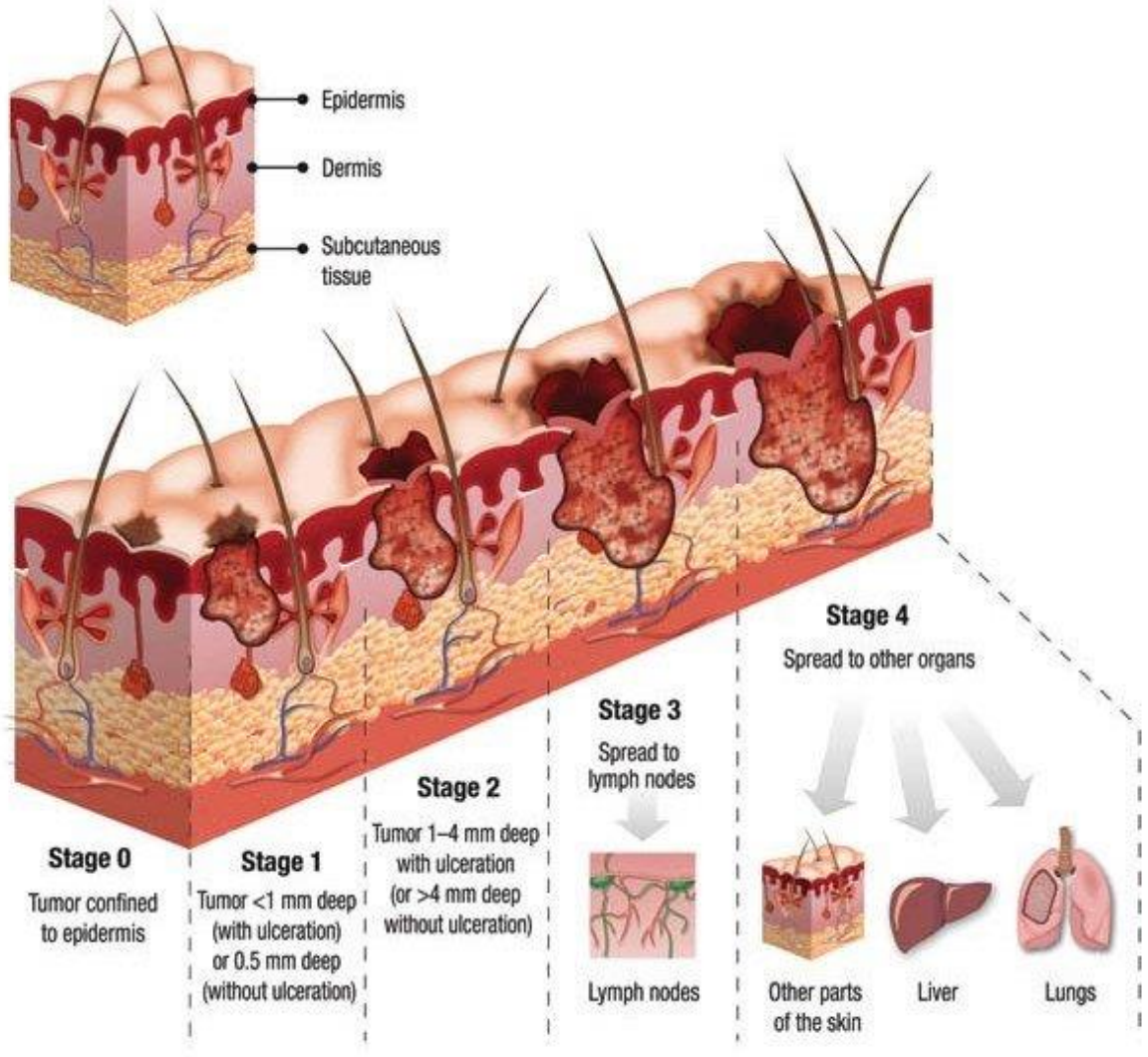


Figure 3. Melanoma cancer stages (Figure from Moqadam *et al., 2018)*[36]

In addition to the tumor stage, the presence of driver mutations should also be investigated as there are drugs targeting the BRAF V600E mutation, usually used in combination with MEK inhibitors. Other approaches are also available such as immune checkpoint blockade (antibodies specific for PD1, PD-L1 or CTLA4), but as it is the case for other drugs, further studies need to focus on determining which patient will benefit from such treatment[38].

For patients that are not eligible for these treatments, chemotherapeutic agents such as temozolomide or dacarbazine can be used in the perspective of reducing the pain and increasing the comfort of the patient but they do not increase the survival[39].

However, chemotherapy is progressively replaced by more adequate approaches such as targeted therapy and immunotherapy.

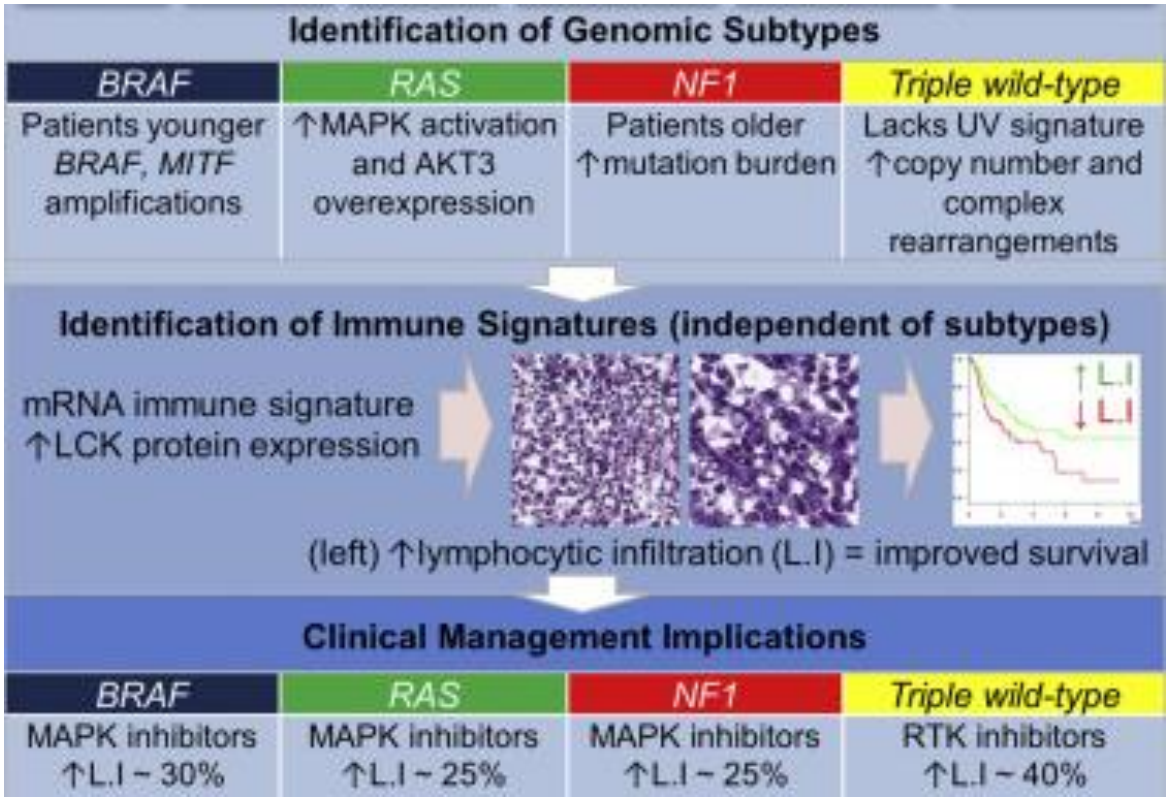


Figure 4. adapted from "Genomic Classification of Cutaneous Melanoma" (Akbani *et al.*, 2015).[39]

Tumor microenvironment

Melanoma is a form of solid tumor. As it is the case for healthy tissues, tumors are not only composed of one type of cells but also other cells responsible for the production of extracellular matrix (fibroblasts), for the formation of blood and lymphatic vessels involved in the delivery of nutrients (endothelial cells) but also immune cells that can be recruited to the tumor site (Figure 5)[40]. The infiltration of the tumor by immune cells can be used to divide the tumors in different subtypes. A first classification divided the tumors into cold (no immune cell infiltration) and hot (immune cells infiltrated) tumors. A more refined approach subdividing the tumors in groups takes the localization of the infiltration also into account[41]. Indeed, tumors with immune cell infiltrations only in the perivascular region do not behave in the same fashion as tumors with diffuse immune cell infiltrations.

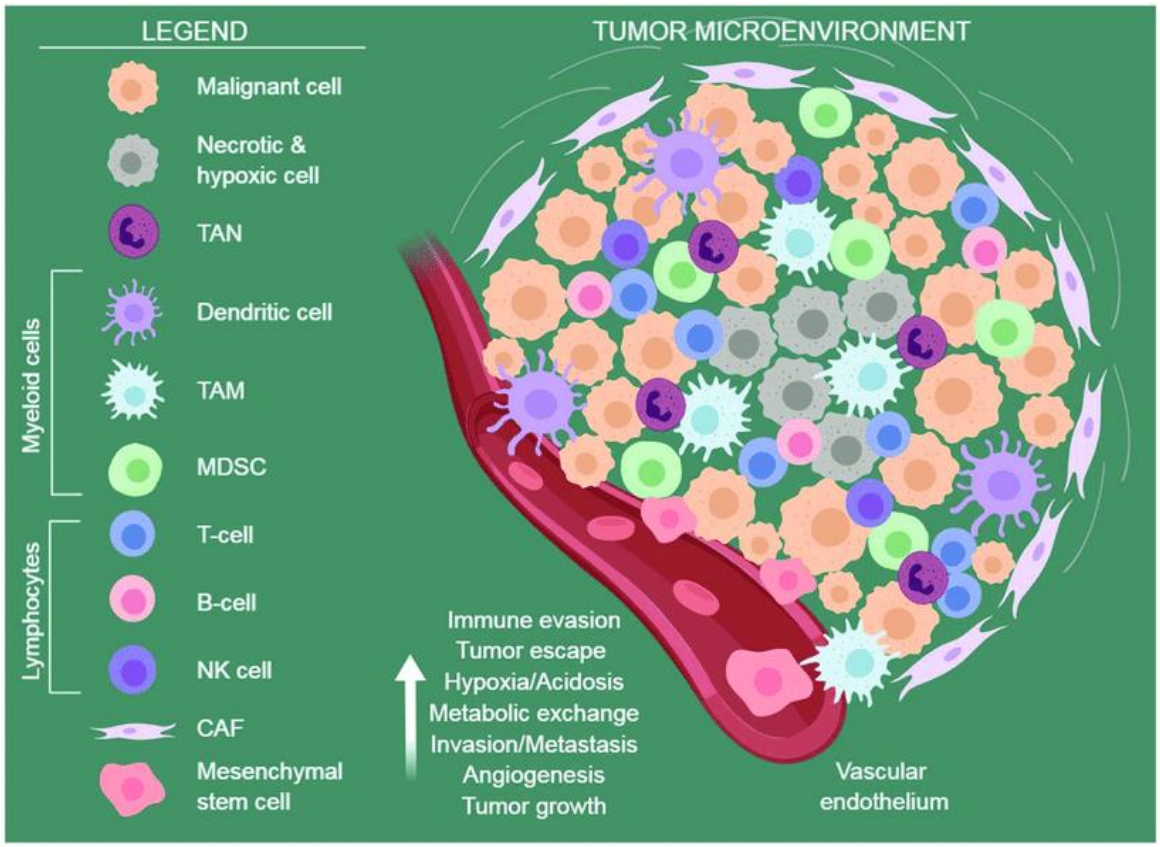


Figure 5. The tumor microenvironment and its immunological and non-immunological components (Figure taken from Audrito *et al.*, 2019) [42].

Infiltration of immune cells in the tumor

The immune cell infiltration of melanoma tumors can be very heterogeneous with many immune cell types present such as granulocytes, dendritic cells, macrophages, myeloid derived suppressor cells (MDSC), T cells, and Natural Killer cells (NK cells) [41]. Immune infiltration is very complex and determining the cell type present is not sufficient to know its role in the tumor. Indeed, immune cells such as macrophages can have very different roles in the tumor. Some macrophages are showing pro-inflammatory phenotype and are thus called M1-like macrophages. They support the immune reaction against the tumor. In contrast, other macrophages have an M2-like phenotype and dampen the immune response[43]. It is necessary to specify that M1 and M2 are not the only possible phenotypes and that they may just be two extremes of a gradient.

High infiltration of melanoma tumors by T cells is generally associated with a better clinical outcome for the patient[44]. This association is strengthened when focusing on CD8+ CTLs and CD4+ Th1 cells. Interestingly, this observation is not restricted to melanoma but remains true across many cancer types[45,46,47] and led to the development of a classification of tumors based on the number and the localization of tumor infiltrating CD8+ T cells as those can be present in the whole tumor or only restricted to the margins[41]. However, not all T cells are beneficial for the

patient. Indeed, the presence of other T cells such as Tregs is associated with a poor prognosis as these cells are potent immunosuppressors[48].

As some of the immune cell types present in the tumor may have pro or antitumor effects depending on their polarization, targeting a whole population of cells would not necessarily benefit the patient.

The role of cytokines in the tumor microenvironment

As the tumor microenvironment is composed of diverse cell types, it is not surprising that different cytokines and chemokines are also present. We can describe a few examples and try to classify them based on their pro- or anti-tumoral role but this classification is complex as their role is not always clear and can highly depend on the context.

Among the anti-tumoral cytokines, IFNγ is one of the most prominent one[49]. Its antitumoral effects are broad. It includes supporting the ongoing immune response through its effect on T cells, increasing antigen presentation as well as altering the panel of antigens presented to more immunogenic peptides. It can also induce the expression of chemokines such as CXCL9 and CXCL10 which are potent recruiter of T cells and thus strengthen the antitumoral immune reaction[50] in addition to its antiproliferative activity on tumor cells[51]. However, IFNγ also leads to the expression of molecules that can dampen the antitumoral activity by directly acting on T cells or by recruiting immunosuppressive cells[52]. Those will be described later in the immune escape mechanisms part.

Tumor Necrosis factor is another prominent cytokine in the tumor microenvironment but its precise role is still under discussion which is in correlation with its many diversified effects. It has been used for decades as treatment for locally advanced melanoma of the extremities[53] but the observation that it is constitutively expressed in different cancer types including breast and ovarian cancer[54] underline its still unclear role. One hypothesis is that in some cancers, it activates the NFkB pathway and thus has a pro-survival effect while in other the pro-apoptotic pathway is the most prominent one. The antitumoral role of TNFα is further questioned by the observation that it may increase cell invasion and angiogenesis[55]. Aberrant constitutive MHC-II expression has been observed in some melanoma, leading to the recruitment of CD4+ T cells that appeared to be mostly monofunctional by only producing TNFα which in turn reduced CD8+ T cell activation[56].

Similarly to TNFα, Lymphotoxin α is also part of the TNF superfamily. It can also be produced by T cells and has pro-apoptotic activity. It has a known role in the development of tertiary lymphoid structures in cancer and thus could support the establishment of an antitumoral immune reaction against tumor cells[57].

Interleukin-2 (IL-2) can be classified among the antitumoral cytokines but this is due to its pro-survival effect. Indeed, T cells, especially in the tumor where they have to face many inhibitory signals and stress, rely on different factors such as IL-2 to maintain their viability and activity[58].

Interleukin- 6 is a pleiotropic cytokine that can be produced by many cell types including melanoma cells themselve, suggesting that its role is more in favor of the tumor which is supported by the correlation between the poor prognosis and the IL-6 level in the serum of melanoma patients[59].

Interleukin-4, Interleukin-5 and Interleukin-13 have been poorly investigated in melanoma so far. Further investigations are still required but it is already known that IL-4 is involved in the NK cell response against the tumor[60] and IL-5 seems to favor the establishment of a pro-tumoral environment by dampening the activation of T cells and M1 macrophages and thus its blockade prevented melanoma metastases in the lung[61]. IL13R is expressed by more than 7% of melanoma and its expression is associated with a decreased proliferation in vitro[62].

In addition to cytokines, we also have many chemokines in the tumor. Some have been used as therapy such as Granulocyte-Macrophage Colony-Stimulating-Factor (GM-CSF) through intra tumoral injections but the efficacy of such interventions remain unclear[63]. Its use in the clinic is due to its role in the maturation of dendritic cells from monocytes and thus remains of interest as an adjuvant for vaccines. It showed interesting results but only when used with antigen-loaded DC or cytolytic virus injected into the tumor[64, 65].

CCL3 and CCL4 can be produced by a wide range of immune cells and in turn can recruit other immune cells such as neutrophils and macrophages[65].

CCL2 is a chemokine that is known for its role in the recruitment of monocytes and dendritic cells. It has been targeted in the clinic but the results are not clear[66]. One hypothesis is that the role of CCL2 may depend on the context in which the recruited cells are found. Indeed, monocytes recruited in a tumor having a strong immunosuppressive TME may lead to the maturation of the monocytes into immunosuppressive cells while monocytes recruited in a tumor where a strong immune response is taking place may differentiate into dendritic cells that will further support the antitumoral immune response[67].

CXCL9 and CXCL10 have a clearer antitumoral role as it can be seen with their strong correlation with good prognosis. This is in line with their role as potent T cell recruiter[68].

Immunotherapy

The correlation between the presence of certain immune cells in the tumor microenvironment and the survival of the patients suggested a role for these immune cells in the fight against the tumor. Immunosurveillance hypothesizes that the immune system is involved in the surveillance and the elimination of potential tumors[69]. This hypothesis would have been strengthened by the observation that there is a higher occurrence of cancer in immunocompromised patients but this has not been seen[70]. In vitro assays have shown that CD8+ T cells have the capacity to kill tumor cells but validating the immunosurveillance hypothesis requires that this kind of event occurs frequently in vivo in absence of intervention. Recent investigations performed on "healthy" tissues such as the esophagus revealed the presence of many abnormal cells, some forming clusters, carrying (driver) mutations that are typically found in tumors[71]. It remains unclear whether these cells are cleared by the immune system or if they just fail to become malignant.

Many investigations such as in vitro assays showing that CD8+ CTLs have the capacity to eliminate tumor cells opened a new chapter in the treatment of cancer as it became clear that boosting the antitumor activity of these tumor-specific T cells could allow tumor elimination. In addition, and in contrast to other cancer therapies such as chemotherapy, the immune system presents the advantage of developing memory cells and thus could potentially act long-term and prevent recurrence of cancer[72]. Indeed, clinical benefits of boosting T cell-based antitumor immune response are achieved by transferring tumor specific T cells and by blocking immune checkpoints. The first approach relies on increasing the tumor-specific T cell per tumor cell ratio while the second approach aims at preventing the inactivation of tumor-specific T cells. Other approaches have also been investigated but with less success so far. It includes vaccination of cancer patients with tumor antigens and adjuvants, using peptides or mRNA[73].

Tumor-specific antigens

The adaptive immune system is very efficient at clearing infectious agents but it strongly relies on the identification of antigens that are absent from self, thus very different from self-molecules. The establishment of a potent antitumoral immune response, supported or not by a medical intervention, also relies on the identification of specific antigens in order to prevent damages to healthy tissues. In this regard, cancers induced by viruses present the advantage of carrying viral antigens which are common between the tumors that have been induced by the same virus and thus similarly targeting treatment could be given to these patients[74]. The transition from healthy to cancerous cells relies on the acquisition of several mutations. These mutations, which are highly abundant in melanoma due to UV-induced DNA damage, give rise to antigens that are not present in healthy tissues and to which no central tolerance has been established[75]. The advantage of these antigens is that they are

tumor specific but the chances of having the same mutation and thus the same antigen in different patients is very low. This strongly limits the use of these antigens as they are highly patient specific. But one could imagine that the advancement in personalized medicine will make it become easier to produce patient-individual therapies[76]. Some genes are only expressed by cells lacking MHC-I or cells only present in immune privileged compartments[77]. As fragments of the proteins encoded by these genes will never be presented on MHC-I by healthy cells, no central tolerance has to be established towards them. However, some cancer cells, through the uncontrolled demethylation of these genes' promoters, may end up expressing antigens that are potentially targetable without targeting healthy tissues[78]. The classes of antigens that have recently been described have the advantage of being only expressed by tumor cells. Some antigens are normally expressed at a low level by healthy cells. One example is the differentiation antigen MelanA. It is slightly expressed by melanocytes but may be expressed at high levels in melanoma. As it is normally only slightly expressed, the tolerance towards this antigen is not very potent and can be disrupted. This means that an immune response could be mounted against MelanA[78], mostly focusing on tumor cells. However, damages to healthy tissues expressing low levels of MelanA may nevertheless occur.

Adoptive cell therapy

High numbers of tumor-specific T cells can boost the antitumoral immune response. However, this requires increasing the numbers of tumor-specific T cells from the patient[79]. One way to bypass the identification of tumor-specific T cells is by directly isolating T cells that infiltrated the tumor tissue knowing that they are tumor-specific due to their localization. These cells need to go through an expansion phase ex vivo in a laboratory incubator before being re-infused to the patients[80]. It is also possible to take T cells directly from the blood of the patient. However, this then requires the selection of tumor-specific T cells and thus the identification of antigens present in the tumor. To avoid this T cell specificity selection, it is also possible to transfect the patient's T cells with TCRs or chimeric antigen receptors (CARs) that are specific for an antigen present on the tumor cells[81]. This approach could be used in melanoma but it requires specific antigens as well as TCRs or CARs and thus is more suited for some other cancers such as B cell lymphoma where targeting CD19/CD20 showed great results[79].

Immune checkpoint blockade

It has been described above that some autoreactive T cells may escape elimination in the thymus and thus that these cells can potentially cause damage to healthy tissue. To prevent such events, the immune reaction is usually inhibited by so-called immune checkpoint receptors expressed on the T

cells[82]. The binding of these receptors to their corresponding ligands will attenuate the T cell response. In a healthy organism, these interactions can prevent damage to healthy tissue but they also limit the activation of T cells during chronic viral infection for example which could prevent clearance of the virus. It is often not clear whether this process takes place in order to prevent an overactivation of the immune system against the virus that could do more harm than good or whether this mechanism is just hijacked by the virus to prevent its clearance. Similarly, the immune checkpoints are also involved in the regulation of the immune reaction against cancer cells. Although a whole panel of these receptors have been described[83], the two major ones that may show great results in the clinic are PD1 and CTLA4[84]. Diverse blocking antibodies have been developed in order to prevent the interaction of these inhibitory receptors with their corresponding ligands. Nivolumab and pembrolizumab are targeting the PD1-PD-L1 interaction while ipilimumab is targeting the CTLA4-CD80/CD86 interaction. All three types of antibodies are now available and used in the clinic for different types of cancer. Interestingly, PD1 and CTLA4 do not come into play at the same time. CTLA4 is mainly involved in preventing the initial activation of the T cells during the T cell-APC interaction while PD1 may attenuate the already ongoing T cell immune response[85]. As both the initiation of an antitumoral immune response and the activation of T cells inside the tumors are often found deficient in cancer patients, both treatments work and the combination of the two blockades can further improve the antitumor immune response in patients with metastatic melanoma[86]. However, as these immune checkpoints are normally involved in preventing the activation of self-antigen specific T cells, blocking them also leads to the activation of self-reactive T cells and thus cause autoimmune damage to healthy tissue[87]. In this regard, a close monitoring of the patient is required and this calls for the development of a new generation of drugs that more specifically target only the antitumor immune response.

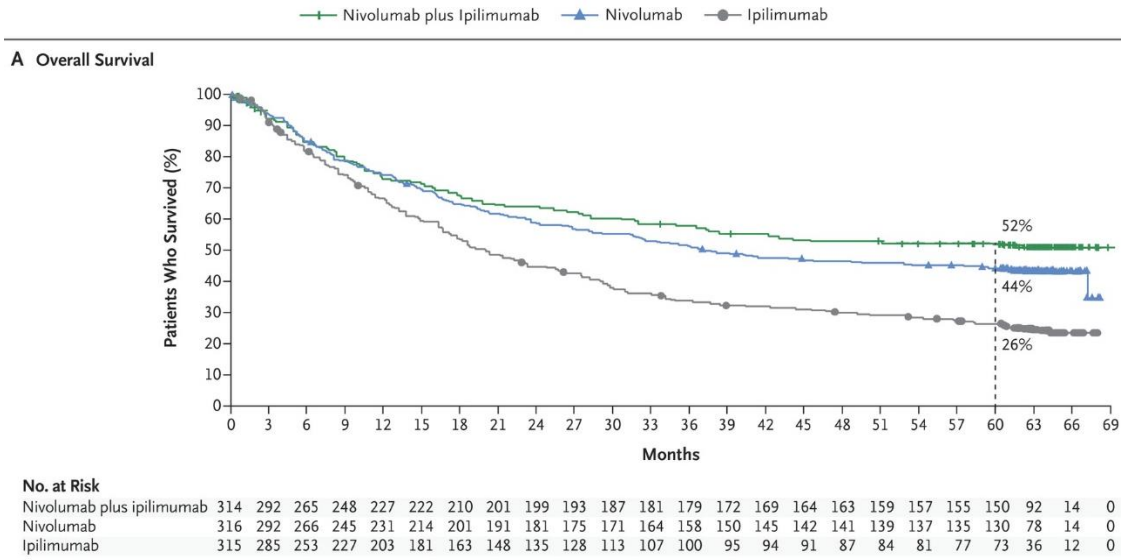


Figure 6. Overall survival of patients with advanced melanoma receiving Nivolumab and Ipilimumab alone or in combination. (Adapted from Larkin et al., 2019)[87]

Immune escape mechanisms

The co-presence and persistence of both the tumor and the antitumoral immune response emphasizes the fact that the immune system is often not able to clear the tumor and that tumor cells can directly or indirectly acquire mechanisms to evade immune mediated elimination. There are several mechanisms that explain this immune evasion. As the antitumoral immune response relies on the recognition of tumor specific antigens, a tumor cell that loses or hides these antigens could escape immune recognition and thus be selected to overgrow and cause cancer relapse or progression. This can occur when cancer cells stop the expression of the antigen or prevent its presentation on the cell surface. These mechanisms have already been described in melanoma with the loss of MelanA[88].

As described in the previous paragraph, the organism has tools to induce peripheral tolerance in the periphery in the case of an auto-immune reaction. The various mechanisms involved in the establishment of peripheral tolerance can be hijacked by tumor cells. One mechanism is the suboptimal stimulation of T cells by APC through the CTLA4-CD80/86 interaction. In the case where an immune response is still established, it can be inhibited directly at the tumor site by the expression of PD-L1 and other inhibitor receptor ligands by tumor cells and consequent inhibition of T cells. Tregs can also come into play by secreting anti-inflammatory cytokines such as IL10 and TGFβ or expressing CTLA4[89]. Other secreted factors can also dampen T cell activity such as Indoleamine 2,3 dioxygenase (IDO) that acts by depleting the tryptophan available for the T cells in the environment and by producing kynurenins which are cytotoxic for T cells[90]. It is challenging to know whether the presence of these mechanisms reflect good prognosis or not, as those are inhibiting the antitumoral immune reaction which reduces its usefulness, but they also suggest that an antitumoral immune response is actually taking place and that appropriate immunotherapy could restore its activity by directly boosting it or by inhibiting inhibitory mechanisms.

Although immunotherapy showed great success in different types of cancer, some patients did not benefit from the treatment and some relapsed, suggesting that there are mechanisms of resistance to this type of intervention. Part of these mechanisms can be similar to the one that are observed in absence of immunotherapy. For example, the loss of MHC-I or the tumor antigens is still effective even when the antitumoral immune response is supported by immunotherapy as there is no target anymore. When facing a stronger antitumoral immune response, the tumor can respond by reinforcing its immunosuppressive tumor microenvironment and this can be achieved by e.g. recruiting immunosuppressive cells though CSF-1 expression in response to the cytokines that are produced by the antitumoral immune response[91]. This direct us to the ability of the tumor cells to

respond to the immunological cues present in the tumor microenvironment. This observation shows that the ability of melanoma cells to respond to cytokines is a key factor influencing the efficacy of immunotherapy. In a murine melanoma model, it has been shown that a tumor cell specific loss of PTPN2, a negative regulator of IFNγ signaling increased the efficacy of PD-1 checkpoint blockade[92]. It is suggested that the loss of PTPN2 prevents the inhibition of the IFNγ signalling and thus leads to a higher antigen presentation and stronger growth inhibition. Similarly, a study in human investigating the response of melanoma patients to PD-1 blockade discovered a genetic alteration in the tumor cells leading to the loss of JAK1 during the treatment[93]. This mutation was not detected in samples taken before anti-PD-1 treatment and occurred at the time of relapse. As cancer treatment relies more and more on immunotherapy, we will observe that some patients are benefitting from it but we will also observe relapse as well as non-responders. This calls for a better characterization of the role of the tumor cells in response to immunological cues present in the tumor microenvironment in order to improve the efficacy of immunotherapy.

All these observations are calling for a better understanding of the role of the different cytokines that can be found in the tumor and how they may shape the tumor microenvironment to influence the outcome of immunotherapy. This research focuses on the effect of different cytokines on the immunological phenotype as well as the diversity of the response of patient derived melanoma cells. Additionally, investigations on the mechanisms explaining this heterogeneity have been performed with the aim of understanding the effects of these mechanisms in the context of immunotherapy.

The following investigations were performed with melanoma cell lines established at the Ludwig Institute for Cancer Research and the University Hospital of Lausanne. The treatment history of the patients was known and was continuously followed beyond the establishment of the cell lines. The presence of absence of some driver mutations was already assessed in previous studies but it was reported that this aspect does not influence the immunological phenotypes of the melanoma cells.[94]

Material and methods

Establishment of cell lines

Tumor specimens from surgery were collected and cut into small pieces and transferred in complete RPMI-FCS (RPMI 1640 GlutaMAXTM-1 (Gibco 61870010) supplemented with 10% heat-inactivated FCS (GIBCO 10270), 1.1uM Arginine (Sigma Aldrich), 0.48uM Asparagine (Sigma Aldrich), 11.25uM Glutamine (Gibco), 10nM Hepes (Gibco), 100U/mL of Penicillin/Streptomycin (Gibco). Tissue pieces were cultured at 37°C and 5% CO_2 until cells attached to the culture dish. Remaining pieces of tumor and dead cells were removed and fibroblasts were eliminated by treating the culture with Geneticin G418 at 25ug/mL for 4 days. This last treatment step could be repeated after letting the cells rest for 2 days in complete RPMI medium.

Cell cultures

Melanoma cell lines have been previously established from surgery specimens with proven metastatic melanoma and were provided by Donata Rimoldi from the Ludwig Center for Cancer Research and the Department of Oncology of the University Hospital of Lausanne (procedure described above). NHM were provided by L. Michalik from the Center for Integrative Genomics of the University of Lausanne. HEMA-LP were purchased from ThermoFisher Scientific (C0245C).

Human melanoma cell lines were cultured in Complete RPMI-FCS (RPMI 1640 GlutaMAXTM-1 (Gibco 61870010) supplemented with 10% heat-inactivated FCS (GIBCO 10270), 1.1uM Arginine (Sigma Aldrich), 0.48uM Asparagine (Sigma Aldrich), 11.25uM Glutamine (Gibco), 10nM Hepes(Gibco), 100U/mL of Penicillin/Streptomycin (Gibco) and 10ug/mL of Ciprofloxacin (Fresenius KABI 15LL514P1). Cells were splitted when approaching 100% confluency and the medium was changed at least twice a week. Splitting ratio and frequency highly varied between cell lines but ranged from 1/10 twice a week to 1/2 every two weeks.

NHM were cultured in Media M2 (Promocell C-24300). Cells were splitted when approaching 100% confluency and the medium was changed at least twice a week. When splitting the cells, the confluency was maintained over 30% as low confluency reduced proliferation.

HEMA-LP were cultured in Medium 254 (ThermoFisher Scientific M254500) supplemented with PMA-Free Human Melanocyte Growth Supplement-2 (ThermoFisher Scientific S-016-5). Medium was changed every 2 or 3 days and the cells were splitted when confluency approached 100%. HEMA-LP growth is confluency-dependent and thus we tried to maintain a confluency between 50% and 100% to increase cell proliferation.

Cells were detached using StemPro Accutase (Gibco A11104-01) for 3 to 5min after a rapid PBS wash. Cell count and viability was assessed with the automated cell counter ADAM MC

(NanoEntek) and the corresponding AccuChip (NanoEntek AD4K-200). Discrimination of cell count and viability relied on propidium iodide staining and image analysis. Propidium iodide stains DNA but can not pass through the membrane and thus only stains the DNA of dead cells.

Cells were incubated at 37°C and 5% CO_2. Cells were not kept in culture for more than 6 months and were discarded if the proliferation or the viability seemed altered. Homemade PCR based mycoplasma test revealed that the cell lines remained mycoplasma free.

Cytokine-stimulation and inhibitor treatment of human melanoma cells and melanocytes

Cells were plated in 12 well plates at least 24h before treatment to have well attached cells at the time of cytokine-stimulation. Cell culture medium (Complete RPMI-FCS or corresponding medium for melanocytes) was removed and a rapid wash with PBS was performed to remove dead or unattached cells. Cytokines were added in Complete RPMI-FCS for melanoma cell lines and melanocytes.

We used the following cytokines: IFNγ (Peprotech AF-300-02), TNFα (Peprotech AF-300-01A), TNFβ (Peprotech 300-01B), IL6 (Peprotech AF-200-06), CCL3 (Peprotech AF-300-08), CCL4 (Peprotech 300-09), GM-CSF (Peprotech 300-03), IL2 (Proleukin), IL4 (Peprotech AF-200-04), IL5 (Peprotech 200-05), IL13 (Peprotech AF-200-13)

Table 1: Seeding of melanoma cells in 12 well plates for cytokine responsiveness assessment

Melanoma cell line	Cells/well		Melanoma cell line	Cells/well		Melanocytes	Cells/well
Me215	210000		T640A	140000		NHM	180000
Me235	110000		T672E	100000		HEMA-LP	180000
Me252	110000		T685A	140000			
Me257	110000		T975A	140000			
Me260LN	130000		T1013A	120000			
Me275	120000		T1015A	110000			
Me290	120000		T1185B	130000			
T311B	110000		T1194B	140000			
T333A	100000		T1257	110000			
T362C	75000		T1349A	180000			
T618A	110000						

Cell numbers were adapted for bigger and smaller plates according to the culture surface of the respective plate format.

Cell culture consumables

Cells were cultured in TPP Tissue Culture Flasks (25cm^2 ref 90026, 50cm^2 ref 90076 or 150cm^2 ref 900121). For experiments, cells were plated in Corning Costar Clear TC-treated Multiple Well

plate (6 well plate with 9.5cm^2/well ref 3506, 12 well plate with 3.69.5cm^2/well ref 3512, 24 well plate with 1.9cm^2/well ref 3527, 48 well plate with 0.95cm^2/well ref 3585)

Flow cytometry

BrefeldinA at 10ug/mL (eBioscience Brefeldin A Solution 1000x 00-4506-51) was added to the culture for 4hours before harvesting the cells for intracellular staining. Cells were collected with accutase after a wash step with cold PBS. Cell number was assessed during the first experiments but this step was removed from the protocol as antibodies were titrated with 500'000 cells which is more than the number of cells present in the well at the time of collection. Viability staining was performed with TO-PRO3 Iodide 1uL/mL (Invitrogen T3605) or Green Fluorescent Reactive dye 1uL/mL (Invitrogen 1831847) in PBS for 30min at 4°C. Cells were washed with FACS buffer (PBS supplemented with 5 mM EDTA, 0.2% BSA and 0.2% NaAzide) and stained with fluorochrome coupled antibodies in FACS buffer for 30min at 4°C. Cells were washed and fixed in FIX buffer (PBS containing 1% Formaldehyde, 2% Glucose, 5 mM NaAzide) for 30min at 4°C before being permeabilized in FACS buffer supplemented with 0.1% Saponin (Sigma Aldrich) and subsequently stained for 30min at 4°C using similar buffer. Afterwards, cells were washed in FACS buffer and kept at 4°C before samples were analyzed at a flow cytometer. Acquisition of the samples was performed using the BD FACS CANTO I machine and the BD FACSDiva v9.0 software. Compensation were performed using OneComp eBeads Compensation Beads (Invitrogen 01-1111-41). Data were analyzed using FlowJo v10. Gating on the cells was achieved by excluding debris and cell doublets thanks to the Forward and Side scatter and dead cells were excluded using the live/dead cell staining. Viability and GMFI values of stainings were extracted for further analysis.

Table 2: Antibodies used for Flow Cytometry

Antigen	Fluorescent dye	Company	Ref
HLA-I	PerCP-Cy5.5	Biolegend	311420
HLA-DR	FITC	Biolegend	307604
PD-L1	PE-Cy7	BD	558017
ICAM	Pacific Blue	Biolegend	353109
CXCL9	PE	Biolegend	357904
CXCL10	PE	Biolegend	519504
CCL2	APC	eBioscience	17-7099-81
ICAM-I	Pacific Blue	Biolegend	353109
IDO	Pacific Blue		

IDO antibody was kindly provided by Benoit Van den Eynde from the Ludwig Cancer Research Institute in Brussels.

Proliferation assay

1 million cells are collected and washed with PBS before being incubated for 7 min at room temperature in the dark with 1mL of Cell Trace Violet (Invitrogen C34557) at 5uM in PBS. Cells are then washed with RPMI-FCS to remove the excess of CTV. Viability and cell count were assessed with the ADAM MC cell counter and cells were plated as indicated in the following table in 12 well plates. In parallel, unstained cells were also plated to be used as CTV- control. A fraction of the cells were directly fixed (30min at 4°C in FIX buffer (PBS containing 1% Formaldehyde, 2% Glucose, 5 mM NaAzide) after CTV staining and kept at 4°C in FACS buffer (PBS supplemented with 5 mM EDTA, 0.2% BSA and 0.2% NaAzide) to be used as CTV+ control at the end of the experiment. Treatment occurred the day after the cells were plated with the cytokines indicated on the figures. At the day of treatment, medium was changed and 3mL of complete RPMI-FCS with cytokines was added to each well. At day 5, cells were detached with accutase after a PBS wash. The viability staining was performed with Green Fluorescent Reactive dye. The staining and fixation were performed as described in the Flow Cytometry section

Table 3: Cell seeding for proliferation assay in 12 well plates

Melanoma cell line	Cells/well		Melanoma cell line	Cells/well
Me215	65000		T640A	45000
Me235	35000		T672E	31000
Me252	35000		T685A	45000
Me257	35000		T975A	40000
Me260LN	40000		T1013A	35000
Me275	37500		T1015A	45000
Me290	35700		T1185B	40000
T311B	35000		T1194B	45000
T333A	30000		T1257	35000
T362C	20000		T1349A	60000
T618A	35000			

Whole exome sequencing

DNA isolation of 3million cells was performed using the DNeasy Blood & Tissue Kit QIAGEN (69504). Similar procedure was performed using blood samples from corresponding patients to have germline DNA. DNA concentration and purity was assessed by Nanodrop to confirm the quality. 260/280 and 260/230 were around 1.8 and between 1.8 and 2.2 respectively. Whole exome sequencing was performed at the Genomic Facility of Lausanne. Paired-end reads sequencing was performed with a read length of 100bp and a minimum mean coverage of 70fold. The whole exome sequencing (WES) of 15 melanoma cell lines and autologous PBMC were performed at the Lausanne Genomic Technologies Facility using the Agilent SureSelect All Exon V8. The

remaining 6 melanoma cell lines and the autologous PBMC were sequenced at the University of Los Angeles using the Nimble Gen SeqCap EZ Human Exome Library version 3.

Analysis of whole exome sequencing data has been performed by Sina Nassiri and Joao Lourenco from the Swiss Institute of Bioinformatics under the supervision of Mauro Delorenzi. PBMC were used to assess whether the genetic alterations that were observed in the Whole Exome Sequencing data were acquired by the tumor cells or if they were already present in the germline DNA indicating that they are not mutations but single nucleotide polymorphism (SNP). Whole exome sequencing data from melanoma cell lines for which we did not have autologous PBMC were compared to a set of reference genomes with the aim of capturing at least part of the SNP and thus limiting the number of mutations reported. We observed that this second approach is less efficient as we report approximately 5 times more mutations in the cell lines that were compared to the set of reference genomes than the one compared to autologous PBMC. Copy number alteration was assessed the following way: Assuming diploidy, the number of reads for each location should be the same. Having less reads than expected indicates that one copy of the gene was loss while having no read indicates that the gene is lost. An increased number of reads indicates that more than two copies of the genes are present. As it is possible that only part of the gene is copied, the mean number of reads for each gene was used to determine copy number. In combination with the sequencing data, this approach can also indicate the frequency of each allele individually. Graphs from Figures 7 and 8 were designed in collaboration with Joao Lourenco.

RNA extraction

Between 10^5 and 2*10^5 cells were plated in 12 well plates on day 0 and treated with cytokines the following day before being harvested at day 2. RNA isolation was performed using the RNeasy Plus Mini Kit (Qiagen 74134) according to the manufacturer´s instruction. RNA quantification and purity were assessed by Nanodrop.

Realtime PCR

Reverse transcription of 100 or 200ng RNA was performed using High Capacity cDNA Reverse transcription Kit (ThermoFisher Scientific 4368814). Reverse transcription was performed as described by the manufacturer's protocol. 100ng (or 200ng by scaling the reagents accordingly) RNA were used for reverse transcription. The volume of the reaction was 20uL (or 40uL) and the samples were kept on ice. The reverse transcription was performed in a thermoblock with a first step of 10min at 25°C, a second step of 120min at 37°C and a third step of 5min at 85°C.The thermoblock then cooled the samples to 4°C. Reverse transcription products were diluted in endonuclease free water to have a final concentration of 1.25ng/uL. Samples were frozen at -20°C.

qPCR was performed using the SYBR Select Master Mix (applied Biosystems 4472903) using 2.5ng of cDNA and 1uM of primers. Primer efficiency was assessed on melanoma cells or PBMC to ensure efficiency and specificity by migrating the final product on agarose gels.

The following primers were used for realtime PCR: GAPDH (For 5'-GGA CCT GAC CTG CCG TCT AG-3' and Rev 5'-CCA CCA CCC TGT TGC TGT AG-3'), S16 (For 5'-TCG GAC GCA AGA AGA CAG CG-3' and Rev 5'-AGC AGC TTG TAC TGT AGC GTG-3'), IFNGR1 (For 5'-CAT CAC GTC ATA CCA GCC ATT T-3' and Rev 5'-CTG GAT TGT CTT CGG TAT GCAT-3'), IFNGR2 (For 5'-TGG ACA AGG ACA GCT CAC CA-3' and Rev 5'-TCA AAG CGT TTG GAG AAC ATC TT-3'), TNFR1 (For 5'-TGC CTA CCC CAG ATT GAG AA-3' and Rev 5'-ATT TCC CAC AAA CAA TGG AGT AG-3'), TNFR2 (For 5'-CTT ACC CCA GCC AGT GTC C-3' and Rev 5'-AAG GAG GTG CTT GGA GCA G-3'), JAK1 exon 7-9 (For 5'-CCT CCT CGT GGG AAG AGA GC-3' and Rev 5'-TCT TGG AAT CCA GTG GAG GC-3'), JAK1 exon 15-17 (For 5'-GGG GGT CCT CTG GAT CTC TT-3' and Rev 5'-GGG GAT CTC GCC ATT GTA GC-3') and JAK1 18-20 (For 5'-GGA GCT GGC TGA CCT CAT GA-3' and Rev 5'-CTG CAG AGC TCA ACC TTC CC-3').

qPCR was performed using the Illumina Eco (Illumina Proprietary EC-900-1001) with the following cycling parameters: initial denaturation step (50C for 120sec, 95C for 600sec) followed by 40 cycles DNA amplification (95C for 10sec and 60C for 30sec). The melting curve was obtained by heating the samples to 95C for 15sec, cooling to 55C for 15sec and heating again to 95C for 15sec. The melting curve protocol was added to estimate primers' specificity by checking that only one peak was present. The PCR product size was further verified by migrating the qPCR product on a home-made 2% agarose (PeqGold 35-1020) gel (1g agarose in 50mL TBE buffer was heated in the microwave until complete agarose dissolution and then SYBR Safe DNA Gel Stain (Invitrogen S33102) was added to visualize the DNA) for 20min at 100V. Primer efficiency was assessed by a 10-fold dilution series using 10ng, 1ng, 0.1ng 0.01ng or 0ng of cDNA for the qPCR reaction and verifying that 3.3 cycles were separating each dilution step. Primers that had an efficiency lower than 90% were not used. JAK1, IFNGR1, IFNGR2, TNFR1 and TNFR2 primers were tested on PBMC while GAPDH and S16 were tested on melanoma cells.

Analysis of the amplification curves was performed using the EcoStudy software v5.0. Ct values were extracted. GAPDH and S16 were used as housekeeping genes for normalization. Normalization was performed as followed:

Normalized expression= $2^{-(Ctgene\ of\ interest - mean(CtS16\ and\ CtGAPDH))}$

Western blot

Cells were plated on day 0 at around 90% confluency and collected the following day. For whole cell protein analysis, the cells were directly lysed in the plates using RIPA buffer (Thermo Fisher scientific 89900) supplemented with proteases inhibitors (cOmplete Protease Inhibitor Cocktail, Roche 11697498001), phosphatase inhibitors (PhosSTOP, Roche 4906845001) and DNase I 100U/mL (Roche 776 785) for 10min on ice after a quick wash with cold PBS. The lysate was then collected and centrifuged for 10 min at 11'000g at 4°C. Supernatant was transferred into new tube and an aliquot was removed for protein quantification with Bradford (Biorad Protein Assay Kit II 5000002). Remaining samples were then mixed with 5X homemade loading buffer (5% β-Mercaptoethanol, 0.02% Bromophenol Blue, 30% Glycerol, 10% SDS, 250mM Tric-Cl pH6.8). Denaturation was achieved by warming the samples for 10min at 95°C. After denaturation samples were stored at -20°C.

For nuclear fractionation, cells were washed with ice cold PBS and detached using accutase. Cells were collected in cold PBS and centrifuged for 5min at 450g at 4°C. Supernatant was discarded. A first lysis step was performed for 10min on ice in PBS supplemented with 0.1% Nonidet P-40 (Cayman Chemical CAY-600009-500) and protease and phosphatase inhibitors (Ref. see above). Samples are then centrifuged for 10min at 4°C and 10'000g and the supernatant is the cytoplasmic fraction. The pellet which represents the nuclear fraction is further lysed for 10min on ice in supplemented RIPA buffer as used for the preparation of whole cell extracts. Nuclear fraction was then sonicated (3sec, on ice, constant power of 2 with the Branson Sonifier 250) to disrupt DNA. Both, cytoplasmic and nuclear fractions are mixed with 5x loading buffer and heated for 10min at 90°C before being stored at -20°C.

Running Gel was prepared by mixing 10% Acrylamide (ProtoGel EC-890), 0.375M Tris HCL pH8.8, 0.001% SDS (AppliChem A3942). 0.001% ammonium persulfate (Thermo Scientific 17874) and 0.001% TEMED. Isopropanol was added on the top of the running gel to avoid bubbles and flatten the gel. Isopropanol was removed once the gel is polymerized and stacking gel (10% Acrylamide, 0.065M Tris HCL pH6.8, 0.001% SDS, 0.001% ammonium persulfate and 0,001% TEMED) was overlayed. 10% acrylamide running gels have been used for all proteins except JAK1 for which 8% acrylamide gels have been used. Migration has been performed using the Mini PROTEAN Tetra Cell system (Biorad 55BR 101679). Migration in the stacking gel was done at 60-80V for approximately 30min while migration in the running gel was performed at 120V for 1h to 1h30 depending on the size of the protein of interest.

Transfer to nitrocellulose membrane has been performed using the Trans-Blot Turbo Transfer System (Biorad 1704150) and the Trans-Blot Turbo RTA Transfer Kit, Nitrocellulose (Biorad 1704271). Transfer setup was 10min at 2.5A and 25V for all proteins except JAK1 for which

transfer time was increased to 15min. Blocking of the membrane was performed with 5% milk powder in TBS for non-phosphorylated proteins or 5% BSA (AppliChem A1391) in TBS for phosphorylated proteins. Membranes were incubated with primary antibodies in 5% BSA in TBS overnight at 4°C with constant agitation. Membranes were washed 3x 10min with TBS-T 0.01% before 1h incubation with the secondary antibody solution (secondary antibody in TBS with 5% BSA) at RT. Revelation was performed using WesternBright ECL HRP substrate (Advansta K-12045) for all antibodies except for JAK1 and nuclear STAT1 for which we used WesternBright Sirius (Advansta K-12043).

Table 4: Antibodies used for western blot

Antigen	Clonality	Species	Reference	Provider	Note	Concentration for use
STAT1	Monoclonal	Rabbit	14994	Cell signalling		1/1000
pSTAT1 (Tyr 701)	Monoclonal	Rabbit	9167	Cell signalling		1/1000
GAPDH	Monoclonal	Rabbit	2118	Cell signalling		1/3000
aTubulin	Monoclonal	Mouse	3873	Cell signalling		1/1000
Lamin A/C	Monoclonal	Mouse	4777	Cell signalling		1/1000
IRF1	Monoclonal	Rabbit	8478	Cell signalling		1/1000
JAK1	Monoclonal	Rabbit	3344	Cell signalling		1/1000
Anti-Rabbit IgG	Monoclonal	Goat	7074P2	Cell signalling	HRP-Linked	1/5000
Anti-Mouse IgG	Monoclonal	Horse	7076P2	Cell signalling	HRP-Linked	1/5000

Cloning of vEGFP and vmCherry vectors

hIRF1-mCherry (VB900088-4049) and hJAK1-EGFP (VB900088-4048) vectors were purchased from Vectorbuilder. Empty vectors containing only the fluorescent proteins mCherry or EGFP were generated by removing IRF1 and JAK1 from the vectors. As the vectors did not contain adequate restriction site, we had to add a new restriction site to the vectors. This was achieved using KAPA HiFi HotStart PCR Kit (KAPABIOSYSTEMS KK2502) with the home designed primers produced by Microsynth For 5'-CTA TAC AAA GTT GGC GGC CG-3' and Rev 5'-ATC GGG CGC GCC GTA CAA AGT GGT GAT GGC CG-3' to insert a second enzyme restriction site for AscI (NEW ENGLAND BioLabs R0558S). PCR was performed as described in the manual and the products were directly digested with AscI in rCutSmart Buffer at 37°C for 30min. AscI was heat inactivated at 80°C for 20min before ligation using T4 DNA ligase. The final product was used to transform competent bacterias as described in the following section. Plasmids collected from the miniprep were digested with AflIII (NEW ENGLAND BioLabs R0541S) and ApaI (NEW ENGLAND BioLabs R0114S) in CutSmart buffer and the size of the products were assessed by gel migration to confirm the the correct plasmids were obtained. Once confirmed that the bacteria contained the right plasmid, bacteria containing the plasmids were thawed and transferred in 2mL of LB medium with 100ug/mL of ampicillin for overnight incubation at 37°C with constant agitation. The following day, the overnight cultures were transferred to 250mL of LB medium with 100ug/mL of

ampicillin for another overnight incubation. Finally, plasmids were isolated using the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen K210006) as described in the manual. Plasmid quantification and purity was assessed by Nanodrop and the plasmid were then kept at -20°C until used for transfection of melanoma cells.

Transformation of competent bacteria

Competent bacteria were thawed on ice for 30min and gently mixed with the 50pg of vectors by flicking the bottom of the tube. Bacteria were incubated on ice for another 30min and then incubated at 42°C for 45sec. Bacteria rested on ice for 2min before being incubated under constant agitation at 37°C for 45min in LB medium. 100uL of bacteria were then seeded on LB Agar plates with 100ug/mL of ampicillin. After incubation overnight at 37°C, bacteria colonies that formed were picked and grew again overnight at 37°C in 2mL of LB medium with 100ug/mL of ampicillin. A fraction of this overnight culture was frozen for preparation of maxiprep. Plasmids were collected using the QIAprep Spin Miniprep Kit (Qiagen 27106) as described in the manual and plasmids concentration were determined by Nanodrop.

Transfection of human melanoma cell lines

Melanoma cells were plated in 24 well plates at a 70% density on day 0 and transfected the following day. On the day of transfection, the medium was replaced with 500uL of fresh complete RPMI-FCS. In parallel, 1.7uL of FuGene HD (Promega E2311) and 1ug of vector were mixed with 25uL of DMEM (Gibco) and incubated for 30min at 24°C. After this incubation period, FuGene HD-vector solution was added to the complete RPMI-FCS and homogenized gently. Cells were incubated overnight with the transfection reagent containing the DNA. 1mL of fresh complete medium was added on the next day. When cytokine treatment was required, it was performed 48h after transfection in new complete RPMI-FCS after washing the cells with PBS.

Statistical analyses

Statistical analyses were performed using Prism v8.2 (GraphPad). Two-way ANOVA was used to analyze the significance of the differences between the conditions except for Figure 9 D and Figure 5. For Figure 9 D, we performed a Mann-Whitney-Wilcoxon test as only two groups had to be compared. For survival of patients, we used a Log-rank (Mantel-Cox) test. When comparing treatment vs control, as in Figure 1 A and 2, two-way ANOVA was followed by a Dunnett test to determine between which group the differences were statistically significant. In other figures, as all conditions were compared, Tukey-Kramer post-hoc tests have been performed with the same goal. The difference between conditions was considered significative when the p-value or adjusted p-

value was lower than 0.05. P-values are shown on the graph or in the table using the following scale: ns = non significative with P>0.05, * P≤0.05, ** P≤0.01, *** P ≤0.001 and **** P ≤ 0.0001.

Table 5: Patients' data: disease stage, treatment, driver mutation and medical interventions

Tumor cell line	Patient	Tumor type	Tumor localization	Diagnosis date	TNM/Stage diagnosis	Sampling date	Previous Therapies (before sampling)	Progression date (from the diagnosis)	Progression-free survival (from the diagnosis) (months)	Progression date (from the sampling)	Progression-free survival (from the sampling) (months)	Death event (Y=1, N=0)	Date of death or last date known alive	Overall survival (from the diagnosis) (months)	Overall survival (from the sampling) (months)	BRAF	NRAS	MEK1	MEK2
Me215	LAU48	Metastases	lymph node	01.09.1992	T1 N0 M0/ I	23.6.1993	Excision primary tumor on right knee (01.09.1992); excision loco regional metastases (01.01.1993); excision of inguinal LN (0N+) and locoregional cutaneous metastases (04.03.1993); ILP and retroperitoneal LN excision (2N+/13)(23.06.1993)	01.01.1993	4.0	28.01.1994	7.2	1	28.04.1995	31.9	22.2	WT	WT	WT	WT
Me235	LAU63	Metastases	lymph node	24.11.1992	pT4N0M0	9.2.1994	Excision nodular lesion on right scapula (24.11.1992); reexcision primary lesion (05.11.1993); Adjuvant treatment wit IFNa-2 (protocol EORTC; 11.02.1993-23.09.1993); excision of a local meta lesion (01.08.1993); Protocol MAGE (16 immunizations; 25.11.1993-15.1.1998); right axillary LN excision (1N+, 02.02.1994)	01.08.1993	8.2	26.10.1995	20,5	1	31.05.2002	114,2	99,7	K601E	WT	WT	E207K
Me252	LAU119	Metastases	lymph node	22.05.1989	pT2a Nx M0/ IB	28.9.1994	Excision lesion left (forearm (22.05.1989); immunotherapy (ganglioside) in 1993 (stopped in Nov 1993 due to absence of development of Ab); left axillary LN excision (2N+/16, 28.09.1994)	01.04.1993	46,4	NA	NA	1	30.05.2015	312,4	248,2	V600E	WT	F53L	WT
Me257	LAU145	Metastases	skin	01.09.1992	IIIA	14.12.1994	Excision of 2 tumor nodules (left leg (01.09.1992); excision metastase on left tibia (21.04.1993); large reexcision primary lesion and excision of nodular dermo-hypodermic metastases (09.06.1993); excision of several cutaneous and subcutaneous nodular metastatic lesions (from July 1993 to April 1994); ILP (TNFa+IFNg+Melphalan, 31.08.1994); excision of a residual cutaneous metastase and of a new metastatic nodule on Achille's tendon (14.12.1994)	21.04.1993	7,6	NA	NA	1	18.03.1996	42,5	15,1	WT	Q61L	WT	WT
Me260.LN	LAU149	Metastases	lymph node	21.12.1977	pT2bN0M0	11.1.1995	Excision lesion on left leg (21.12.1977); LN excisionr; 1 metastase with capsular effraction (07.06.1994); Protocol EORTC IFNa2a+IL-2 (19.08.1994-12.11.1994)	01.06.1994	197,4	12.12.1997	35,0	1	29.07.2004	319,4	114,6	V600E	WT	WT	WT
Me275	LAU50	Metastases	lymph node	21.10.1992	pT4N0M0/ IIB	5.7.1995	Excision lesion on right ankle (21.10.1992); ILP with TNF, IFN, Melphalan and right iliac LN excision (0N+/5, 07.07.1993); right inguino-crural LN excision (1N+/14, 05.07.1995)	01.01.1993	2,4	13.11.2003	100,4	0	08.07.2017	296,7	264,3	V600E	WT	WT	WT
Me290	LAU203	Metastases	lymph node	01.09.1992	pTxN2aM0/ IIIB	1.5.1996	Excision ulcerated lesion on right leg (01.09.1992); Excision intransit metastases (06.10.1995); ILP with TNF, Melphalan and inguino-crural LN excision (big metastatic lesion and several tumoral LN, >1N+, 01.05.1996)	06.10.1995	37,1	NA	NA	1	26.07.2010	214,9	170,9	WT	n.d.	WT	WT
T311B	LAU311	Metastases	lymph node	18.02.1998	pT3N0M0	17.3.1999	Excision lesion on left shoulder (18.02.1998); reexcision primary lesion (18.03.1998); EORTC18951 DTIC, CDDP, IFNalpha +IL2 (15.10.1998-13.01.1999; 4 cures); left axillary LN excision (1N+/16, 17.03.1999)	29.09.1998	7,3	31.03.1999	0,5	1	26.05.1999	15,2	2,3	WT	Q61L	WT	WT
T333A	LAU333	Metastases	skin	08.08.1994	pT2NxM0	25.2.1999	Excision intersacrupelvic lesion(08.08.1994); reexcision primary lesion (24.08.1994); Dacarbazin+cisplatin (1.10.1998, 3 cycles); Homoimotherapy Nolvadex (1.12.1998); excision right sub-clavicular metastase and radical right cervical lymphadenectomy (0N+/7) (25.02.1999); Protocol LUD 96-007 (03.06.1999-02.2004, 16 vaccines)	01.10.1998	49,8	NA	NA	0	21.02.2013	222,6	168,0	WT	Q61K	E203K	WT
T362C	LAU362	Metastases	skin	31.10.1997	pT2b N2b M0/ IIIC	23.2.2000	Excision primary lesion left leg (31.10.1997); excision sentinel LN positive for micrometastases (21.11.1997); Inguinal LN excision (09.12.1997, 3N+/3); excision satellite metastases (03.02.1999) and in transit metastases (09.04.1999); Treatment immunoglobulin (Sandoz, 07.1999); treatment ILP with TNF and left retroperitoneal lymphadenectomy (22.09.1999); evaporation by CO2 laser of metastases on left leg (23.02.2000)	03.02.1999	15,1	20.04.2000	1,9	1	09.02.2001	39,3	11,6	WT	WT	WT	WT
T618A	LAU618	Metastases	skin	10.07.1998	pT4a pN0 pM0/ IIC	20.7.2004	Biopsy lesion left feet (10.07.1998); excision primary lesion and excision left inguinal sentinel LN non met (05.08.1998) (Aduant treatment IFNa (02.11.1998-04.06.1999); ILP of left leg with TNF, IFNg and Melphalan (août 2001, 5 LN excised w/o tumor); Protocol 96-010 Melan-A+Flu +/-adjuvant, +/-IL2 (13.12.2001-09.10.2002, 6 vaccines); Protocol LUD 00-018 CpG 7909, peptides, Montanide (26.05.2003-24.05.2004, 12 vaccines); radiotherapy on met on left calf (June 2004); resection subcut nodular metastase right thigh (20.07.2004)	18.06.2001	35,3	20.10.2004	3,0	1	11.05.2006	94,1	21,7	WT	Q61R	WT	WT
T640A	LAU640	Metastases	skin	13.05.1996	pT3 N0 M0/ II	26.9.2001	Excision primary lesion right leg 13.05.1996; excision of in transit subcut metastases (26.09.2001)	31.08.2001	63,6	05.03.2002	5,3	1	18.11.2002	78,2	13,7	WT	WT	WT	WT

Tumor cell line	Patient	Tumor type	Tumor localization	Diagnosis date	TNM/Stage diagnosis	Sampling date	Previous Therapies (before sampling)	Progression date (from the diagnosis)	Progression-free survival (from the diagnosis) (months)	Progression date (from the sampling)	Progression-free survival (from the sampling) (months)	Death event (Y=1, N=0)	Date of death or last date known alive	Overall survival (from the diagnosis) (months)	Overall survival (from the sampling) (months)	BRAF	NRAS	MEK1	MEK2
T685A	LAU685	Metastases	skin	22.01.2002	pT1 N0 M1/ IV	12.4.2002	Excision primary right pretragian tumor (22.01.2002); excision right pretragian metastatic lesion and right cervical lymphadenectomy (12.04.2002)	12.03.2002	1,6	12.09.2002	5,0	1	06.09.2004	31,5	28,9	WT	WT	WT	S154F
T975A	LAU975	Metastases	brain	03.03.2004	pT4aN1b M0/ IIIB	12.9.2005	Excision right pectoral lesion (03.03.2004); excision axillary sentinel LN (2 micromet et 1 macromet at right, sentinel neg at left) + reexcision primary (07.04.2004); right axillary LN excision (26.05.2004 (0N+/17); excision right pectoral metastase and left axillary LN excision (0N+/7) (09.03.2005); Protocol LUD 00-018 CpG 7909, peptides, Montanide (14.04.2005-05.09.2005, 4 vaccines); excision cerebral metastase (12.09.2005)	01.02.2005	11,0	10.10.2005	0,9	1	25.11.2005	20,8	2,4	WT	Q61L	WT	WT
T1013A	LAU1013	Metastases	lymph node	29.09.1999	T1aN0M0/ I	7.6.2007	Excision of the primary lesion at the left lumbar region (29.09.1999); treatment with interferon-alpha (01.10.1999); excision of a left inguinal adenopathy (04.09.2001); left radical inguino-iliac curettage 7N+/19N (06.02.2002); le 08.04.2002 inclusion in the LUD 96-010 clinical trial (vaccination with Melan-A ELA, Flu and Montanide); excision of two nodules on the inner and anterior sides of the left thigh (02.04.2003); isolated left lower limb perfusion applying TNF and Melphalan (06.08.2003); le 20.10.2003 inclusion in the LUD 00-018 clinical trial (Melan-A ELA, CpG and Montanide); resection of the left external inguinal metastasis (22.10.2003); excision of 2 metastases in the soft tissues at the right flank and under the left buttock (11.12.2003); radiotherapy on the left iliac region (09.12.2003-01.04.2004); radiotherapy in the dorso-lumbar region with 1x8Gy and 1x6Gy (19 and 26.04.2004); 7x chemotherapy with DTIC-Cisplatin (03.05.2004); le 07.03.2005 inclusion dans le CePO-ITA01/01 adoptive transfer trial; 3x treatment avec Cisplatine 150mg and Temo 300mg (23.06.2005, 04.08.2005 and 14.09.2005); radiotherapy stereotaxic (13.07.2005); treatment avec Temo (01.10.2005); right temporal craniotimia and excision of right temporal metastasis (16.10.2006); new right temporo-parietal craniotimia and resection of relapse of right temporal metastasis (04.12.2006); radiotherapy of the whole brain with boost on resection cavity (10.12.2006); excision of right temporal metastase (07.06.2007)	04.09.2001	23,2	12.07.2007	1,2	1	29.07.2007	94,0	1,7	V600E	WT	WT	WT
T1015A	LAU1015	Metastases	visceral (lung)	25.11.2004	pT2aN0b M1a /IV	4.12.2007	Excision lesion on right clavicle and meta nodule on scapula (25.11.2004); large reexcision lesion on clavicle (09.12.2004); Protocol LUD-00-018 CpG 7909, peptides, Montanide (14.03.2005-15.01.2007, 20 vaccines); excision subcut nodule on right scapula (04.12.2007)	29.11.2005	12,1	13.06.2008	6,3	1	22.05.2009	53,9	17,6	V600E	WT	WT	WT
T1185B	LAU1185	Metastases	lymph node	01.12.2005	pT2aN1a M0/ IIIA	30.9.2009	Excision lesion on left leg (01.12.2005);excision left inguinal sentinel LN (1N+/2) and large reexcision of primary lesion (29.12.2005); left inguinal LN excision (0N+/7, 23.01.2006); excision of 4 subcut in transit metastases on left leg (28.11.2006); ILP with TNF, IFN, Melphalan and left ilio-obturator LN excision (1N+/4, 14.02.2007); Protocol LUD 01-003 ELA, Mage-A10, NY-ESO-1, adjuvant (11.09.2007-17.02.2009, 13 vaccines); excision 3 nodules leg (24.11.2008); excision 5 subcut metastastic lesions on left leg (24.02.2009); Protocol CePO-ITA-01/02 adoptive transfer (26.05-2009-04.08.2009, 7 reinfusions); excision multiple subcut meta lesions on left leg (16.06.2009); excision subcut met lesions on abdomen and left leg (07.07.2009); excision multiple in transit meta lesions on left leg, left bottom, abdomen (07.08.2009); ILP treatement on left leg and excison subcut metas on left leg and left ilio-obturator LN (1N+, 30.09.2009)	21.11.2006	11,7	NA	NA	1	24.03.2011	63,7	17,8	V600E	WT	WT	WT
T1194B	LAU1194	Metastases	lymph node	26.02.2007	b pN3 M0/	11.4.2007	Excision lesion right heel (26.02.2007); biopsy adenopathy (30.03.2007); reexcision primary lesion and right inguino-crural LN excision (4N+/8, with capsular effraction in 2 LN, 11.04.2007)	30.03.2007	1,1	03.08.2007	3,7	0	16.08.2007	5,6	4,2	V600E	WT	WT	WT
T1257A	LAU1257	Primary	rectal	13.07.2007	pT2N3Mx R0 (Staging CA canal	19.7.2007	Diagnostic with colonoscopy (30.06.2007); biopsy inguinal metastases (13.07.2007)	02.06.2008	10,7	02.06.2008	10,5	1	24.12.2009	29,4	29,2	WT	WT	WT	WT
T1349A	LAU1349	Metastases	lymph node	30.09.2003	T3N1M0/ III	15.7.2009	Excision lesion right calf (30.09.2003); excision right inguinal sentinel LN (1N+/3, 12.11.2003); Protocol Jandus 87/06: right inguino-crural LN excision (2N+/14, with capsular effraction, 15.07.2009)	12.11.2003	1,4	25.04.2012	33,4	0	20.11.2017	169,8	100,3	V600E	WT	WT	WT

Table 6: Median survival of patients, grouped based on the responsiveness of their melanoma cell lines to IFNγ, TNFα or IFNγ plus TNFα

Reporter	Responsiveness	Median survival [Month]		
	Fold of control	IFNg	TNFa	IFNg + TNFa
HLA-I	>2	29,63	20	29,63
	<2	18,2	29,27	8,133
HLA-DR	>2	29,63	6,3	29,27
	<2	17,83	22,47	20,15
PD-L1	>2	29,63	29,63	29,27
	<2	10,12	18	14,38
CXCL9	>2	142,9	na	22
	<2	22,47	na	22,47
CXCL10	>2	9,017	10,12	29,27
	<2	29,27	29,27	6,3
CCL2	>2	9,017	29,27	22
	<2	29,27	17,83	25,87
IDO	>2	29,27	na	29,27
	<2	20,23	na	18
Mean responsiveness	>2	22	21,6	29,27
	<2	25,87	22,47	6,3

Table 7: Statistical analysis of the responsiveness of melanoma cells to increased doses or combination of cytokines

Cell line	Reporter	HLA-I							HLA-DR						
	Treatment	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL
Me257	IFNg 100ng/mL	ns							ns						
	TNFa 50ng/mL	****	****						ns	*					
	TNFa 100ng/mL	****	****	ns					ns	*	ns				
	IFNg 10ng/mL TNFa 10ng/mL	ns	ns	****	****				****	****	****	****			
	IL6 40ng/mL	****	****	ns	ns	****			ns	ns	ns	ns	****		
	IL6 100ng/mL	****	****	ns	ns	****	ns		ns	ns	ns	ns	****	ns	
	CTRL	****	****	ns	ns	****	ns	ns	ns	*	ns	ns	****	ns	ns
T618A	IFNg 100ng/mL	ns							ns						
	TNFa 50ng/mL	****	****						****	****					
	TNFa 100ng/mL	****	****	ns					****	****	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				****	****	****	****			
	IL6 40ng/mL	****	****	*	**	****			****	****	****	****	****		
	IL6 100ng/mL	****	****	**	**	****	ns		****	****	****	****	****	ns	
	CTRL	****	****	***	****	****	ns	ns	****	****	ns	ns	****	****	****
T975A	IFNg 100ng/mL	ns							ns						
	TNFa 50ng/mL	****	****						ns	*					
	TNFa 100ng/mL	****	****	ns					ns	*	ns				
	IFNg 10ng/mL TNFa 10ng/mL	ns	ns	****	****				ns	ns	****	****			
	IL6 40ng/mL	****	****	ns	ns	****			ns	**	ns	ns	****		
	IL6 100ng/mL	****	****	ns	ns	****	ns		ns	**	ns	ns	****	ns	
	CTRL	****	****	ns	ns	****	ns	ns	ns	**	ns	ns	****	ns	ns

Cell line	Reporter	PD-L1							CXCL9						
	Treatment	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL
Me257	IFNg 100ng/mL	****							ns						
	TNFa 50ng/mL	****	****						ns	ns					
	TNFa 100ng/mL	****	****	ns					ns	ns	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				****	****	****	****			
	IL6 40ng/mL	****	****	ns	**	****			ns	ns	ns	ns	****		
	IL6 100ng/mL	****	****	ns	*	****	ns		ns	ns	ns	ns	****	ns	
	CTRL	****	****	ns	**	****	ns	ns	ns	ns	ns	ns	****	ns	ns
T618A	IFNg 100ng/mL	ns							ns						
	TNFa 50ng/mL	****	****						ns	ns					
	TNFa 100ng/mL	****	****	ns					ns	ns	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				****	****	****	****			
	IL6 40ng/mL	****	****	****	****	****			ns	ns	ns	ns	****		
	IL6 100ng/mL	****	****	****	****	****	ns		ns	ns	ns	ns	****	ns	
	CTRL	****	****	****	****	****	***	**	ns	ns	ns	ns	****	ns	ns
T975A	IFNg 100ng/mL	****							ns						
	TNFa 50ng/mL	****	****						ns	ns					
	TNFa 100ng/mL	****	****	ns					ns	ns	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				***	**	****	****			
	IL6 40ng/mL	****	****	*	**	****			ns	ns	ns	ns	****		
	IL6 100ng/mL	****	****	ns	*	****	ns		ns	ns	ns	ns	****	ns	
	CTRL	****	****	ns	*	****	ns	ns	ns	ns	ns	ns	****	ns	ns

Cell line	Reporter	CXCL10							CCL2							IDO						
	Treatment	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL	IFNg 10ng/mL	IFNg 100ng/mL	TNFa 50ng/mL	TNFa 100ng/mL	IFNg 10ng/mL +TNFa 10ng/mL	IL6 40ng/mL	IL6 100ng/mL
Me257	IFNg 100ng/mL	ns							ns							ns						
	TNFa 50ng/mL	*	***						***	**						****	****					
	TNFa 100ng/mL	**	***	ns					****	****	ns					****	****	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				ns	ns	*	**				ns	ns	****	****			
	IL6 40ng/mL	**	****	ns	ns	****			ns	ns	***	****	ns			****	****	ns	ns	****		
	IL6 100ng/mL	*	**	ns	ns	****	ns		ns	ns	****	****	ns	ns		****	****	ns	ns	****	ns	
	CTRL	**	***	ns	ns	****	ns	ns	ns	ns	***	****	ns	ns	ns	****	****	ns	ns	****	ns	ns
T618A	IFNg 100ng/mL	ns							ns							ns						
	TNFa 50ng/mL	ns	ns						****	****						ns	ns					
	TNFa 100ng/mL	ns	ns	ns					****	****	ns					ns	ns	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				****	****	****	****				****	****	****	****			
	IL6 40ng/mL	ns	ns	ns	ns	****			ns	ns	****	****	****			ns	ns	ns	ns	****		
	IL6 100ng/mL	ns	ns	ns	ns	****	ns		ns	*	****	****	****	ns		ns	ns	ns	ns	****	ns	
	CTRL	ns	ns	ns	ns	****	ns	ns	**	**	****	****	****	ns	ns	ns	ns	ns	ns	****	ns	ns
T975A	IFNg 100ng/mL	ns							ns							ns						
	TNFa 50ng/mL	***	****						****	****						****	****					
	TNFa 100ng/mL	***	****	ns					****	****	ns					****	****	ns				
	IFNg 10ng/mL TNFa 10ng/mL	****	****	****	****				****	****	****	****				ns	ns	****	****			
	IL6 40ng/mL	****	****	ns	ns	****			ns	ns	****	****	****			****	****	ns	ns	****		
	IL6 100ng/mL	****	****	ns	ns	****	ns		ns	ns	****	****	****	ns		****	****	ns	ns	****	ns	
	CTRL	****	****	ns	ns	****	ns	ns	ns	ns	****	****	****	ns	ns	****	****	ns	ns	****	ns	ns

There were no significative differences between the treatments for Me215 and T311B.

Table 8: Statistical analysis of the expression of the IFNγ and TNFα receptors. Effect of treatment and comparison between cell lines

	Receptor	TNFR1			TNFR2			IFNGR1			IFNGR2		
Cell line	Treatment	IFNg	TNFa	IFNg+TNFa	IFNg	TNFa	IFNg+TNFa	IFNg	TNFa	IFNg+TNFa	IFNg	TNFa	IFNg+TNFa
	TNFa	ns			ns			ns			ns		
	IFNg+TNFa	ns	ns		ns	ns		ns	ns		ns	ns	
Me215	Ctrl	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	TNFa	**			ns			ns			**		
	IFNg+TNFa	****	ns		ns	ns		ns	ns		***	****	
Me260LN	Ctrl	ns	****	****	ns	ns	ns	ns	ns	ns	***	****	ns
	TNFa	ns			****			****			ns		
	IFNg+TNFa	ns	ns		****	****		***	****		ns	ns	
T311B	Ctrl	*	ns	ns	ns	**	****	ns	**	***	ns	ns	ns
	TNFa	****			ns			ns			ns		
	IFNg+TNFa	ns	****		****	****		***	****		**	*	
T362C	Ctrl	****	ns	****	****	****	****	***	***	***	ns	ns	**
	TNFa	ns			**			**			****		
	IFNg+TNFa	*	ns		**	ns		**	ns		****	ns	
T618A	Ctrl	*	**	****	ns	****	****	ns	***	***	ns	****	****
	TNFa	ns			ns			ns			ns		
	IFNg+TNFa	ns	ns		****	****		***	****		ns	ns	
T672E	Ctrl	*	ns	ns	ns	ns	***	ns	ns	***	ns	ns	ns

Receptor	TNFR1					TNFR2					IFNGR1					IFNGR2				
Cell line	Me215	Me260LN	T311B	T362C	T618A	Me215	Me260LN	T311B	T362C	T618A	Me215	Me260LN	T311B	T362C	T618A	Me215	Me260LN	T311B	T362C	T618A
Me260LN	****					**					**					ns				
T311B	****	**				ns	****				ns	****				ns	ns			
T362C	**	****	ns			****	****	****			****	****	****			ns	ns	ns		
T618A	*	****	ns	ns		ns	**	ns	****		ns	**	ns	****		ns	ns	ns	ns	
T672E	**	****	ns	ns	ns	ns	***	ns	****	ns	ns	***	ns	****	ns	ns	ns	ns	ns	ns

Statistical analysis was performed using a one-way Anova followed by a Tukey-Kramer multiple comparison test. Adjusted p.values are represented as follow:

ns = non significative with P>0.05, * P≤0.01, ** P≤0.001, *** P ≤0.0001 and **** P ≤ 0.00001.

Results

Most T cell-derived cytokines do not seem to affect melanoma cells

Based on the observations from previous studies, melanoma cells are not immunologically inert as they are able to respond to some immunological cues present in the tumor microenvironment.[94] The first aim was to determine to what cytokines melanoma cells are able to respond. To this end a rapid test using flow cytometry was set up using a selected panel of reporter proteins that have been shown to play a role in the immunology of melanoma. The panel consists of immune genes that have both pro-tumoral (PD-L1, IDO) anti-tumoral (MHC-I, MHC-II, CXCL9, CXCL10) or yet undefined roles (CCL2).

Patient-derived melanoma cell lines were seeded and treated with different cytokines that are known to be produced by T cells (IFNγ, TNFα, LTα, CCL3, CCL4, IL2, IL4, IL5, IL13, GM-CSF). Individual cytokines or cytokine combinations were added for 48h to the culture and the level of expression of the reporters was assessed by flow cytometry (Figure 7).

This initial screening using 5 melanoma cell lines gave some indication regarding the ability of the melanoma cells to sense and respond to activated T cells present in the tumor microenvironment. However, we should keep in consideration that the readouts selected are only capturing part of the phenotype of the melanoma cells and that an absence of changes does not exclude that other parameters of the cells are affected by the treatment. Based on the reporters selected, we see that most of the cytokines do not alter the phenotype of the melanoma cells. Importantly, we find that IFNγ, TNFα, TNFβ and to a minor extent IL6 lead to an increased expression of one or more of the analyzed immune genes. Interestingly, we can already note here that the overall responsiveness as well as the quality of the response of the different melanoma cells is very heterogenous. For instance, IL6 induced a significant increase in the expression of MHC-II and PD-L1 in the cell line T618A while the other cell lines tested remained unaffected.

A strong TNFα mediated upregulation of CCL2 is seen for Me252 and T618A cells. CCL2 upregulation upon TNFα treatment in the T1257 cells was lower than in the two previous ones but still significant. The two remaining cell lines remained unresponsive to TNFα. TNFβ has the same pattern of responsiveness as TNFα but the magnitude of the response is decreased. The cell lines Me215 and T311B that did not respond to TNFα remained also unresponsive to TNFβ.

The cell lines that responded to TNFα also responded to IFNγ but the genes affected are not entirely similar, as expected. HLA-I was upregulated in Me252, T618A and T1257. HLA-DR

seems to be slightly induced in Me252 and T1257 while we see a more than two-fold increase in T618A. The PD-L1 upregulation pattern is similar in all 3 responsive cell lines. To our surprise, we do not see CXCL9 and CXCL10 upregulation although both of these chemokines are supposed to be induced by IFNγ.[50]

Overall, we see that three of the five cell lines present a similar pattern of responsiveness to the cytokines we tested. It is also interesting to note that 2 cell lines remained unresponsive.

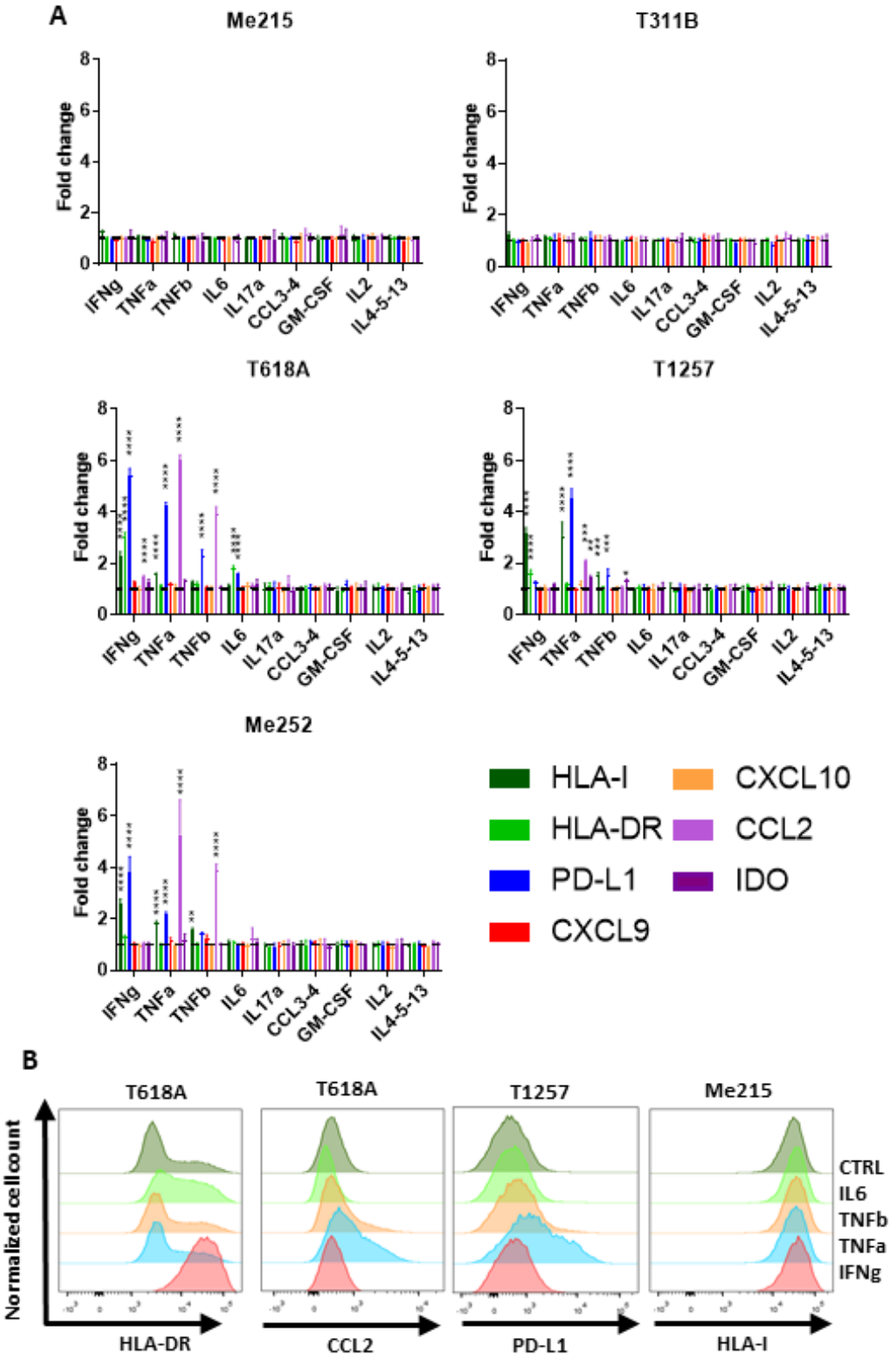


Figure 7. Upregulation of immune genes by melanoma cells after cytokine treatment

Patient derived tumor cell lines were seeded as described in Table 1 and treated the following day with the indicated cytokines using the following concentrations: IFNγ 10ng/mL, TNFα 50ng/mL, TNFβ 40ng/mL, IL6 40ng/mL, IL2 200U/mL, GM-CSF 40ng/mL, CCL3 40ng/mL, IL4 25ng/mL, IL17A 20ng/mL, CCL4 40ng/mL, IL5 40ng/mL, IL13 40ng/mL. Cells were collected 48h later and the expression level of different markers were investigated by flow cytometry. The analysis only

includes live single cells thanks to a live/dead marker and doublet exclusion. (A) Bar graphs showing the upregulation of the different proteins depicted as fold change relative to the untreated control (represented by the dotted line) with standard deviation (n≥3, Dunnets multiple comparison test: p.value <0.05=*, P<0.01=**, <0.001=***, <0.0001=****). (B) Representative histograms showing the expression level of HLA-DR, CCL2, PD-L1 and HLA-I in T618A, T1257 and Me215 cells +/- cytokine treatment.

Increasing the cytokine dose does not increase responsiveness, while some cytokine-combinations do

To ensure that the lack of responsiveness was not due to an insufficient concentration of the cytokines, we increased the doses from the initial dose (10ng/mL for IFNγ, 50ng/mL for TNFα and 40ng/mL of IL6) to 100ng/mL focusing on the cytokines that induced the expression of certain marker proteins in the first screening. We report in Figure 2 that an increased dose of IL6 did not further increase the level of expression of HLA-DR or PD-L1 in the T618A cells. Other reporters remained unaffected in the T618A, and the other cell lines did not become responsive to high dose of IL6. Increasing the dose of IFNγ only led to minor increase in the expression level of the reporter proteins compared to the previously used dose for most of the cell lines. Indeed, PD-L1 upregulation is stronger with a higher dose of IFNγ in the T975A and Me257 cells. Increasing the dose of TNFα did also not lead to an increased responsiveness. Similarly, to IFNγ, the cell lines that were not responding to the low dose of TNFα did also not respond to the high dose. Interestingly, the non-responding cell lines Me215 and T311B remained unresponsive to all cytokines tested even to the high dose. These results suggest that the maximal effect by the different cytokines was achieved even with the low dose. In this assay, we also combined IFNγ and TNFα as we assume that they may be present at the same time in the tumor microenvironment. In contrast to the dose increase that only led to minor changes, the combination of low doses of IFNγ and TNFα turns out to be the most efficient treatment to induce the expression of several reporters including PD-L1, HLA-I, HLA-DR and CCL2. However, there may be some heterogeneity due to the fact that some cell lines already reached the maximal upregulation with the low dose of one of the cytokines alone. Noticeably, upregulation of CXCL9 and CXCL10 was absent or low in the presence of each of the cytokines alone while the combination of IFNγ and TNFα led to a strong production of these chemokines.

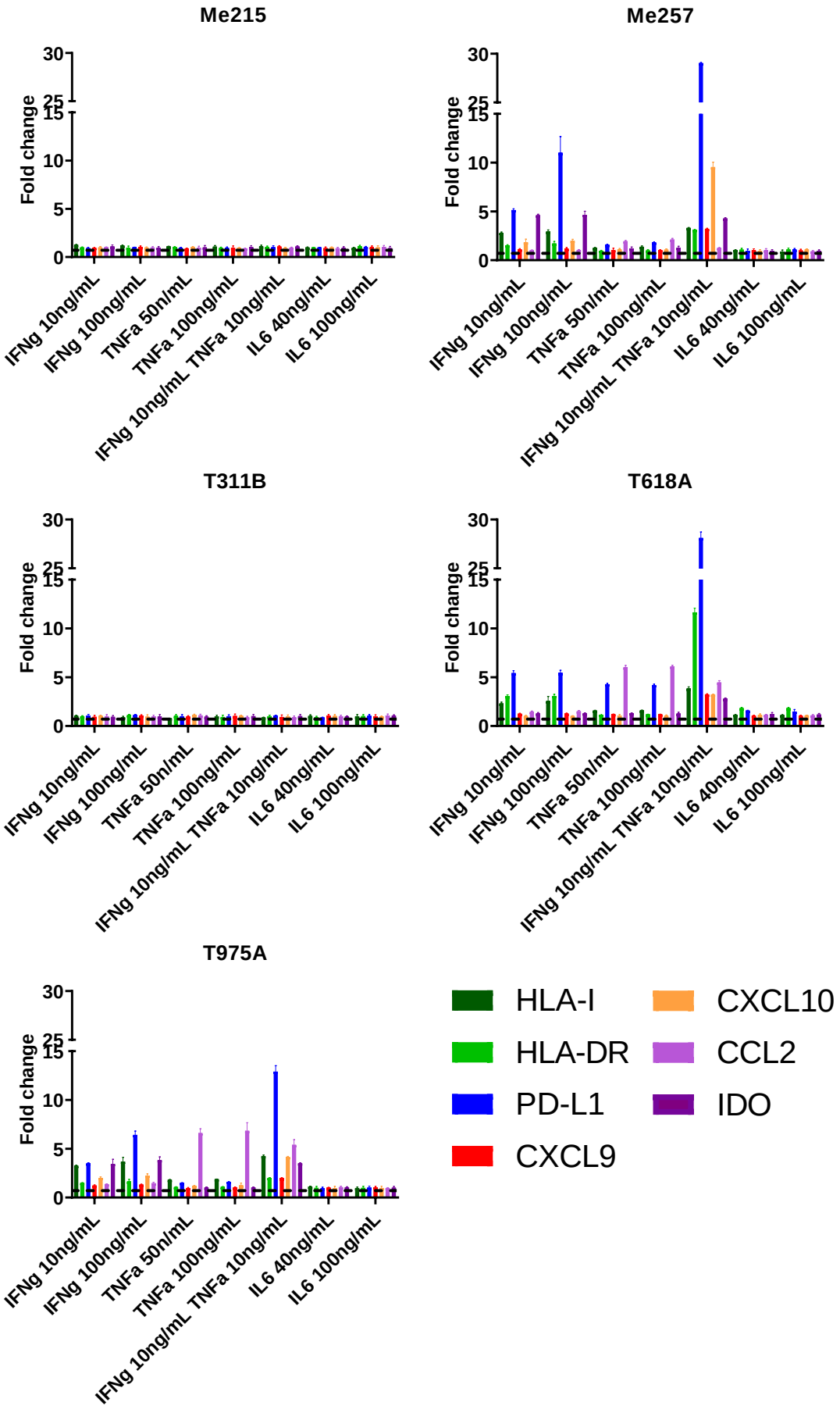

Me215
Me257
T311B
T618A
T975A
Fold change
30
25
15
10
5
0
IFNg 10ng/mL
IFNg 100ng/mL
TNFa 50n/mL
TNFa 100ng/mL
IFNg 10ng/mL TNFa 10ng/mL
IL6 40ng/mL
IL6 100ng/mL
HLA-I
HLA-DR
PD-L1
CXCL9
CXCL10
CCL2
IDO

Figure 8. Upregulation of immune genes after treatment with high doses or combination of cytokines

Patient derived tumor cell lines were seeded as described in Table 1 and treated the following day with different doses and combinations of cytokines as indicated on the graphs. The level of expression of the reporters was assessed by flow cytometry after 48h of stimulation. The analysis only includes live single cells thanks to a live/dead marker and doublet exclusion. Bar plots depict the responsiveness as fold change relative to the untreated control (represented by the dotted line) with standard deviation. n≥3. Statistical analysis is reported in Table 7.

Screening of 21 patient-derived human melanoma cell lines

To assess whether the observations obtained during the initial screening were representative for a larger cohort, we used 21 human melanoma cell lines obtained from 21 different patients and investigated the change in reporter protein expression using the low dose of IFNγ and TNFα as well as the combination of both cytokines. We also investigated whether normal human melanocytes would respond in a similar manner and thus included the human melanocytes NHM and HEMA-LP (Figure 9). Although we still see heterogeneity in the amplitude of the responsiveness, we can observe a pattern that is shared between most of the cell lines including the melanocytes. IFNγ is a potent inducer of HLA-I, HLA-DR, PD-L1 and IDO. TNFα induces CCL2 and also HLA-I and PD-L1 to some extent in some cell lines (e.g. T1257A). Although some rare cell lines already present a low upregulation of CXCL9 and CXCL10 with IFNγ alone, the addition of TNFα leads to a strong upregulation of both chemokines. Some cell lines present characteristics that are deviating from the "standard" response. One example is the T1185B cell line that responds as expected when looking at all the reporters except HLA-DR. The T1349A also presents an altered responsiveness with most of the reporters not upregulating but we still appreciate a marginal increase in HLA-I. The observations of partially affected responsiveness could be of interest in order to dissect the response to different cytokines and identify specific mechanisms affecting only some cytokine induced genes that could potentially be involved in immune evasion. However, here we will focus mostly on 2 cell lines, namely Me215 and T672E that present a globally altered responsiveness. Indeed, both, Me215 and T672E cells were classified as very poor responders based on their inability to significantly upregulate any of the selected reporters. Two further cell lines are also showing interesting patterns as both T311B and T1349A present a very reduced responsiveness.

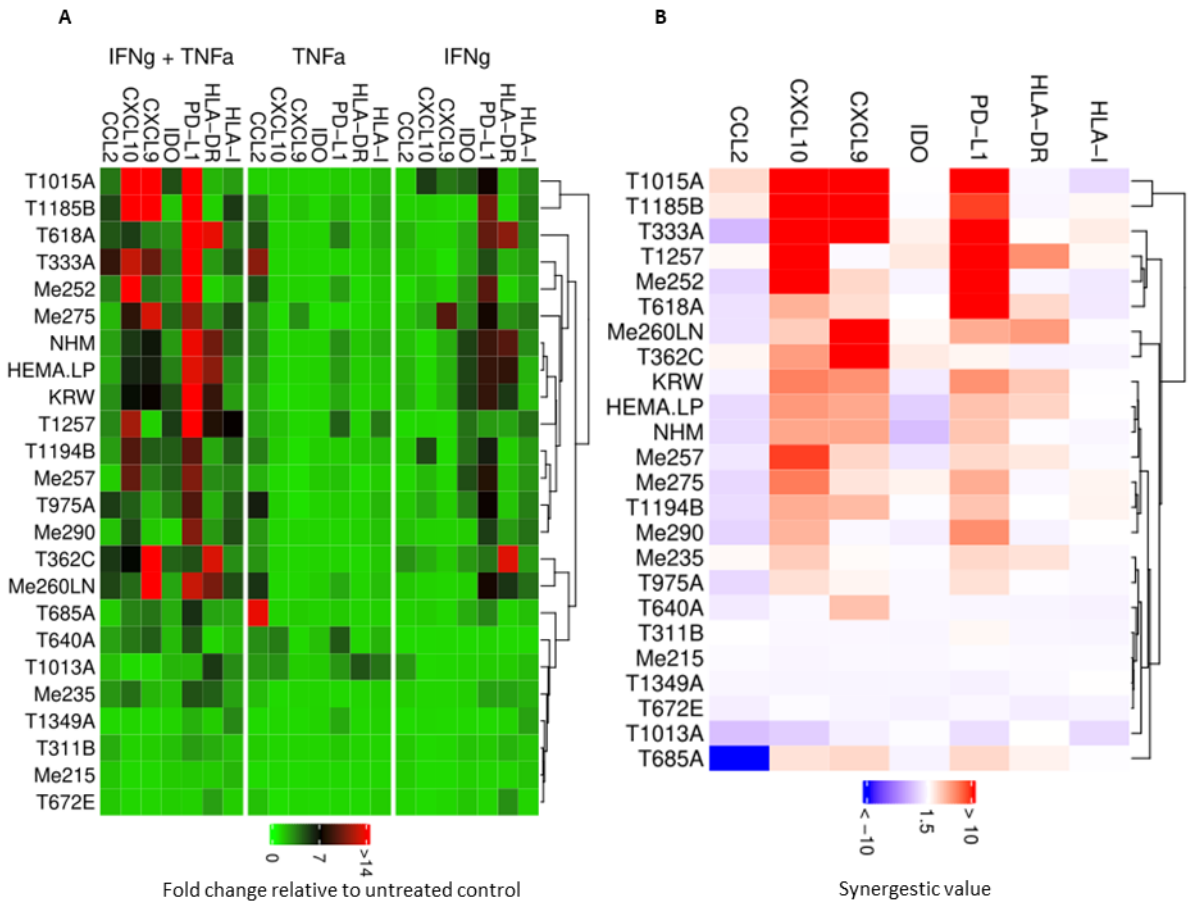


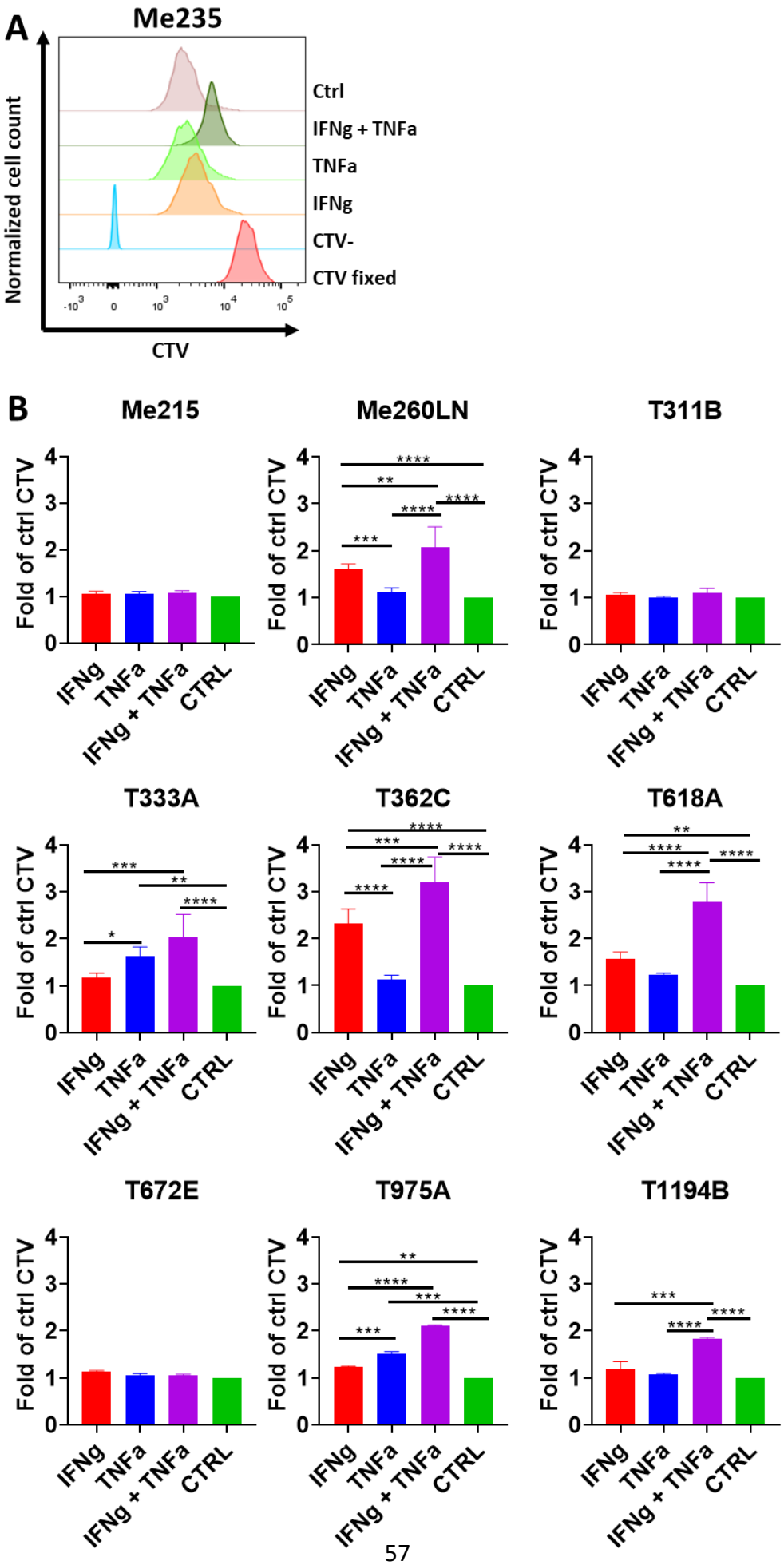
Figure 9. Upregulation of reporter proteins as fold of untreated control after 48h of treatment with cytokines alone or in combination

Patient-derived tumor cell lines or primary melanocytes (HEMA.LP and NHM) were seeded as described in Table 1 and treated the following day with the following cytokines: IFNγ (10ng/mL), TNFα (40ng/mL), or IFNγ and TNFα (both 10ng/mL). (A) The expression level of the different reporter proteins was assessed by flow cytometry after 48h of stimulation. The analysis only includes live single cells thanks to a live/dead marker and doublet exclusion. Heatmap shows the effect of cytokine treatment that were calculated as fold change relative to the untreated controls. Fold change is represented using a color scale. These values were used to establish a hierarchical clustering. Each experiment has been performed at least 3 times. (B) To extract the synergistic value obtained by combining IFNγ and TNFα, we subtracted the fold change value obtained with each cytokine individually from the fold change value of the combination of both cytokines using the following model: Fold change value of control – ((fold change value of IFNγ-1)+(fold change value of TNFα))=synergistic value. These values are shown in a heatmap. Data are ordered based on hierarchical clustering.

IFNγ and TNFα as modulators of cell proliferation and viability

One proposed antitumoral effect of IFNγ relies on its ability to limit tumor cell proliferation.[51] We loaded the melanoma cells with a fluorescent dye called Cell Trace Violet and assessed its dilution by flow cytometry after 4 days of culture with the different cytokines (Figure 10). The dilution of the fluorescent dye among the dividing cells gives an indication on the number of cell divisions that occurred during the assay and thus the anti- or pro-proliferative activity of the cytokines. The results depicted in Figure 10B report that IFNγ affected the proliferation of several cell lines (Me260LN, T362C, T618A), while some other cell lines were not affected (Me215, T311B, T672E). Although TNFα has been mostly reported for its direct activity in the killing of melanoma cells,[18] the antiproliferative activity of TNFα alone remained low but was still noticeable on some cell lines as Me235 (Figure 10A), T975A and T333A. As for the expression level of some of the reporters, we observe that the combination of IFNγ and TNFα has often a stronger antiproliferative activity than the cytokines alone (Figure 10C). Interestingly, the proliferation of the T1194B was very poorly affected by the cytokines alone but the combination decreased its proliferation two-fold.

As mentioned, TNFα is known for its cytotoxicity and we noticed during the experiment that in some cell lines the treatment induced cell death. We thus decided to assess the ability of both cytokines to induce cell death. This was achieved by using Green Fluorescent Reactive Dye. This dye reacts with free amines that are only present in low amount on the cell surface of viable cells. In contrast, dead cells are not maintaining their membrane integrity and thus allow the entry of the dye that can also react with free intracellular amines resulting in a strong staining of dead cells. Our results shown in Figure 10C illustrates that the viability remained unchanged in several cell lines including Me215 and T672E. In contrast, some cell lines like the Me260LN showed a clear decrease in cell viability after exposure to TNFα.


A
Me235
Normalized cell count
CTV
Ctrl
IFNg + TNFa
TNFa
IFNg
CTV-
CTV fixed
B
Me215
Me260LN
T311B
T333A
T362C
T618A
T672E
T975A
T1194B
Fold of ctrl CTV
IFNg
TNFa
IFNg + TNFa
CTRL

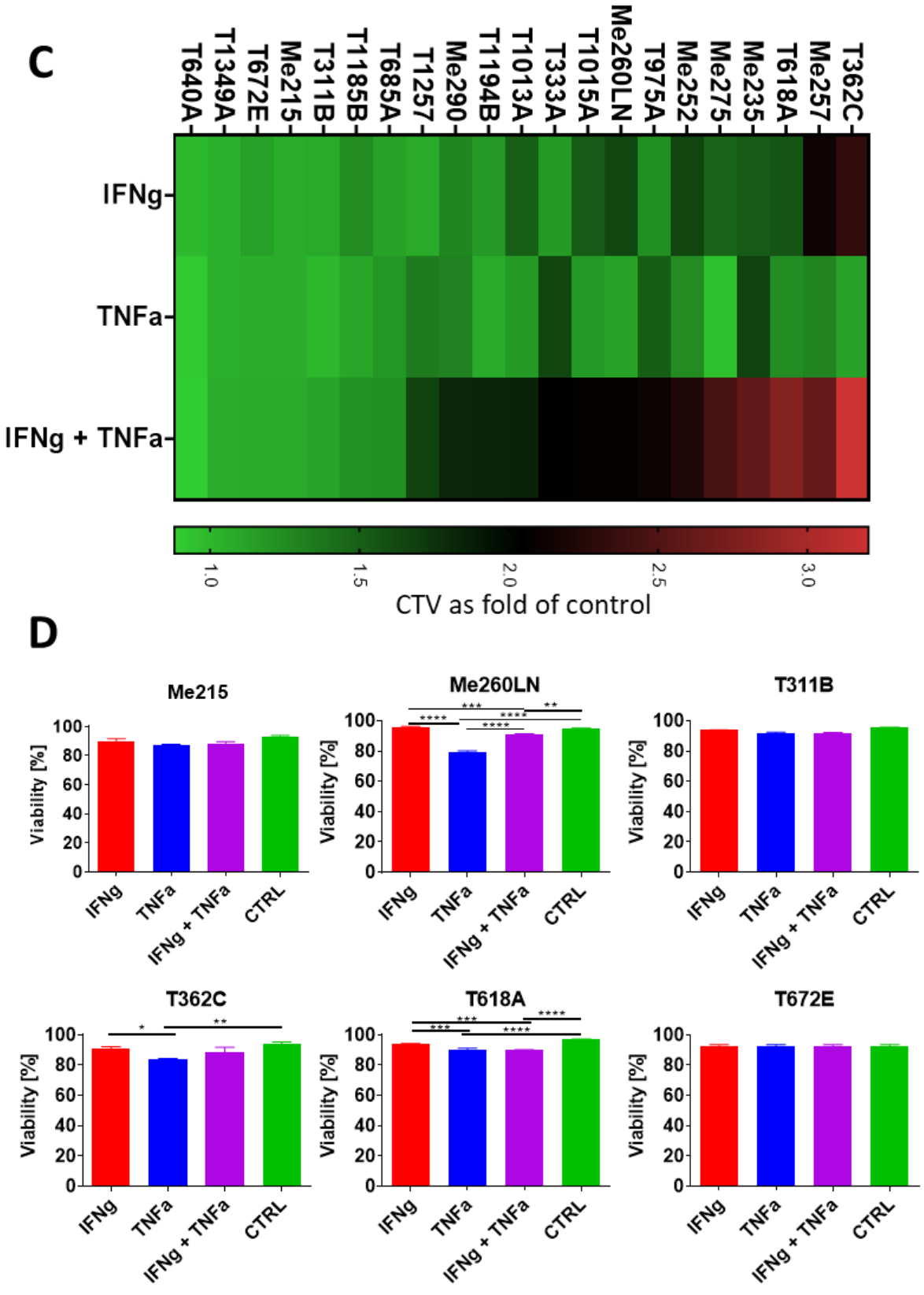


Figure 10. Effect of cytokines on cell proliferation and viability of human cancer cells

(A, B). Cells were loaded with Cell Trace Violet (CTV), a fluorescent marker, and seeded at low confluency so they would not reach 100% confluency before the end of the experiment. Cells were treated with the described cytokines (IFNγ 10ng/mL alone and 10ng/mL in combination with

TNFα, TNFα 50ng/mL alone and 10ng/mL in combination with IFNγ) one day after seeding. Cells were cultured for 96h. At the end of the experiment, we analyzed the dilution of CTV in the cell lines which gives an indication on the proliferation of the cells. (A) Representative histogram showing CTV loaded or unloaded (CTV-) Me235 cells +/- cytokine stimulation. Fixed CTV of non-proliferated cells was used as labeling control, Dead cells were gated out using a live/dead cell marker. (B) GMFI of CTV from treated and control samples were compared and fold change relative to control is shown to illustrate the antiproliferative capacity of the cytokines. (C) Representation of the antiproliferative capacity of the cytokines as heatmap using the CTV loading as fold of control for scale. (D). Cells were seeded as in Figure 1 and treated with IFNγ (10ng/mL), TNFα (50ng/mL) or IFNγ and TNFα (both 10ng/mL). Cell viability was assessed by flow cytometry using green fluorescent reactive dye at 48h after treatment. ($n \geq 3$, Dunnets multiple comparison test: p.value: ns = non significative with $P>0.05$, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$, error bars indicate standard deviation).

Melanoma cell responsiveness and patient survival

As we hypothesized that the ability of melanoma cells to respond to cytokines present in the tumor microenvironment may be relevant for immune escape, we compared the responsiveness of the melanoma cells to the survival of the corresponding patients. We used the fold change of control values from the previous screening to determine the responsiveness of the melanoma cells. As our reporters can have both anti- and pro-tumor roles, we assessed the correlation of their upregulation after cytokine treatment with the survival of the patients individually and also by grouping the cell lines using the mean responsiveness. Responding cell lines where the ones where cytokine treatment induced a more than two-fold increase in the gene expression (Figure 11). It was previously shown that the level of expression of HLA-I was correlated with the survival of the patient[95]. Here we report that a similar observation can be made with the ability of melanoma cells to upregulate HLA-I by the combination of IFNγ and TNFα. A similar trend can be seen with IFNγ alone but it was not significative. Interestingly, when looking at PD-L1 upregulation with IFNγ treatment alone, we see a trend indicating that the ability to upregulate PD-L1 is associated with a good prognosis. As PD-L1 is known for its role in dampening T cell activation, this observation raises questions that will be discussed latter. No correlation between the survival of the patients and the responsiveness to TNFα was observed in our cohort but the fact that TNFα alone had only minor effects on the melanoma cells based on our reporters led to widely unequal group sizes. Overall, although we see some trends, we do not see many correlations between the responsiveness and the survival which could be partially due to the size of our cohort. When combining the responsiveness of all the reporters to each treatment, we did not see any correlation with the survival.

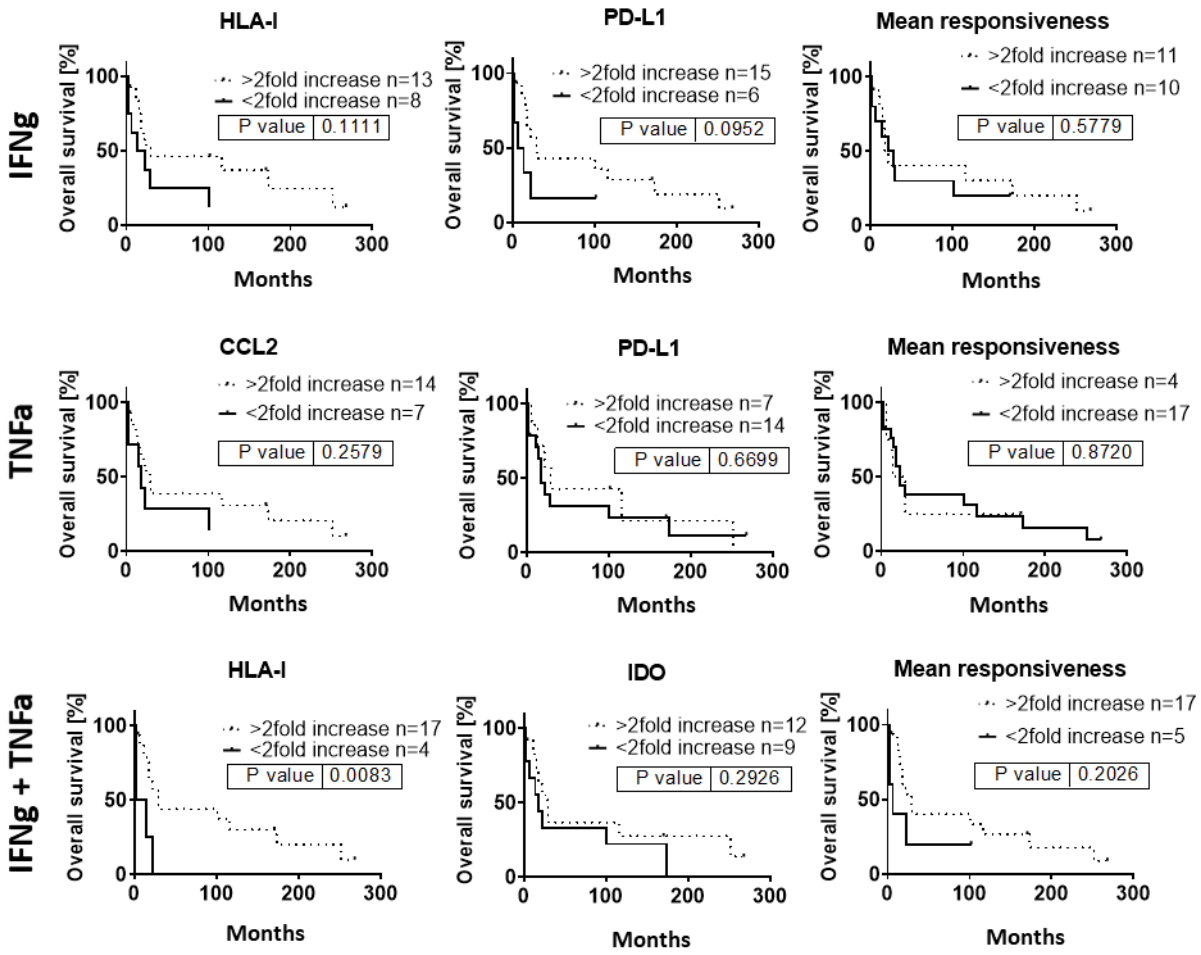


Figure 11. Patients' survival and melanoma cell responsiveness

Using the fold change of control values, melanoma cell lines were grouped for each reporter based on their ability to respond. Cells for which we saw a more than two-fold upregulation are considered as responders. We also grouped all reporters together by calculating the mean upregulation of all reporters to the corresponding cytokine. This data depicted in the lower line indicate whether a general responsiveness would also be associated with the prognosis of the patients. The survival time indicated in months represents the time interval from sampling of the tumor material used to generate our cell lines until the death of the patients.

Expression of cytokine receptors

These initial results showed that there is some heterogeneity in the responsiveness to the cytokines IFNγ and TNFα and that some of the investigated cell lines present a very altered or abrogated responsiveness. The literature reports some examples of the importance of responding to IFNγ for the efficiency of immunotherapy as well as some ways by which the tumor cells could acquire loss of responsiveness[93]. Based on this observation, we selected two non-responsive cell lines for further investigation, namely Me215 and T672E. One explanation for a lack of responsiveness would be the absence of the corresponding cytokine receptors. To this end we assessed the mRNA

expression level of TNFR1 and TNFR2 as well as IFNGR1 and IFNGR2 in several cell lines (Figure 12). The non-responsive cell line Me215 expressed TNFR1 at a lower level than all the other cell lines tested. This observation is of interest as TNFR1 signaling tends to be pro-inflammatory[96]. Both non-responding cell lines Me215 and T672E express TNFR2 at low levels but this can not be an explanation for the lack of responsiveness as the responding T362C shows similar level of expression. We also observe that TNFα treatment increases the expression of TNFR1 in the Me260LN and T618A. Interestingly, treating the cells with the combination of IFNγ and TNFα leads to the upregulation of TNFR1 (Me260LN, T362C and T618A) and TNFR2 (Me260LN, T311B, T362C and T618A). This could be an explanation for the synergistic effect we observe with the reporters used in the previous experiments.

In absence of treatment, all cell lines express similar levels of IFNGR1. In contrary, IFNGR2 is expressed at a lower level in the Me215 but further investigations are still required to assess whether this decreased receptor expression is responsible for this lack of responsiveness. When looking at cytokine treatment, we observe that TNFα increased the expression of IFNGR1 and IFNGR2 in several cell lines and that the combination of IFNγ and TNFα often strengthened the upregulation (Me260LN, T362C, T618A) as previously seen for the TNF receptor expression.

The upregulation of several receptors that we observe may be one of the explanations for the synergistic effect of the combination of IFNγ and TNFα. Indeed, by increasing the expression of their own receptors, the combination of both cytokines can increase the responsiveness of the melanoma cells to themselves, but more investigations should be performed to confirm this hypothesis.

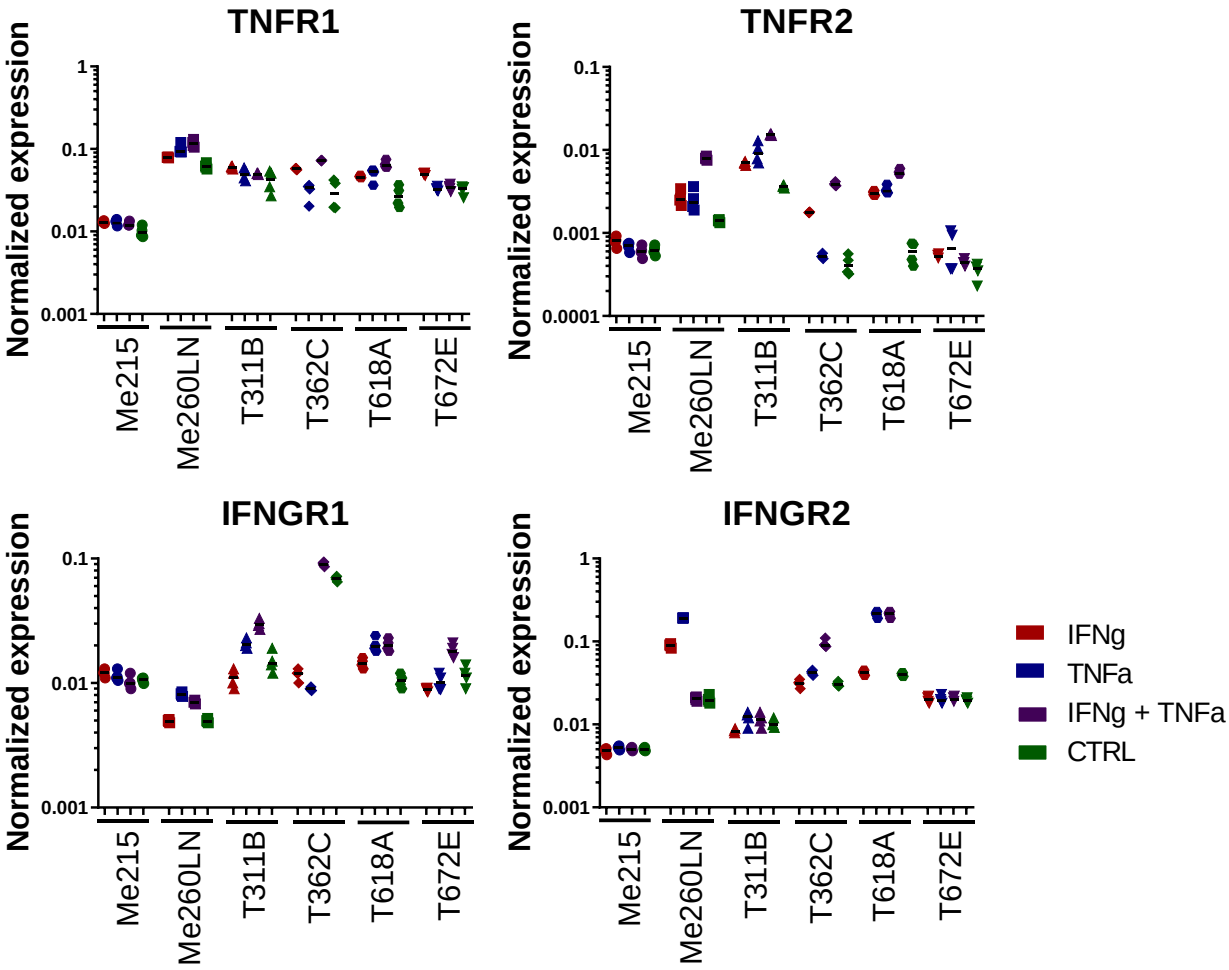


Figure 12. Level of expression of the receptors for IFNγ and TNFα

Patient derived melanoma cells were cultured for 24h with IFNγ (10ng/mL), TNFα (50ng/mL), IFNγ (10ng/mL) and TNFα (10ng/mL) or untreated. RNA isolation was performed and transcript levels of TNFR1, TNFR2, IFNGR1 and IFNGR2 were determined by real-time PCR. Expression level was normalized to the expression of the housekeeping genes GAPDH and S16. n=4. Statistical analysis is presented in Table 8.

Whole exome sequencing of melanoma cells

With the aim to unravel mechanisms explaining the heterogeneity in the cytokine responsiveness, we performed a whole exome sequencing (WES) analysis to identify molecular alterations in the IFNγ and TNFα signaling pathways. We isolated the DNA from early passaged melanoma cells and sequenced it. For 14 of 21 patients, we also had PBMC that were used for whole exome sequencing. As it seems that most of the identified mutations altering the function of the tumor cells are affecting the exomes, we opted for Whole Exome Sequencing instead of Whole Genome Sequencing. The raw data only gives a sequence without any indication on what is normal and what

was altered during the tumorigenesis. To identify the alterations, we have to compare the sequences obtained with the DNA extracted from PBMC or with a set of reference human genomes. The advantage of using PBMC DNA from autologous patients is that by comparing the DNA sequence from the tumor cells to the DNA sequence of PBMC, and by only looking at what is different, we get rid of the Single Nucleotide Polymorphism (SNP). SNP are germline substitutions of single base pairs in the genome and may differ from one individual to another. As not all SNP are present in the set of reference genomes used for the 7 remaining cell lines, the results also contain many SNP that we cannot differentiate from Single Nucleotide Variants (SNV). In addition to Single Nucleotide Variants, the whole exome sequencing also allows us to identify Copy Number Alterations. Both informations are important, as a defective pathway may be the result of a gene deletion or the loss of function of a protein due to a mutation in an exon. Before continuing our analysis, we wanted to verify that the data we obtained were similar to other publicly available data. To evaluate whether the amount and the type of SNV in our cohort is representative, we compared the WES data we obtained from our cohort to the melanoma dataset of the TCGA database. In general, Melanoma is known for having higher numbers of mutations compared to other cancers[97]. We can see that we have similar amounts of mutations in our cohort and in the TCGA dataset (Figure 13A). When looking at our cohort, we can observe that the numbers of mutations are heterogeneous. One part of the explanation may be due to the level of sun exposure[98] as we can see that, although generally predominant, the proportion of C to T transition is heterogenous among our cell lines (Figure 13B). As different causes are leading to different mutations, we can expect a high heterogeneity in the proportion of each SNV type. This is confirmed by the proportion of each SNV type that we can find in the different cell lines from our cohort (Figure 13B). We can thus not compare each cell line individually to the TCGA dataset and have to combine all our cell lines. The proportion of the combined SNV types from our cohort depicted in Figure 13C below the TCGA data shows some similarity with what can be found in the TCGA. Based on the observation that we have similar amounts of SNV and that the proportion of each type of SNV are similar in our cohort and in the TCGA, we assume that our cohort is representative for larger melanoma cohorts.

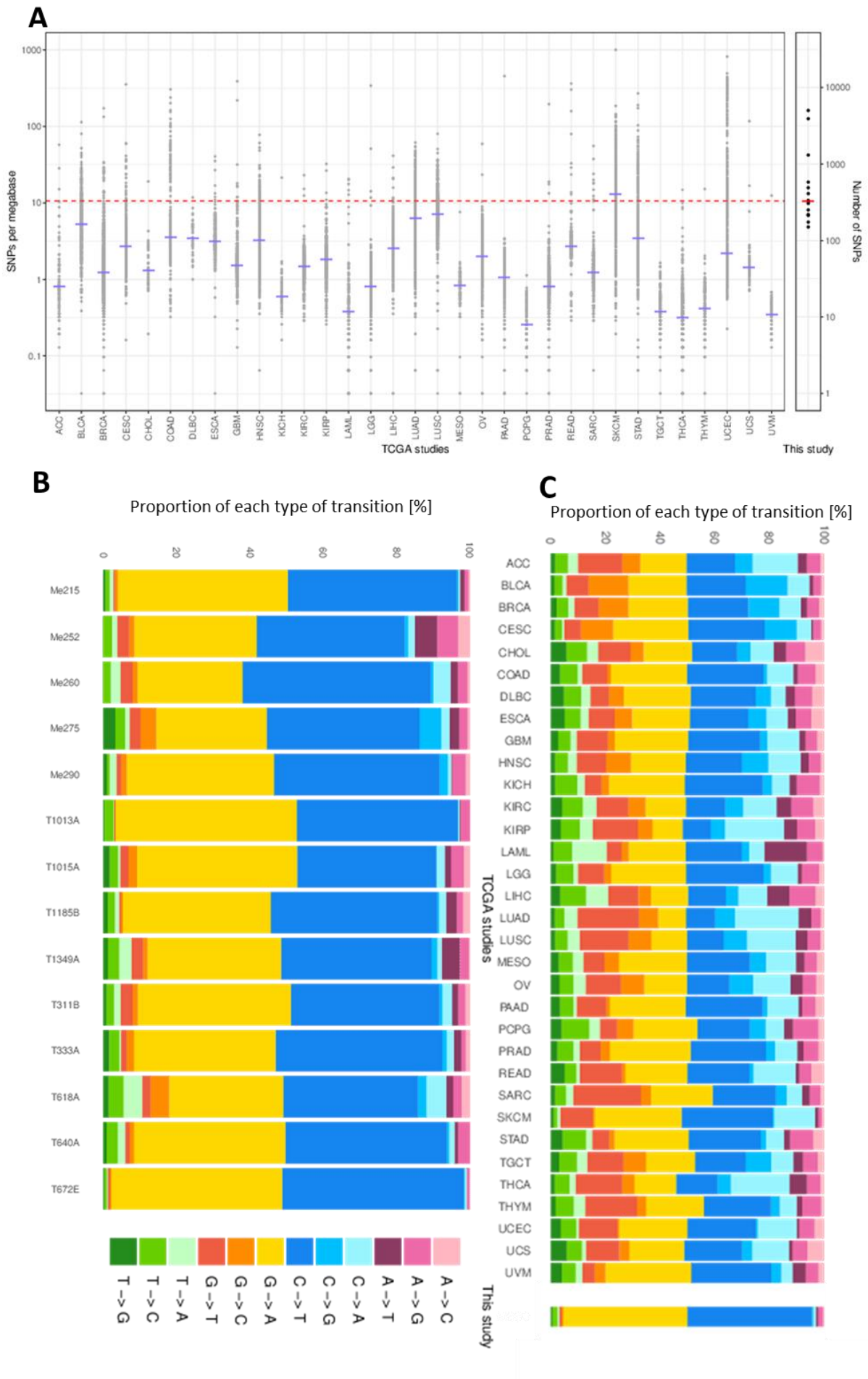
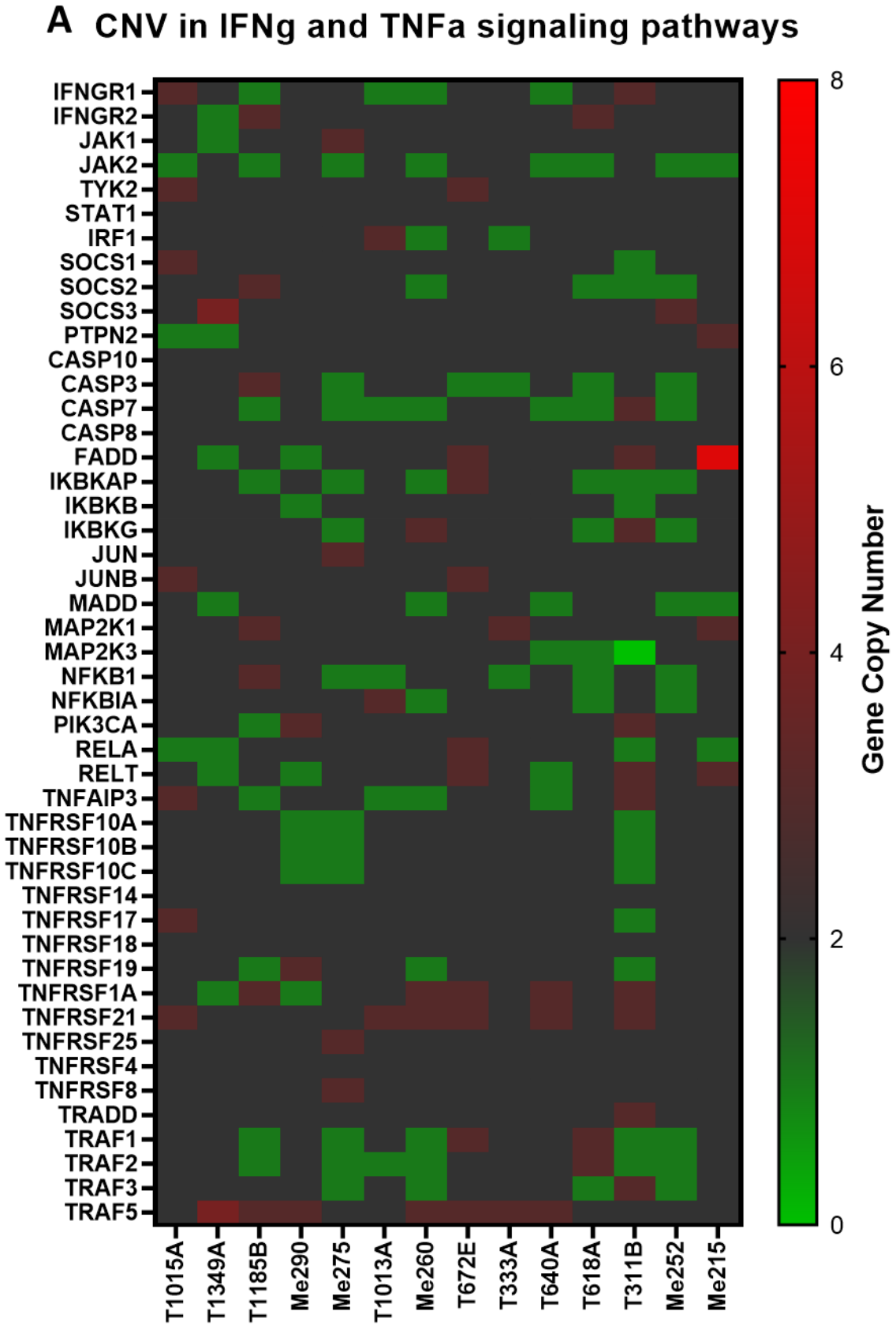

A
SNPs per megabase
Number of SNPs
TCGA studies
This study
ACC BLCA BRCA CESC CHOL COAD DLBC ESCA GBM HNSC KICH KIRC KIRP LAML LGG LIHC LUAD LUSC MESO OV PAAD PCPG PRAD READ SARC SKCM STAD TGCT THCA THYM UCEC UCS UVM
B
Proportion of each type of transition [%]
Me215 Me252 Me260 Me275 Me290 T1013A T1015A T1185B T1349A T311B T333A T618A T640A T672E
T->G T->C T->A G->T G->C G->A C->T C->G C->A A->T A->G A->C
C
Proportion of each type of transition [%]
TCGA studies
This study

Figure 13. Representativity of the cohort of patients included in this study

WES data from the melanoma cell lines were analyzed using the matching DNA from PBMC or a set of reference genomes when no PBMC were available to determine the number, position and type of SNV. (A) This graph presents the number of SNVs among different cancer types as well as the mutational load in the cell lines included in our study. Percentage of each SNV type identified in our cell lines (B), different cancer types (source TCGA database) as well as the combination of SNV found in all our cell lines (C).

Mutations in the JAK1 gene in a non-responding cell line

To identify mutations that could explain the lack of responsiveness of some of our melanoma cell lines. We defined a list of genes involved in the IFNγ or TNFα signaling pathway by merging the lists of IFNγ and TNFα signaling pathway from Qiagen and KEGG (hsa04630). As the loss of both copies of a gene is an obvious mechanism by which a cell line can lose its ability to respond to a cytokine, we first looked at gene copy loss. We did not find loss of key genes in the IFNγ and TNFα signaling pathways in the Me215 and T672E. In a second step, we looked at gene mutations and found that the JAK1 gene in the non-responding T672E cell line had one mutation in the coding sequence and an additional mutation in the splice acceptor variant region (Figure 14B). The mutation in the coding sequence changes the codon coding for a tryptophan at position 467 to a stop codon. This happens in the SH2 domain of the protein and quite early in the sequence. Protein structure modelling shows that this genetic alteration results in a loss of the pseudo-kinase and the kinase domain which leads to the production of an inactive form of the protein. Further analysis of the sequencing data suggests that the other copy of the JAK1 gene is still present in this cell line and thus it may still express a functional JAK1 protein. We did not report any mutation of interest in the Me215 cells suggesting that the lack of responsiveness may not be caused by a genetic alteration.


A CNV in IFNg and TNFa signaling pathways
IFNGR1
IFNGR2
JAK1
JAK2
TYK2
STAT1
IRF1
SOCS1
SOCS2
SOCS3
PTPN2
CASP10
CASP3
CASP7
CASP8
FADD
IKBKAP
IKBKB
IKBKG
JUN
JUNB
MADD
MAP2K1
MAP2K3
NFKB1
NFKBIA
PIK3CA
RELA
RELT
TNFAIP3
TNFRSF10A
TNFRSF10B
TNFRSF10C
TNFRSF14
TNFRSF17
TNFRSF18
TNFRSF19
TNFRSF1A
TNFRSF21
TNFRSF25
TNFRSF4
TNFRSF8
TRADD
TRAF1
TRAF2
TRAF3
TRAF5
T1015A
T1349A
T1185B
Me290
Me275
T1013A
Me260
T672E
T333A
T640A
T618A
T311B
Me252
Me215
8
6
4
2
0
Gene Copy Number

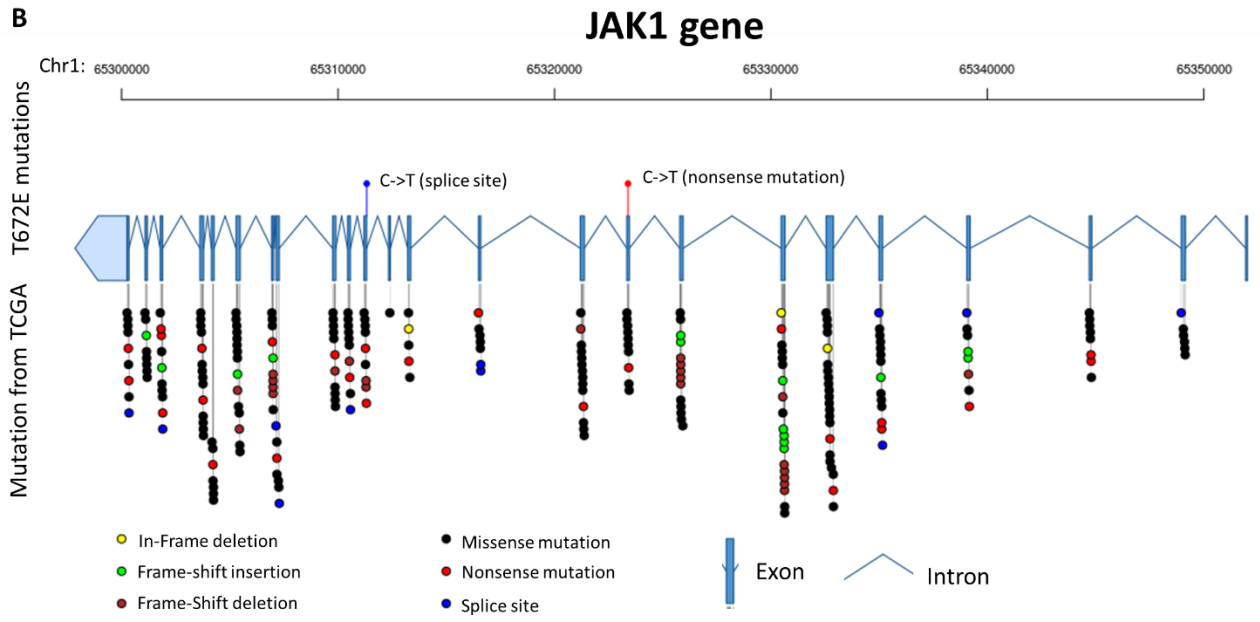


Figure 14. WES data from TCGA and one of our cell lines: No copy loss, but mutations in JAK1 in the cell line T672E

Whole exome sequencing (WES) data were used to identify genes from the IFNγ of TNFα signaling pathway that could be altered and explain the lack of cytokine responsiveness in the corresponding melanoma cell line. (A) Heatmap showing the loss of key genes involved in the IFNγ and TNFα signaling pathway based on the analysis of allele frequency of the whole exome sequencing data. (B) Schematic representation of JAK1 gene structure containing exons (boxes) and introns (lines). Circles shown below the different exons indicate the frequency and type of mutations that have been found in the TCGA database in all types of cancer. Mutations that were identified in the T672E cell line are shown above the gene structure at the corresponding region. In red the mutation in the exon and in blue the mutation in the intron.

Mutations in JAK1 gene are associated with decreased mRNA expression

To assess the effect of the second mutation that occurred in the intronic region neighboring exon 15 of JAK1, we investigated the length of different parts on the JAK1 mRNA expecting that the mutation may lead to an altered splicing (Figure 15). To do so, we designed primer pairs that specifically amplified the region where the mutation occurred as depicted in the schematic representation of the JAK1 gene in Figure 15A. One control primer pair was placed on a region that was located before both mutations (Exon 7-9) while a second control primer pair amplified a region located at the end of the JAK1 gene (Exon 18-20). The last primer pair focused on amplifying the exons that were neighboring the mutation in the intron (Exon 15-17). Thus, an abnormal length of this last PCR product would indicate that the mutation leads to an aberrant mRNA and thus a nonfunctional JAK1 protein. We first screened 6 cell lines as well as PBMC with the two control

primer pairs to assess the level of expression of JAK1 (Figure 15B). We observe that 5 melanoma cell lines are expressing JAK1 at similar levels but lower than PBMC. Interestingly, the non-responding cell line T672E shows a more than tenfold decrease in the JAK1 mRNA expression. Using the T618A cell line as control, we controlled that the length of the mRNA from the T672E was the one that was expected. This was confirmed by migrating the PCR products on an agarose gel (Figure 15C). We continued the investigation by looking at the primer pair focusing on the splice acceptor variant. We performed a real-time PCR and found that there was a more than tenfold decrease of the expression of JAK1 in the T672E in comparison to the control T618A (Figure 15D). However, when migrating the PCR product on gel, we did not find an aberrant length for the T672E suggesting that the product remained functional but only expressed at a very low level. We can hypothesize that this decreased JAK1 expression is the explanation why we do not see any response in the T672E. We still decided to sequence the PCR products obtained from the PCR to ensure that the mutation did not led to minor changes in the sequence that could not be detected with our investigation as changes of 1 or 2 base pairs are not noticeable on gels. Similarly, if the sequence is changed but the length is not altered, the investigation we performed would not reveal it. The sequencing confirmed that the JAK1 mRNA was normal.

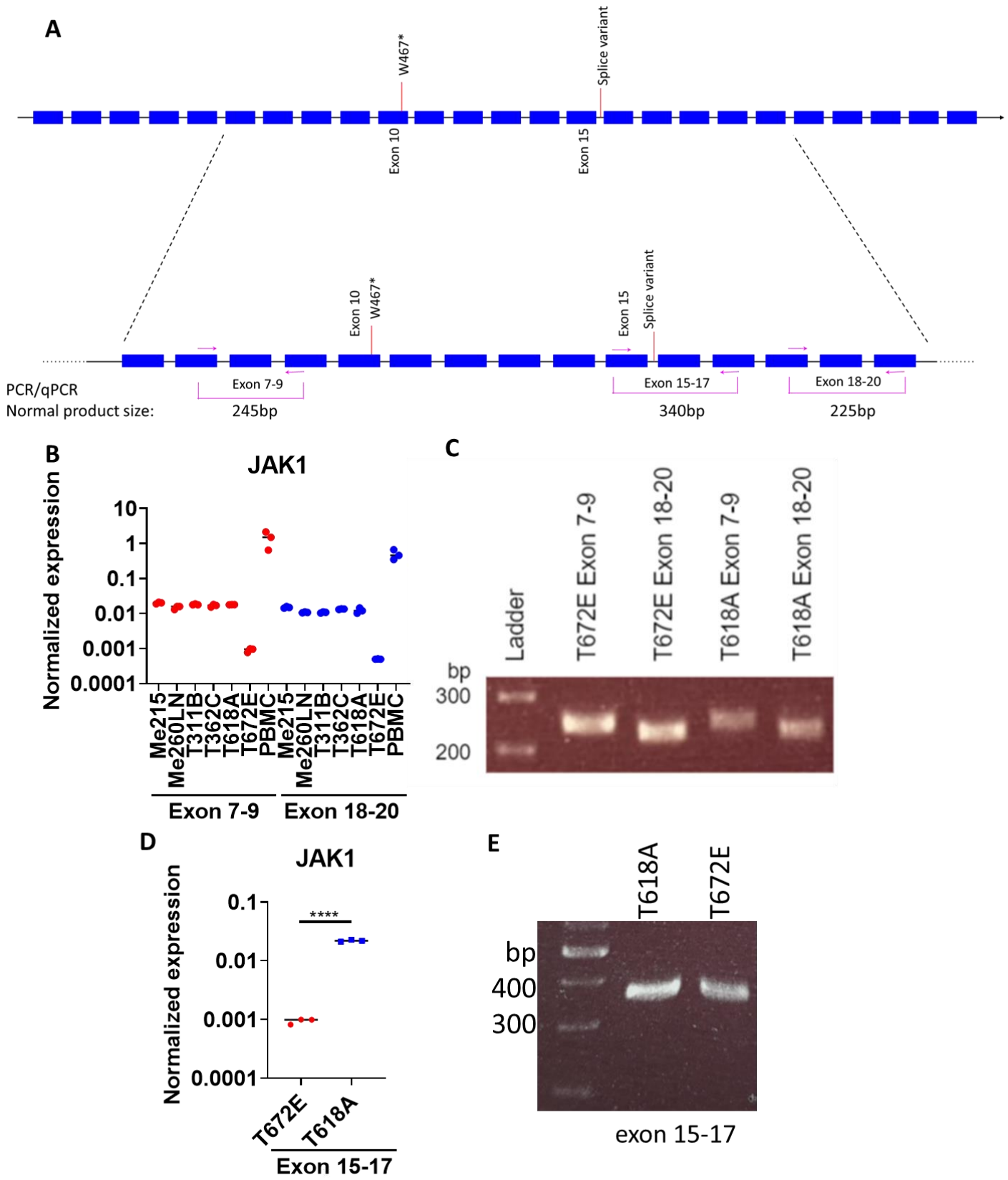


Figure 15. JAK1 gene integrity and level of expression in the T672E cell line

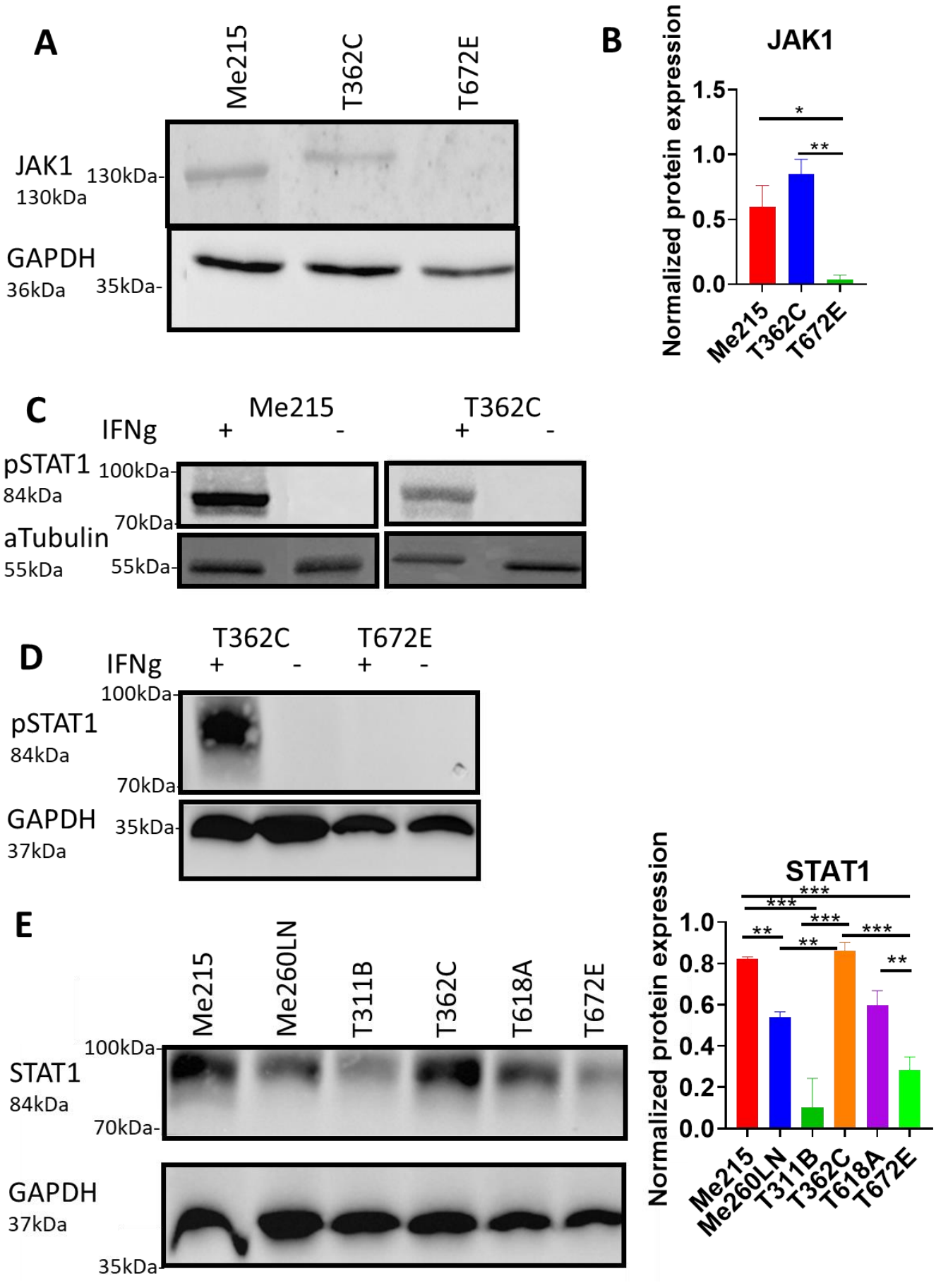
mRNA from cancer cells was isolated and used to assess the integrity of the JAK1 mRNA in the T672E cell line as well as the level of expression. (A) Schematic representation of the JAK1 gene indicating the locations of the mutations, the primers used for the PCR as well as the expected product size. (B) The level of expression of JAK1 was assessed in 6 melanoma cell lines and human PBMC by real-time PCR using primers specific for the exons 7 to 9 and 18 to 20. The graph presents the normalized JAK1 expression using GAPDH as housekeeping gene. (C) The size of the PCR products obtained from this PCR for T618A and T672E analyzed on a gel. (D) Level of expression of the JAK1 RNA (exon 15 to 17) was assessed by real-time PCR using GAPDH as housekeeping gene for normalization. (E) PCR products were analysed on agarose gel.

Lack of JAK1 in the T672E and poor IRF1 upregulation in the Me215 cells

As the mRNA level of JAK1 was strongly decreased in the T672E, we hypothesized that it may lead to the absence of the JAK1 protein and thus abrogate the IFNγ signaling pathway. In order to determine the protein level of JAK1 we performed a western blot analysis using cell lysates of different cell lines including the non-responding Me215 and T672E and the responding T362C. (Figure 16 A and B). JAK1 protein was present in both the Me215 and T362C but not in the T672E. To ensure that the downstream signaling was abrogated in the T672E, and to investigate whether the pathway was still functional in Me215, we dissected the IFNγ signaling pathway. The binding of IFNγ to its receptor leads to the recruitment of JAK1 and JAK2. Once activated, JAK1 and JAK2 phosphoralytes STAT1 on Tyrosine 701. pSTAT1 forms a homodimer that can translocate in the nucleus. We assessed the level of STAT1 phosphorylation in the Me215 and T672E using the T362C as responding control. Our results show that the pathway seems to be still functional in the Me215 as we see a nice phosphorylation of STAT1 after 10min of stimulation with IFNγ (Figure 16C). This is not the case in the T672E (Figure 16D). To ensure that the lack of JAK1 was the reason for the deficient STAT1 phosphorylation and not a reduced constitutive expression of STAT1, we assessed the level of STAT1 in several cell lines (Figure 16E). STAT1 was still present in the T672E but at a lower level than in the other cell lines. Interestingly, the non-responding cell line T311B also shows a decreased STAT1 expression compared to the cytokine-responsive cell lines analyzed.

To further investigate the reason why we do not observe a response in the Me215 upon IFNγ treatment, we assessed the translocation of STAT1 in the nucleus. To this end we stimulated the Me215 and T362C cells for 30min with IFNγ, performed a whole cell lysate and separated the nuclear and cytoplasmic fraction. As for the previous step, the pathway remained functional at this level as we still observe a translocation of STAT1 to the nucleus after IFNγ treatment in the Me215 (Figure 16F). Once in the nucleus, STAT1 induces the expression of IRF1 which in turn is

responsible for the induction of many genes including the reporters we investigated. We assessed the level of expression of IRF1 as well as its induction in the Me215 and other cell lines (Figure 16G). Although we still see a marginal increase in IRF1 after 3h and 5h of IFNγ stimulation, the response remained way lower than in the responding T362C and T618A. The T672E cells seem to express a constitutively low level of IRF1 that is not increased in presence of IFNγ. We can hypothesize that the reduced upregulation of IRF1 upon IFNγ treatment explains the lack of responsiveness of the Me215 cell line but further investigations are still required.


A
Me215
T362C
T672E
JAK1
130kDa
130kDa
GAPDH
36kDa
35kDa
B
JAK1
Normalized protein expression
1.5
1.0
0.5
0.0
*
**
Me215
T362C
T672E
C
Me215
T362C
IFNg
+
-
+
-
pSTAT1
84kDa
100kDa
70kDa
aTubulin
55kDa
55kDa
D
T362C
T672E
IFNg
+
-
+
-
100kDa
pSTAT1
84kDa
70kDa
GAPDH
37kDa
35kDa
E
Me215
Me260LN
T311B
T362C
T618A
T672E
100kDa
STAT1
84kDa
70kDa
GAPDH
37kDa
35kDa
STAT1
Normalized protein expression
1.0
0.8
0.6
0.4
0.2
0.0
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Me215
Me260LN
T311B
T362C
T618A
T672E

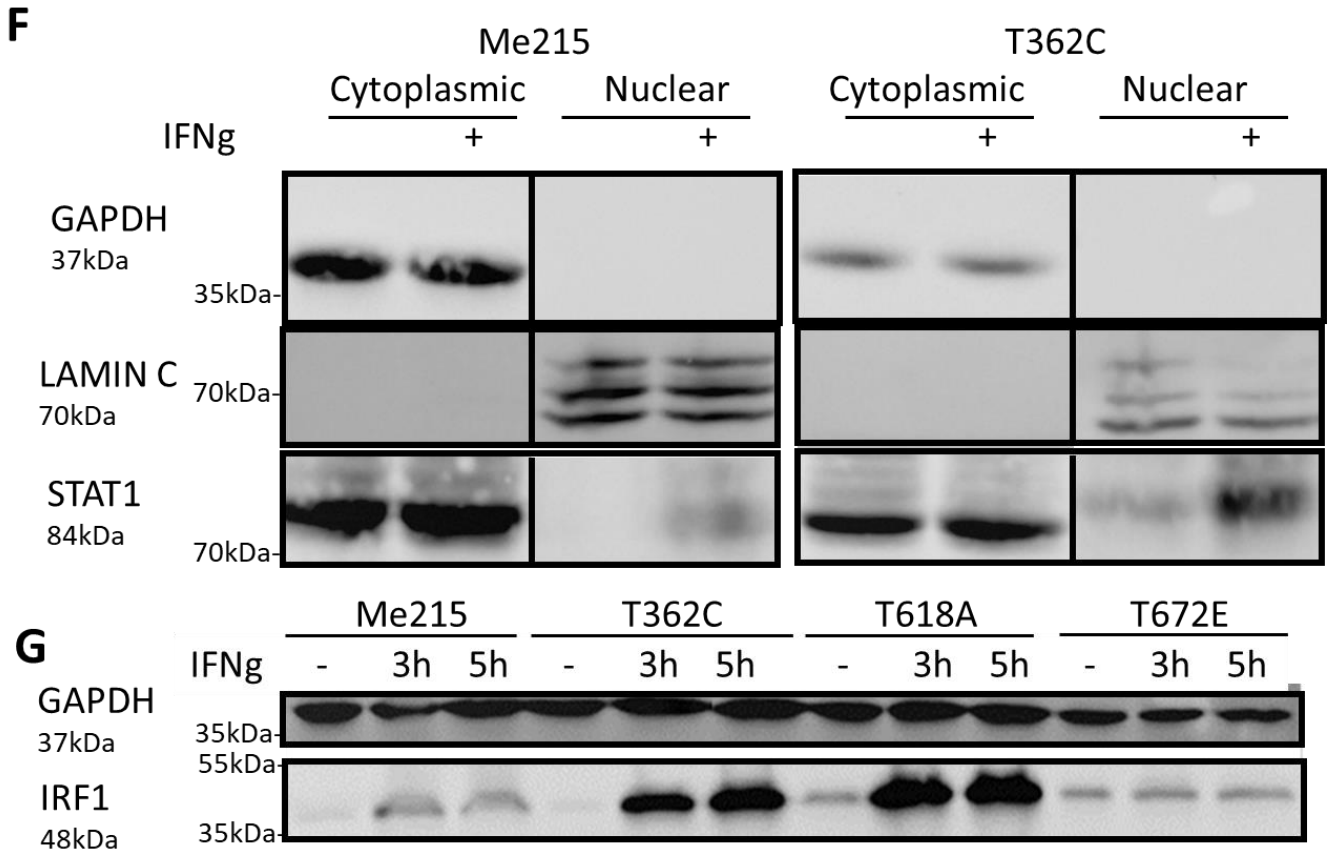


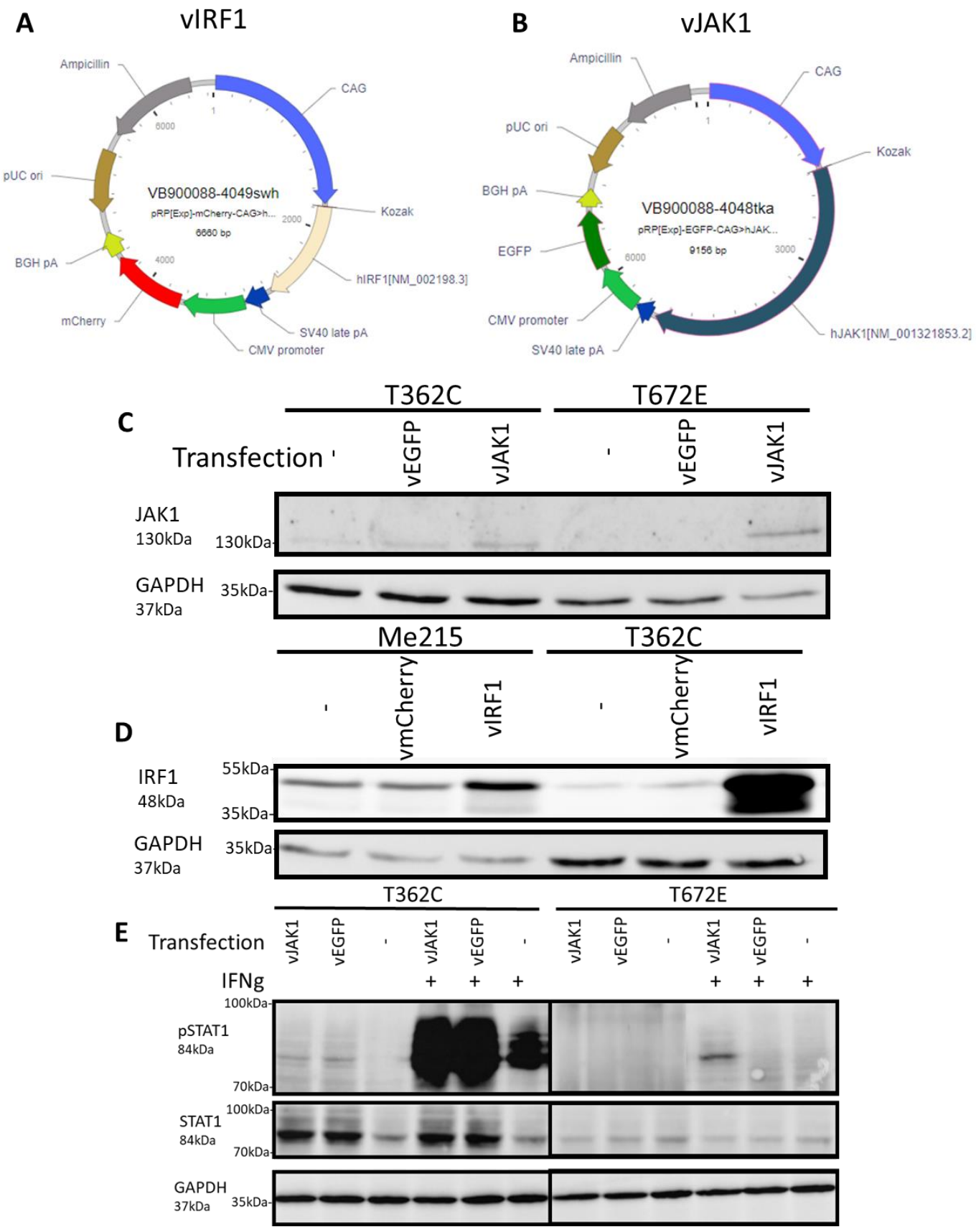
Figure 16. Characterization of the IFNγ signaling pathway in Me215 and T672E by western blot

Melanoma cells were seeded and treated the following day with IFNγ at 10ng/mL for all experiments that required cytokine. Preparation of whole cell lysate, cytoplasmic fraction and nuclear fraction has been performed as described in the material and methods. (A) Representative experiment of JAK1 expression levels. (B) Bargraph shows JAK1 expression level normalized to GAPDH. (C and D) Phosphorylation of Tyrosine 701 of STAT1 after 10 minutes of stimulation with IFNγ. (E) Bargraph shows STAT1 constitutive expression in different melanoma cell lines. (F) STAT1 level in the cytoplasmic and nuclear fractions of Me215 and T362C after 30minutes of IFNγ stimulation. (G) IRF1 protein level after 3h or 5h of stimulation with IFNγ. (n=4, p.value: ns = non significative with P>0.05, * P≤0.05, ** P≤0.01, *** P ≤0.001)

JAK1 transfection restores IFNγ signaling in T672E

To confirm that the defect in JAK1 is responsible for the lack of IFNγ responsiveness, we transfected the T672E cell line with a vector containing JAK1 (vJAK1) and EGFP using an EGFP-only vector (vEGFP) as control (Figure 17A). We performed the same experiment with an IRF1 (vIRF1) and mCherry (vmCherry) vector for the Me215 cell line (Figure 17B) in order to see whether it could lead to the upregulation of IFNγ induced genes and thus confirm that the lack of responsiveness is due to a defective IRF1 expression. The successful transfection was confirmed by

the expression of JAK1 and IRF1 investigated by western blot analysis for both cell lines (Figure 17C and D). JAK1 level was similar to what can be found in the responding T362C. To determine whether the reconstitution of JAK1 lead to a regain of IFNγ signaling, we assessed the STAT1 phosphorylation upon IFNγ treatment(Figure 17E). The phosphorylation of STAT1 was not very strong but still present in the T672E cells transfected with vJAK1 while no phosphorylation was found in the cells transfected with the vEGFP. One reason for the minor effect could be due to the low transfection efficiency which means that only part of the cells that were used to prepare cell lysate were successfully reconstituted (Figure 17F and G). As PD-L1 is one of the genes for which we saw the strongest upregulation after IFNγ treatment in responding cell lines, we assessed its level of expression in vJAK1-transfected IFNγ-treated T672E (Figure 17H and J). Interestingly, we see some level of upregulation of PD-L1 only in vJAK1-transfected IFNγ treated T672E. No changes were observed with vEGFP transfection. However, the PD-L1 upregulation remained lower than the control responding cell line. Maybe other mechanisms are coming into play or this responsiveness is due to the heterogeneity that we observe among all cell lines. IRF1 transfection in the Me215 did not lead to an upregulation of PD-L1 in both the Me215 and T362C cell lines (Figure 17I and K).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pUC ori
Kozak
BGH pA
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pRP[Exp]-mCherry-CAG>h...
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B
vJAK1
Ampicillin
CAG
pUC ori
Kozak
BGH pA
EGFP
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SV40 late pA
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9156 bp
C
T362C
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-
vEGFP
vJAK1
JAK1
130kDa
130kDa
GAPDH
37kDa
35kDa
D
Me215
T362C
-
vmCherry
vIRF1
-
vmCherry
vIRF1
IRF1
48kDa
55kDa
35kDa
GAPDH
37kDa
35kDa
E
T362C
T672E
Transfection
vJAK1
vEGFP
-
vJAK1
vEGFP
-
vJAK1
vEGFP
-
vJAK1
vEGFP
-
IFNg
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STAT1
84kDa
100kDa
70kDa
GAPDH
37kDa
35kDa

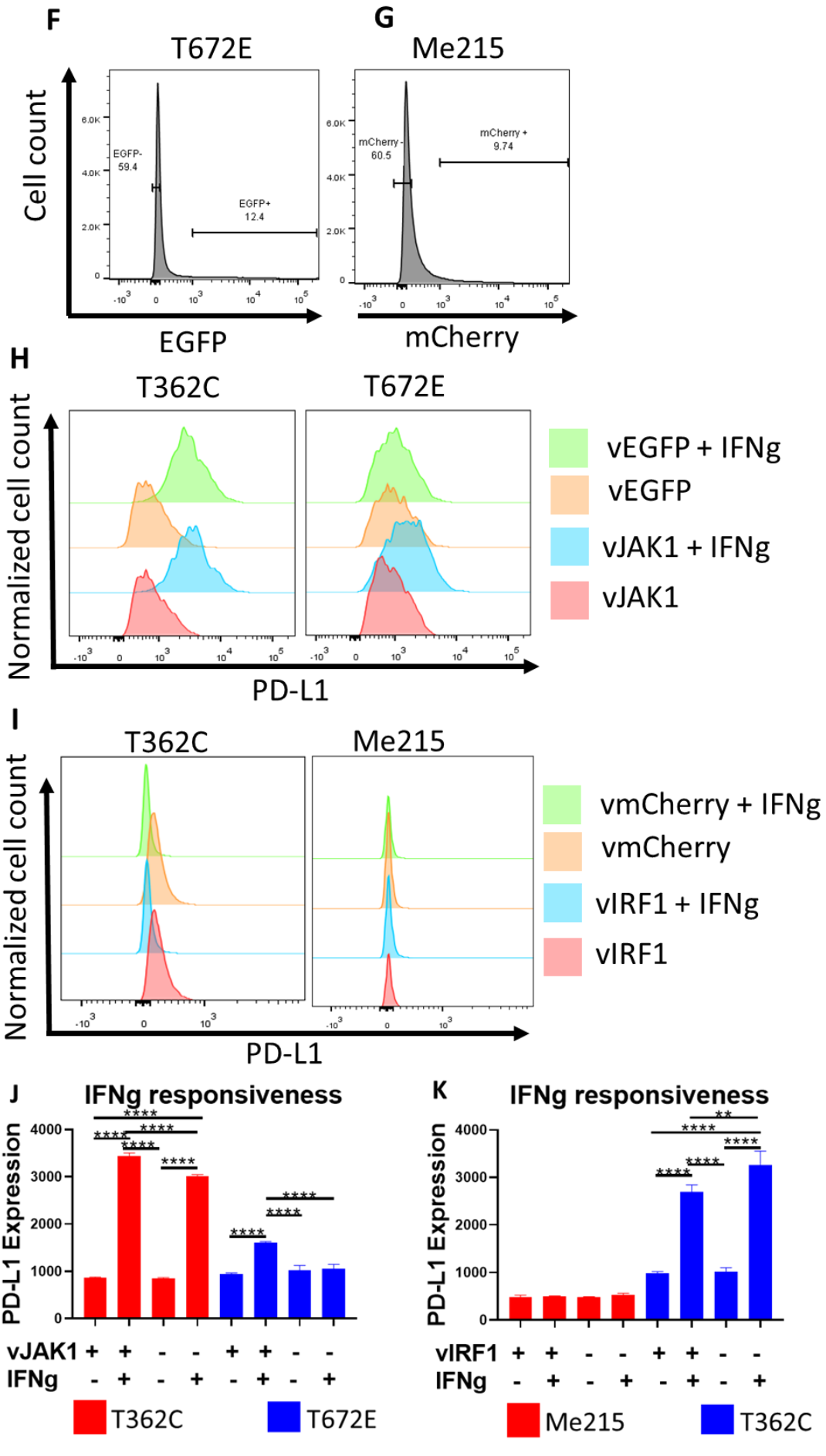
F
T672E
Cell count
EGFP-
59.4
EGFP+
12.4
EGFP
G
Me215
mCherry -
60.5
mCherry +
9.74
mCherry
H
T362C
T672E
Normalized cell count
PD-L1
vEGFP + IFNg
vEGFP
vJAK1 + IFNg
vJAK1
I
T362C
Me215
Normalized cell count
PD-L1
vmCherry + IFNg
vmCherry
vIRF1 + IFNg
vIRF1
J
IFNg responsiveness
PD-L1 Expression
vJAK1
IFNg
T362C
T672E
K
IFNg responsiveness
PD-L1 Expression
vIRF1
IFNg
Me215
T362C

Figure 17. Restoring the IFNγ signaling pathway with JAK1 or IRF1 transfection

Cells were transfected with IRF1-mCherry (A) or JAK1-EFGP (B) vectors from VectorBuilder using the FuGene HD transfection reagent. Transfection was done the day following the cell seeding, and treatment and cell collection was performed 2 days after transfection to ensure that the proteins of interest were expressed. JAK1 and IRF1 expression were confirmed by Western Blot 2 days after transfection with vJAK1, vEGFP (C), vIRF1, vmCherry (D). T362C and T672E cells were treated for 10min with 10ng/mL of IFNγ 2 days after transfection with vJAK1 or vEGFP. Protein expression of STAT1 and STAT1 phosphorylation on Tyrosine 701 were assessed by western blot. (E) T672E, Me215 and T362C were transfected with the vectors described above and treated at day 2 post transfection for 48h with IFNγ at 10ng/mL. Transfection efficiency was assessed by measuring the proportion of EGFP+ (F) or mCherry+ (G) using flow cytometry. (H) Representative experiment showing PD-L1 expression levels in vJAK1 or vEGFP transfected T362C and T672E cells +/- IFNγ (10ng/ml) treatment. (I) Representative experiment showing PD-L1 expression levels in vIRF1 or vmCherry transfected Me215 and T362C cells +/- IFNγ. (J and K) Barplot representation of the PD-L1 levels after vJAK1, vEGFP, vIRF1 or vmCherry transfection of the Me215, T362C and T672E cell lines +/- IFNγ treatment. Mean and standard deviation, n=3, p.value: ns = non significative with $P>0.05$, * $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$ and **** $P\leq 0.0001$.

Attempts to identify the mechanisms responsible for the lack of cytokine responsiveness in Me215 cells

As we identified that the Me215 cell line poorly upregulated IRF1 after IFNγ stimulation, we wanted to assess whether it was still able to increase ICAM expression as this reporter induction is IRF1 independent[99]. Me215 and T362C cells were treated with IFNγ for 48h and ICAM expression was assessed by flow cytometry. We observed that ICAM expression increased in the Me215 and the T362C cells after IFNγ treatment while PD-L1 did not increase in the Me215 cells (Figure 18A). This confirms that the IFNγ signaling pathway is still active in the Me215 but the IRF1-dependent gene regulation seems to be altered. As we did not find any mutation in IFNγ key genes, we hypothesized that Me215 only poorly upregulated IRF1 upon IFNγ treatment because of aberrant Histone Deacetylation activity leading to the inaccessibility of IRF1 gene or its promotors. To test this hypothesis, we used the HDAC inhibitor CUDC-101 aiming to restore IFNγ responsiveness in the Me215 and determined the upregulation of IFNγ reporter proteins by flow cytometry. Our results show that IFNγ mediated HLA-I upregulation was only seen in Me215 when cells were treated with CUDC-101 (Figure 18B). Further investigations are required to confirm our hypothesis. We are currently performing an ATAC sequencing analysis of different cancer cell lines including the Me215. We hope that these results will indicate whether the lack of responsiveness in the Me215 is due to epigenetic dysregulations.

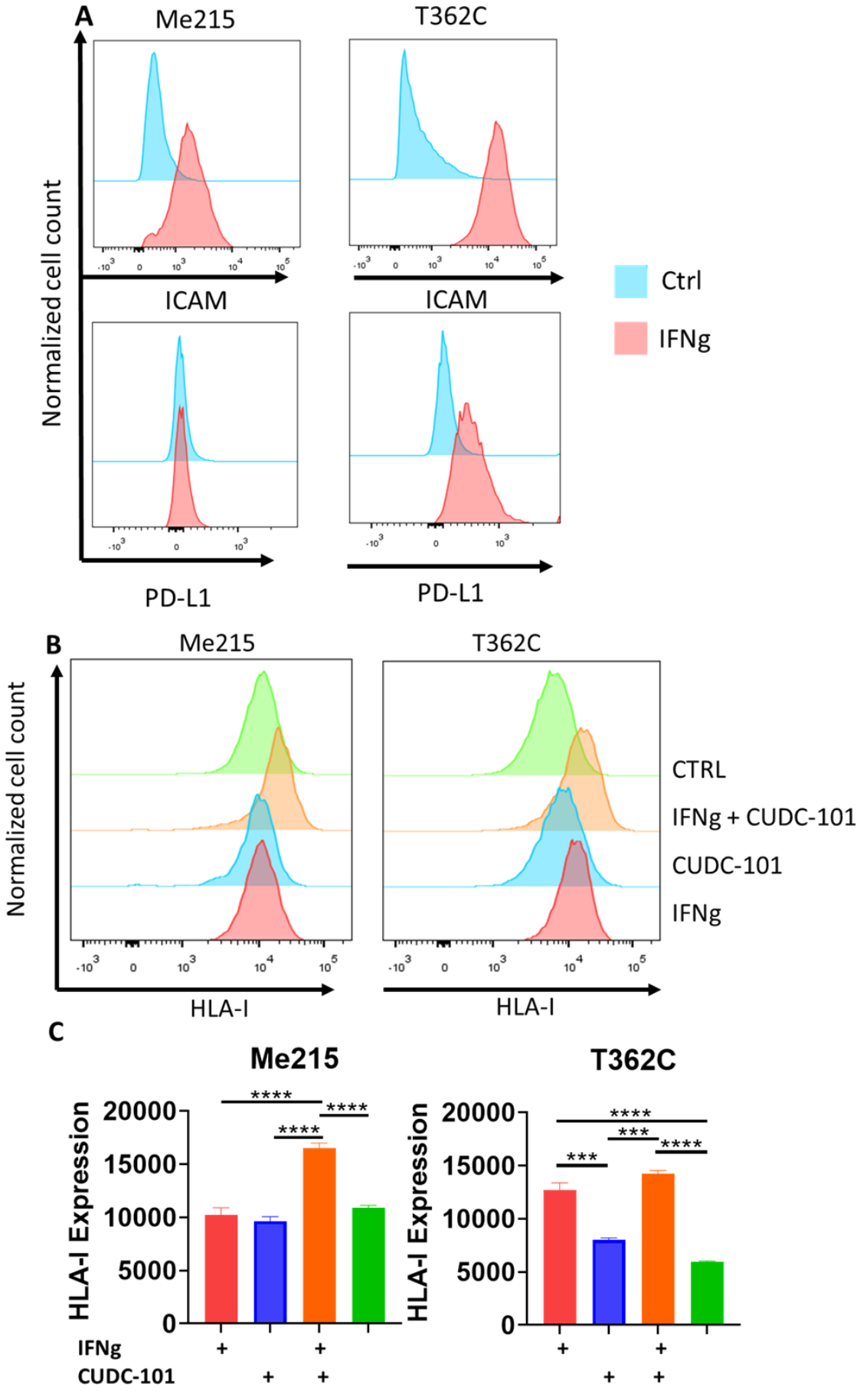
A
Me215
T362C
Normalized cell count
ICAM
ICAM
Ctrl
IFNg
PD-L1
PD-L1
B
Me215
T362C
Normalized cell count
CTRL
IFNg + CUDC-101
CUDC-101
IFNg
HLA-I
HLA-I
C
Me215
T362C
HLA-I Expression
20000
15000
10000
5000
0
****
****
****
***
***
****
IFNg
CUDC-101

Figure 18. Me215 cell line as partial IFNγ responder and restoration of its responsiveness
Cells were plated on day 0 as indicated in Table 1 and treated the following day with IFNγ at 10ng/mL. (A) Level of expression of ICAM and PD-L1 were assessed by flow cytometry after 48h of treatment. (B) Cells were plated and treated the following day with CUDC-101 (Selleckchem S1194) at 1uM for 24h before adding IFNγ at 10ng/mL. HLA-I expression was assessed by flow cytometry after 48h of cytokine stimulation. (C) Barplot representation of the level of expression of HLA-I by Me215 and T362C treated with IFNγ and/or CUDC-101 as in the experiment described above. Mean and standard deviation, n=2, p.value: ns = non significative with $P>0.05$, * $P\leq0.05$, ** $P\leq0.01$, *** $P\leq0.001$ and **** $P\leq0.0001$.

Discussion

We initially hypothesized that melanoma cells are actively participating in shaping the tumor microenvironment by responding to the immunological cues present. As tumor cells arise from healthy cells, we expect to see conserved behaviors that may either favor or de-favor the elimination of the melanoma cells by the immune system. However, it is clearly a possibility that melanoma cells either acquire new mechanisms that suppress the anti-tumoral immune response or most importantly, that they are able to lose mechanisms that would normally support the antitumoral immune response. Previous researches have shown that the phenotype of melanoma cells exposed to CTLs could be largely reproduced by IFNγ and TNFα treatment[94] suggesting that a big portion of the changes induced by the CTLs is mediated by cytokines and thus that a better characterization of the role of the cytokines is required. According to our rational that T cells are the prominent cell population that exert anti-tumor activity, we focused our investigation on the effects of T cell derived cytokines on the immunological phenotype of melanoma cells. We selected a panel of cytokines and used a set of reporter proteins based on what was recently found to be upregulated upon coculture of melanoma cells with CTLs or IFNγ plus TNFα treatment[94]. The panel included proteins that were previously reported to support anti-tumoral immune reactions such as HLA-I[100], HLA-II[101], CXCL9[68] and CXCL10[68] or proteins involved in the inhibition of antitumoral immune cells such as PD-L1[102], IDO[103] and CCL2[67] although the role of CCL2 is not very clear yet. We selected these reporters as they are key players in crucial points of the anti-tumoral immune response. HLA-I and HLA-II are involved in antigen presentation (influencing both the level of antigen presentation and the panel of antigens presented) which is required for elimination of tumor cells by CTLs, while CXCL9 and CXCL10 are major chemokines involved in the recruitment of CTLs. On the opposite side of the spectrum of the anti-tumoral immune response, we have PD-L1 that is strongly involved in the inhibition of T cell activity as is IDO, while CCL2 has been reported to be involved in the recruitment of immunosuppressive cells. We are aware that this panel of reporter proteins may not capture the whole response of melanoma cells to the cytokines we exposed them to but as those were upregulated in many of the cell lines that were co-cultured previously with CTL[94], we expect to capture relevant parameters of the melanoma cells' immune phenotype.

Our initial screening indicates that most of the patient-derived melanoma cell lines did not respond to many of the cytokines we selected. We do not exclude the possibility that this lack of observed responsiveness was due to the panel of reporter proteins we used. We could imagine that other immune genes were induced by these cytokines and that they could lead to different events in the melanoma cells ultimately influencing the immune response inside the tumor microenvironment.

We did not assess cell proliferation and cell viability which are both relevant information that could also be influenced by these cytokines. In this regard, a broader screening of the effects of cytokines on the phenotype of melanoma cells could be of interest. RNAseq in combination to a viability assay and a proliferation assay could be a good approach. However, this would be only a first step to obtain indications on the relevant cytokines as molecular characterization of the response is still required.

To summarize the results of this initial screening, we found no changes in the expression of the selected reporters upon exposure to IL2, IL4, IL5, IL13, GM-CSF, CCL3 and CCL4. IL6 induced HLA-DR upregulation but only in one of the 5 cell lines. IFNγ induced mostly HLA-I, HLA-DR and PD-L1 upregulation. TNFα induced CCL2 but also MHC-I and PD-L1 in some cell lines. TNFβ induced the same genes as TNFα. Interestingly, 2 cell lines did not respond to any of the cytokines. We saw no or only poor upregulation of CXCL9 and CXCL10 upon IFNγ treatment which was surprising as these chemokines are supposed to be induced by IFNγ[50]. We cannot exclude that the lack of responsiveness could be due to other parameters. Indeed, we focused on a single timepoint and it is possible that induction of some proteins requires more than 48h. Additionally, we added the cytokines only a single time, at the beginning of the culture period. The cytokine concentration is likely to decrease over time through degradation and capturing by the cells.

We continued the investigation by increasing the doses of cytokines to ensure that the lack of responsiveness was not due to doses that were too low as well as to assess whether we could increase the response. As it was shown in the previous research that the combination of IFNγ and TNFα was required to reproduce the phenotype of the melanoma cells in presence of CTLs, we also combined both cytokines. We found that increasing the dose of IL6 did not further increase HLA-DR expression. Similarly, increasing the dose of IFNγ and TNFα did not or only poorly increase the responsiveness of the melanoma cells. It is possible that the maximal response for each cytokine alone was already achieved with the low dose or that increasing the dose led to some level of cytotoxicity that reduced the cell fitness and thus their ability to respond. In opposition to what was observed with increased doses, combination of cytokines boosted the responsiveness and led to strong upregulation of CXCL9 and CXCL10. The combination of IFNγ and TNFα at low dose were frequently stronger than the high dose single cytokine treatment suggesting a synergy between these cytokines. However, the 2 cell lines that did not respond previously remained unresponsive.

We continued our investigation by screening the responsiveness of 21 patient-derived melanoma cell lines and 2 human melanocytes to IFNγ, TNFα and the combination of both. Melanocytes were included to see how “healthy” cells should normally respond to the cytokines and investigate which cells show aberrant phenotypes. This screening clarified some of the observations that were made

during the initial screening. IFNγ induces the upregulation of MHC-I, MHC-II and PD-L1 in many cell lines. We also observe some IDO, CXCL9 and CXCL10 upregulation. It is not entirely clear whether the response of the melanoma cells to IFNγ is supporting the immune response or not. Indeed, increased antigen presentation and T cell recruitment favors tumor cell elimination while upregulation of inhibitory mechanisms dampen T cell activity. There seems to be a balance between the pro and antitumor effects of IFNγ although the signaling may still favor one side. This balance may explain why it is not optimal to use IFNγ as treatment alone. Indeed, simply increasing the level of expression of genes on both sides of the balance may not lead to beneficial outcome[104]. Systemic IFNγ treatment has been used with the aim of converting cold tumors into hot tumors. Characterization of the tumors showed an increased antigen presentation associated with some level of infiltration by T cells but also an increased expression of the immune checkpoint protein PD-L1[105]. The addition of immune checkpoint blockade could shift the balance towards an IFNγ signaling that support the antitumoral immune response. In this regard, PD1/PD-L1 blockade would make more sense as the inhibition seems to occur at the tumor site, but one could still expect benefits from combining PD-1 and CTLA4 blockade.

TNFα mostly induced CCL2 but also MHC-I and PD-L1 in some cell lines. CCL2 is a chemokine that is involved in the recruitment of a wide range of immune cells including monocytes, basophils, T lymphocytes and NK cells[106]. The role of CCL2 in melanoma remains partially unclear as it has been shown to influence the immune status of the tumor through the recruitment of mononuclear[107] cells and polarization of macrophages towards a M2 phenotype[108], but also the recruitment of cytotoxic T cells[109]. This pleiotropic role of CCL2 may explain why clinical trials targeting CCL2 were not very successful. A hypothesis is that CCL2 blockade leads to an improved antitumoral immune response that is dampened by other inhibitory mechanisms such as PD1/PD-L1[110, 111]. We showed that TNFα and IFNγ, which can be both expressed by tumor-specific T cells, are inducing the expression of CCL2 and PD-L1, respectively. CCL2 has been proposed as a mechanism of resistance to PD1 blockade through the recruitment of immunosuppressive cells, while PD1/PD-L1 interaction could dampen the anti-tumoral immune response supported by CCL2 blockade. In this regard, it is not surprising that CCL2 blockade may enhance the immunotherapeutic effect of PD1 blockade[110, 111].

The combination of both IFNγ and TNFα showed great upregulation of most of the reporter proteins. CXCL9 and CXCL10 were nicely induced by IFNγ plus TNFα indicating that both cytokines are required. By making a hierarchical clustering of the cell lines based on the responsiveness, we observe a gradient of responsiveness. The observation that the melanocytes were among the responding cell lines indicates that some of the melanoma cell lines decreased or lost the ability to respond. Although the responsiveness is an heterogenous continuum, we could

clearly classify T311B and T1349A as poor responders and Me215 and T672E as non-responders. This means that 4 out of 21 melanoma cell lines show a strongly altered response to IFNγ and surprisingly these 4 cell lines also have a decreased TNFα responsiveness. It would be interesting to see if this decreased response to both cytokines is something unique or whether it is frequently happening together. We could hypothesize that the decreased responsiveness to one of the cytokines confers an advantage to the tumor cells but also impacts the other pathway, and if so by which mechanism(s). It is a possibility that these phenotypes have been selected by a strong anti-tumoral immune response. As mentioned previously, losing the ability to respond to TNFα may protect from its cytotoxicity while not responding to IFNγ offers many advantages including decreased antigen presentation. Verifying this hypothesis would require to have access to cell lines or at least tumor samples taken at earlier time points to see whether these altered response appeared in presence of an anti-tumoral immune response and whether their appearance is associated with a decrease in the activation and/or numbers of tumor-specific immune cells. We think that broader screening should be performed to investigate how frequent this reduced responsiveness is and how it may influence the results of immunotherapy. In addition, some cell lines only lost the ability to upregulate certain proteins while still upregulating the others. Both cases are of interest. As the response to IFNγ and TNFα includes the expression of proteins that have pro- and anti-tumoral roles, losing the ability to upregulate proteins having anti-tumoral activity while still upregulating proteins that protect tumor cells might be a great advantage for the tumor. Investigating the role of these particular alterations will be discussed.

Besides influencing the immunological phenotype of the melanoma cells, it has previously been reported that IFNγ has an antiproliferative activity while TNFα has a direct cytotoxic effect on tumor cells[112]. We assessed the effect of the two cytokines on the proliferation and the viability of the melanoma cells. By assessing the proliferation, we can report that IFNγ alone already reduced the proliferation of some cell lines and that a few cell lines were also affected by TNFα. As for the reporters, the combination of both cytokines had the strongest anti-proliferative activity and most of the cell lines were affected. Here again, we report a great heterogeneity in the response of the melanoma cells with some cell lines that were not affected among which we find the one that were previously reported as poor (T1349A and T311B) or non (Me215 and T672E) responders. We can hypothesize that the tumor cells acquired mechanisms that protect them against the anti-proliferative activity of the cytokines which confers them an advantage but it is also possible that this is only a side effect of the loss of the ability to upregulate immune genes. It would be interesting to develop a melanoma mouse model that can reproduce the resistance to the antiproliferative activity of the cytokines while maintaining the upregulation of the different

immune genes that we previously investigated. In combination with another model reproducing the inhibition of proliferation while being unable to upregulate immune genes, this model would allow us to assess whether the resistance to the inhibition of proliferation is really a relevant mechanism of resistance to immunotherapy.

We assessed the effect of the cytokines on the viability of the cells. We found that TNFα was decreasing the viability in some cell lines while IFNγ had no effect. We do not seem to have a synergy of the cytokines when looking at the combination. Again, we see some heterogeneity in the viability of the cells after treatment with some not being affected by the cytokines. We can hypothesize that some cell lines lost the ability to respond to TNFα or at least are resistant to the cytotoxic activity of TNFα while maintaining a functional signaling pathway. Tumor immune evasion has been shown to occur thanks to loss of TNFα sensitivity in a melanoma mouse model[112]. Our observations indicate that loss of TNFα sensitivity is also happening in melanoma patients, but do not give further indication on the clinical impact of this lack of responsiveness. We do not know whether this phenomenon is relevant for patients. Finding patient derived melanoma cell lines that became resistant to TNFα-induced cell death while still maintaining CCL2 upregulation would be a better indication of the relevance of this process. This raises a general question regarding the experiments that we performed in vitro: What concentration of cytokines should be used to mimic what is occurring in patients. In our assay, we used 50ng/mL of TNFα. It would be interesting to know which cytokine concentration is physiologically relevant. This would require quantification of the cytokines in different locations of the tumor. Indeed, it is probable that the concentration of TNFα and IFNγ vary a lot depending on the distance to T cells. Improving in vitro assays calls for a better characterization of the cytokine concentration in the tumor in vivo.

To learn more about an eventual relevance of this hypothesize, it will be necessary to better characterize the role of TNFα in the tumor microenvironment and also of the concentration of TNFα and IFNγ that can be found in absence of immunotherapy. Indeed, the doses we are using in our assays were decided based on their ability to reproduce the phenotype of the melanoma cells in presence of CTL in vitro but it is possible that in the tumor, the cytokine concentrations are different. We do not have the technologies to assess the concentration of the cytokine inside the tumor, or more specifically in the proximity of activated T cells. We only have information on the presence and the concentration of the soluble factors in the tumor by characterizing liquid biopsies of the tumor-draining lymphatic vessels[113]. A study reported that TNFα concentration in the tumor draining lymph was around 3pg/mL which is lower than the concentration we used. However, we can hypothesize that this is due to the dilution of TNFα and that the concentration in the proximity of activated T cells is higher.

Now that we characterized the responsiveness of the melanoma cells to IFNγ and TNFα, we took advantage of the data we have about our cohort of patients to see whether we have an association between the ability of the cell line to upregulate each reporter protein and the survival of the corresponding patient. Only HLA-I upregulation in presence of IFNγ and TNFα together was associated with a prolonged survival of the patients. We see some trends when looking at the median survival of each group but they should not be interpreted as our cohort is quite small and the heterogeneity makes it hard to find significative associations especially with groups that can be of very different size. This calls for a better characterization of the ability of tumor cells to respond to cues present in the tumor microenvironment at the molecular level as this would give indications on the anti- or pro-tumoral role of some proteins or pathways. In addition, these data could also give indications on the reason why some treatments such as IDO blockade[114] or CCL2 blockade[111] alone are giving the expected results in the clinic.

The screening we performed identified 2 cell lines (T672E and Me215) that were not responding to IFNγ and TNFα and we were interested in determining what were the mechanisms involved in this absence of response as well as what were the consequences. One mechanism by which the cells can lose the ability to respond is by not expressing the receptors. We assessed the level of expression of TNFR1, TNFR2, IFNGR1 and IFNGR2 by qPCR. T672E expressed the receptors at similar levels to what was found in responding cell lines. TNFR1 and IFNΓR2 were expressed at lower levels in the Me215. This observation does not mean that a reduced receptor level is responsible for the lack of responsiveness. Further characterization of the pathway activation is still required. Interestingly, treatment of the cells with IFNγ, TNFα or the combination led to changes in the level of expression of the receptors. Indeed, the treatment induced an upregulation of receptor expression in some cell lines which could explain cellthe synergistic effect that we observed when looking at the reporters. We can hypothesize that IFNγ or TNFα signaling alone are strong enough to induce CXCL9 and CXCL10 in some cell lines but that by increasing the number of receptors at the cell surface, the signaling became strong enough to induce the expression of CXCL9 and CXCL10 or further upregulate PD-L1. We should still take into consideration that these data were obtained by qPCR which does not give an indication on the localization of the receptors so it should first be confirmed that these treatments lead to an increased number of receptors at the cell surface.

As the level of expression of the receptors was not explaining the absence of responsiveness of the cell line T672E, we decided to determine putative genetic alterations in the IFNγ and TNFα signaling pathways. We performed a whole exome sequencing experiment and looked at complete loss of copy of genes in both pathways as this might give a clear indication on why cells do not

respond to the cytokines. We confirmed that at least one copy of each key gene of the IFNγ and TNFα signaling pathway was still present. We then looked at mutations and found that the cell line T672E had one mutation in exon10 and a second mutation in an intron neighboring the exon15 of the JAK1 gene. The mutation in exon10 leads to a stop codon that renders the protein non-functional but the effect of the second mutation was not clear. As it could interfere with the splicing of the RNA, we performed qPCR to look at the level of expression of JAK1 as well as the length of the mRNA produced. First of all, JAK1 was expressed at a similar level in all melanoma cell lines except in the T672E which was very low. However, when looking at the length of the mRNA to assess whether the splicing would give aberrant mRNA, we did not find any alteration. No specific mutations were observed in the Me215 cell line.

To assess the consequences of the mutation in exon 10 of JAK1 found in the T672E cells and the low level of cytokine receptors expression in the Me215, we characterized the activation of the IFNγ signaling pathway at the molecular level. When first looking at JAK1, we did not detect JAK1 in the T672E. This was associated with an absence of STAT1 phosphorylation upon IFNγ treatment and thus indicates that the lack of JAK1 was responsible for the lack of response to IFNγ in T672E. In contrary, when characterizing the IFNγ pathway activation in the Me215, we observed the phosphorylation of STAT1 as well as its translocation to the nucleus upon IFNγ treatment. These observations indicate that the low level of IFNGR2 receptor expression was not sufficient to prevent IFNγ signaling. Normally, STAT1 translocation into the nucleus leads to IRF1 upregulation which in turn induces the expression of a panel of genes. We found that IRF1 upregulation in the Me215 was very weak in comparison to responding cell lines and thus hypothesize that this low IRF1 upregulation was responsible for the lack of response observed.

JAK1 was reconstituted in the T672E by transfecting the cells with a JAK1-EGFP vector. After confirming the expression of JAK1 and the phosphorylation of STAT1 upon IFNγ treatment by western blot, we showed that the cells were able to respond to IFNγ again by upregulating PD-L1 although the upregulation was not as high as the one that was observed in the control cell line but this could be simply part of the heterogeneity that we usually see in such responses. A similar experiment has been performed with the Me215 with an IRF1-mCherry vector. Although IRF1 was expressed in the transfected Me215, there was no upregulation of PD-L1 both in absence or in presence of IFNγ. This indicates that there are other mechanisms responsible for the IFNγ non-responsiveness of the Me215 cells.

The reporters we used to determine cytokine responsiveness are induced via IRF1 which was only poorly expressed in the Me215. So we decided to assess the level of expression of ICAM-1 as it is induced by IFNγ in an IRF1-independent manner. When the Me215 was treated with IFNγ, PD-L1 expression remained unchanged but ICAM-1 increased. The observation that Me215 is still able to upregulate ICAM-1 but that IRF1 transfection does not increase PD-L1 expression indicates that there might be several mechanisms explaining the lack of responsiveness. The first one is the inability to upregulate robustly IRF1 upon IFNγ treatment but even when IRF1 is induced, we do not see any changes in PD-L1 expression suggesting that a second alteration is present in this cell line. As we found no specific mutation in the Me215, we hypothesize that an epigenetic dysregulation could prevent the induction of IRF1 as well as the induction of the downstream genes of IRF1. We treated the Me215 with an HDAC inhibitor before the addition of IFNγ and found that this led to an increased PD-L1 expression. We still need to confirm these results as one of the three experiments was not conclusive, but they seem to confirm that there is an aberrant HDAC activity in this cell line that prevents the upregulation of IRF1. We only assessed PD-L1 upregulation after HDAC inhibition and IFNγ treatment, and still need to confirm that it also leads to the upregulation of other reporters. The role of IRF1 in the tumor is context dependent. IRF1 has the potential to mediate T cell blockade by inducing the upregulation of PD-L1[115]. However, this observation and thus this role of IRF1-induced PD-L1 is less relevant in presence of PD-L1 blockade. We do not have much data on the frequency of alterations of IRF1 in melanoma. Our data indicates that epigenetic dysregulation may be a factor. Other researchers showed that activation of the Wnt/β-Catenin signaling pathway may lead to IRF1 degradation[116]. The Wnt/β-Catenin pathway activation in melanoma cells has been shown to affect CTL activity in a co-culture model. It was suggested that this was due to IL10 expression by the melanoma cells, but IL10 blockade did not fully restore T cell activation[117]. We would suggest to assess the IFNγ responsiveness of melanoma cell lines that present an active Wnt/β-Catenin pathway. This is relevant due to the observation that activation of this pathway has been associated with inhibition of anti-tumor immunity[118] and resistance to immunotherapy[119]. Wnt/B-Catenin pathway is known to be active in colorectal cancer but genetic and epigenetic alterations have also been found in melanoma[120, 121]. HDAC inhibitors are now used in the clinic and have shown their ability to enhance the response to immunotherapy although the mechanisms are not entirely clear yet. A study reported that HDAC treatment of melanoma cells lead to a decreased PD-L1 expression and increased HLA-I expression. We did not find a similar phenotype as HDAC inhibitor alone had no effect[122]. However, they also indicate that the combination of an HDAC inhibitor with anti-PD-1 in a B16 mouse model was associated with an increased plasma level of CXCL9 and CCL2 which would fit with the observation that HDAC inhibition may improve the response to IFNγ by the tumor cells and thus a higher expression of

these chemokines. Interestingly, combination of an HDAC inhibitor with anti-CTLA4 did not have the same effect[122]. One explanation would be that HDAC inhibition improves the response of melanoma cells to IFNγ which also leads to the upregulation of PD-L1 that is not blocked and thus dampens T cell activation, preventing further induction of CXCL9 and CCL2. Further investigations are required to assess how frequent IRF1 downregulation occurs in cancer patients, and the mechanisms leading to this phenotype, but we can hypothesize that it could contribute to tumor immune evasion even in presence of immunotherapy.

To summarize these observations, we report that one cell line (T672E) did not respond to IFNγ because it acquired a mutation in the JAK1 gene that abrogated downstream IFNγ signaling. The other non-responder (Me215) has aberrant HDAC activity that hampers its response to IFNγ by preventing the induction of IRF1 and downstream genes but not the IFNγ-induced IRF1-independent ICAM-1 protein upregulation. Together, these observations underline the diversity by which the tumor cells can alter their phenotype/function and thus calls for a better characterization of the immunological phenotype of the melanoma cells at all levels.

In this new era of immunotherapy, the immunological phenotype of melanoma cells is becoming more and more relevant and many studies are now focusing on it to improve the efficacy of such treatments[123]. The importance of IFNγ is regularly pointed out in this regard[123]. Indeed, IFNγ induces many changes on the melanoma cells and these changes can be both pro- or anti-tumoral. Immunotherapies such as PD-L1 blockade shifts the balance of IFNγ signaling towards its anti-tumoral effect and thus it is not surprising that IFNγ signaling in vivo and in vitro is associated with good response to therapy[123]. In addition, we can hypothesize that IFNγ signaling in tumor cells is important for the maintenance of a potent antitumoral immune response as IFNγ induces CXCL9 and CXCL10 which recruit T cells that can in turn produce IFNγ, resulting in a positive feedback loop. In an environment that is stressful for the T cells[124], constant arrival of fully functional T cells may be important.

We identified cell lines that do not respond to IFNγ. It can be a total loss of response, a decreased response or only the lack of upregulation of some specific proteins. The patients that bear these alterations may not benefit from immune checkpoint blockade or other kind of immunotherapies. It is important to find ways to bypass these mechanisms of resistance to restore the efficacy of immunotherapy. In a melanoma mouse model of resistance to PD1 blockade based on tumor cell-specific JAK1/JAK2 knockout, tumor elimination has been achieved by combining PD-1 blockade to intratumoral injection of a TLR9 agonist.[125] This treatment activated both the innate and adaptive

immune response, and tumor clearance was mediated by CD8+ T cells and NK cells. Another mouse model of resistance to PD1 blockade relied on B2M knockout and in this case tumor clearance could be achieved by CD122-preferential IL2 agonist treatment which boosted the CD4+ T cell and NK cell response.[125] Here we have two examples of genetic alterations associated with resistance to immunotherapies. With the fact that cancer treatment relies more and more on immunotherapy, we will for sure find other mechanisms of resistance that we will have to overcome. Thus the characterization of these mechanisms and phenotyping of melanoma cells are required. In this study, we focused on the complete IFNγ non-responder tumor cells identified in the screening, as those are clearly associated with unfavorable clinical outcome. But it is also possible for some tumor cells to only lose the ability to upregulate one or a few specific genes. We can imagine a case in which the tumor cell only loses the ability to upregulate CXCL9 and CXCL10 while maintaining PD-L1 expression. This could limit T cell recruitment. Our approach does not allow to assess the effect of this kind of functional alteration, and thus other methods should be used. A cohort of patients receiving immunotherapy should be established and followed to find alterations that are associated with lack of responsiveness to immunotherapy. A feasible strategy is to investigate cases of relapse coinciding with particular mutations in tumor cells. Here one can compare the phenotype of the tumor cells before and after the relapse, possible guiding towards the key mechanisms[93]. This strategy requires deep characterization of the melanoma cells as the defect can be at the level of the genes, the epigenome, the RNA or the protein. Ideally, the alteration should be reproduced in a mouse model for confirmation. Mouse models could also be used for large-scale screening in which you randomly insert mutations in tumor cells and assess the effect of these mutations on the response to immunotherapy. Such a screening has already been performed[92] by injecting melanoma cells that bared different mutations and assessing the differences in the frequency of these mutations after treatment of immunocompetent mice with anti-PD1 and GVAX and untreated immunodeficient mice. The authors found that mutations in the gene coding for PTPN2 was associated with a decreased tumor growth in presence of an anti-tumoral immune response supported by immunotherapy while it had no effect on tumor growth in immunodeficient mice. This indicates that the role of PTPN2 is only relevant in presence of an antitumoral immune response which makes sense as PTPN2 is a protein tyrosine phosphatase that is induced by IFNγ but also inhibits IFNγ signaling, resulting in a negative feedback loop that prevents the increase of antigen presentation. It is possible to identify other genes that could influence the efficacy of immunotherapy using this approach, but we can hypothesize that it would not allow to identify alterations in pathways involved in chemotaxis or other indirect factors. Indeed, having an heterogenous mix of cells baring different mutations means that some cells could compensate the mutation-induced changes of other cells. One example could be that a tumor cell

that lost the ability to express CXCL9 and CXCL10 has an advantage, but neighboring cells will still express these cytokines and thus recruit T cells, preventing the mutated cell to take advantage of its alteration. However, these events also happen in patients, which could maybe explain why we find more alterations that protect tumor cells from direct T cell attack (alterations in JAK1, IRF1, MHC-I, PTPN2) in comparison to alterations that limit T cell recruitment (alterations in CXCL9, CXCL10 expression) in patients.

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Grasso, C. S. *et al.* Conserved Interferon-γ Signaling Drives Clinical Response to Immune Checkpoint Blockade Therapy in Melanoma. *Cancer Cell* **38**, 500-515.e3 (2020).

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Torrejon, D. Y. *et al.* Overcoming Genetically Based Resistance Mechanisms to PD-1 Blockade. *Cancer Discov* **10**, 1140–1157 (2020).

Contributions to publications :

1.

Grasso, C. S.; Tsoi, J.; Onyshchenko, M.; Abril-Rodriguez, G.; Ross-Macdonald, P.; Wind-Rotolo, M.; Champhekar, A.; Medina, E.; Torrejon, D. Y.; Shin, D. S.; Tran, P.; Kim, Y. J.; Puig-Saus, C.; Campbell, K.; Vega-Crespo, A.; Quist, M.; **Martignier, C**.; Luke, J. J.; Wolchok, J. D.; Johnson, D. B.; Chmielowski, B.; Hodi, F. S.; Bhatia, S.; Sharfman, W.; Urba, W. J.; Slingluff, C. L.; Diab, A.; Haanen, J. B. A. G.; Algarra, S. M.; Pardoll, D. M.; Anagnostou, V.; Topalian, S. L.; Velculescu, V. E.; Speiser, D. E.; Kalbasi, A.; Ribas, A. Conserved Interferon-γ Signaling Drives Clinical Response to Immune Checkpoint Blockade Therapy in Melanoma. *Cancer Cell* **39**, 122 (2021).

My role in this paper was mostly through discussions, exchange of data from ongoing experiments as well as samples.

2.

Gestermann, N.; Saugy, D.; **Martignier, C.**; Tillé, L.; Fuertes Marraco, S. A.; Zettl, M.; Tirapu, I.; Speiser, D. E.; Verdeil, G. LAG-3 and PD-1+LAG-3 inhibition promote anti-tumor immune responses in human autologous melanoma/T cell co-cultures. *Oncoimmunology* **9**, 1736792 (2020).

For this article, I performed and helped for experiments in which we cocultured patient-derived melanoma cell lines with autologous Tumor Infiltrating Lymphocytes (TILs) that were expanded in vitro. This includes CTV or CSFE loading of the cells, cell plating, treatment, collection of samples, staining for flow-cytometry and acquisition of the samples by flow cytometry. I also performed ELISA to detect IFNγ in the coculture experiment and qPCR to determine PD-L2 and L-Sectin expressions by the tumor cells. I contributed to the maintenance of the melanoma cells in culture and the expansion of TILs.

3.

Neubert, N. J., Schmittnaegel, M., Bordy, N., Nassiri, S., Wald, N., **Martignier, C**., Tillé, L., Homicsko, K., Damsky, W., Maby-El Hajjami, H., Klaman, I., Danenberg, E., Ioannidou, K., Kandalaft, L., Coukos, G., Hoves, S., Ries, C. H., Fuertes Marraco, S. A., Foukas, P. G., De Palma, M., Speiser, D. E. T cell-induced CSF1 promotes melanoma resistance to PD1 blockade. *Sci Transl Med* **10**, eaan3311 (2018).

For this article, I performed ELISA to determine whether there was a correlation between CSF1 plasma level and stage of cancer (Figure 1). I also setup and performed the transwell/coculture experiment to show that coculturing MelanA+ melanoma cells with MelanA-specific CD8+ T cells could induce the expression of CSF1 by melanoma cells in a contact-independent manner that could be blocked by blocking IFNγ and TNFα. (Figure 4)

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Neubert, N. J., Tillé, L., **Martignier, C.**, Fuertes Marraco, S. A. & Speiser, D. E. Chapter Twenty-Four - Analysis of cancer cell-intrinsic immune regulation in response to CD8+ T cell attack. in *Methods in Enzymology* (eds. Galluzzi, L. & Rudqvist, N.-P.) vol. 631 443–466 (Academic Press, 2020).

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Professional experience

March 2017 – Presently:	PhD Thesis in the laboratory of Prof. D. Speiser
May 2016 – February 2017:	Master Thesis in the laboratory of Dr. G. Verdeil / Prof. D. Speiser
October 2015:	Introductory course in laboratory animal science module 1
July – December 2015:	Pre-Master project in the laboratory of Prof. V. Katanaev
Summers 2009 – 2015:	Summer work at the Distillery of Bassins
Summer 2013:	Presentation of the distillation process at Forest Fair Luzern
September 2010 :	Internship at the Cabinet Vétérinaire du Boiron (Nyon)
August 2009 :	Internship at the Clinique Vétérinaire du Châtelard (Bière)

Education

2017 – Present:	PhD in Cancer and Immunology at the University of Lausanne focusing on mechanisms of resistance to immunotherapy
2015 – 2017:	Master in Medical Biology at the University of Lausanne with specialisation in Immunology and Cancer
2012 – 2015:	Bachelor in Biology at the University of Lausanne
2009 – 2012:	Bilingual Maturité (French-German) at the Gymnase of Nyon with specialisation in Biology, Chemistry, Physic and advanced Mathematics
1998 – 2009:	Compulsory school with specialisation in Mathematics and Physic from 2006 to 2009

Publications

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Skills

Languages:	French : Mother tongue English : Speaking and writing German : Speaking and writing
Laboratory Technics:	Microscopy, Flow Cytometry, PCR, RTPCR, Cloning, Western Blot
Softwares:	Microsoft Office, OpenOffice, Prism, FlowJo, ImageJ, R

Teaching

2018-2021	Teaching assistant for Practical course for 3rd year biology students at the University of Lausanne, Switzerland
2016-2019	Teaching Biology Support course for high school student

Conferences

October 2020:	Poster presentation at the Society For Melanoma Research 17th International Meeting, Virtual

November 2020:	Swiss Cancer Center Leman Retreat, Virtual
September 2019:	Poster presentation at the Swiss Society of Allergology and Immunology Annual Meeting, Lugano
March 2019:	Poster Presentation at the Keystone Symposia: Cancer Metastasis: The Role of Metabolism, Immunity and the Microenvironment, Florence
September 2018:	ISREC-SCC Symposium, Lausanne
August 2018:	Poster presentation at the Swiss Society of Allergology and Immunology Annual Meeting, Interlaken
November 2017:	Poster Presentation at the Swiss Cancer Center Leman Retreat, Lausanne
November 2016:	Swiss Cancer Center Leman Retreat, Lausanne

Qualities and Hobbies

Curious, enthusiastic and sociable

Running, Cycling, Swimming and other Sports

Reading, Politics and Travelling