Study of two barriers in Melanoma Immunotherapy; Myeloid Derived Suppressor Cells and PD-L1/P-D1 Inhibitory Interaction

Estudio de dos barreras de la inmunoterapia en melanoma; células mieloides supresoras y la interacción inhibidora PD-L1/PD-1

Universidad Pública de Navarra

2018

Fundación Miguel Servet
Navarra Biomed

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upna
Universidad Pública de Navarra
Nafarroako Unibertsitate Publikoa

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La autora de esta tesis doctoral ha contribuido en los siguientes aspectos a la realización de los siguientes artículos científicos:

PDL1 Signals through conserved sequence motifs to overcome interferon-mediated cytotoxicity:

- Clonaje de mutantes
- Realizacion experimentos *invitro*
- Realizacion experimentos *invivo*
- *Analisis estadisticos*
- Escritura del manuscrito

A core of kinase-regulated interactomes defines the neoplastic MDSC lineage:

- Preparacion de muestras para analizar mediante proteomica cuantitativa
- No realizó el estudio proteomico con el espectrómetro de masas ni su posterior análisis bioinformatico.
- Realizacion experimentos *invitro*
- *Analisis estadisticos*
- *Estudio de los interactomas*
- Escritura del manuscrito

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ACKNOWLEDGEMENTS

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En primer lugar y sobre todo quiero agradecer a mi director el Dr. David Escors por su apoyo y guía durante mi tesis. Por contagiarme con su infinito entusiasmo por la investigación y siempre recordarme lo importante e interesante que es el tema de la inmunoterapia en cancer y la gran relevancia de nuestro área de trabajo hoy en dia. La gran habilidad de David para dirigir el trabajo, sus buenísimos consejos, y todos los conocimientos que me ha enseñado así como la capacidad de mirar las cosas desde puntos de vista no esperados y sacar probecho de todos los resultados obtenidos han sido indispensables para poder realizar mi tesis y formarme como una investigadora capaz y eficiente. Él es una fuente de inspiración no solo por su gran experiencia cientifica en inmunologia sino tambien por su calidad humana a la hora de dirigir y tratar a su grupo de investigación.

Trabajar en el laboratorio con David me ha enseñado también lo mucho que cuesta empezar a formar un grupo y cómo con constancia, paciencia, trabajo en equipo y ayuda entre compañeros se pueden conseguir grandes cosas con pocos recursos. También quiero agradecer a su mujer y co-directora la Dra. Grazyna Kochan por toda su ayuda durante mi trabajo experimental y la gran cantidad de conocimientos científicos que me ha enseñado también relacionados con otros ámbitos como las proteínas, baculovirus o química entre otros… su actitud siempre dispuesta a trabajar y su pasión por la ciencia me ha inspirado en muchos momentos de debilidad o duda.

Quiero agradecer en especial al Dr. Hugo Arasanz, por su ayuda experimental en nuestros inicios del doctorado, por hacer tan agradable el trabajo a su lado y por todos los conocimientos médicos y cerveceros que me ha dado. También quiero dar gracias a Miren Zuazo por todo el apoyo moral en días bajos y las risas (también con Hugo) que hemos tenido estos años, porque muchas veces las dos nos entendíamos a la perfección y sin ellos el dia a dia en el labo habría sido mucho mas duro.

La Dra. Idoia Blanco es una gran compañera y amiga a la que doy gracias por todo su cariño y la alegria que siempre me ha trasmitido asi como por darme la seguridad que aveces me faltaba a creer en mi trabajo. No me olvido de dar las gracias a la persona que me hizo ver que el doctorado era mi opción de futuro y me motivo y ayudo para empezar en ello, Noemi Perez. Y también Theresse Lienchtenstein que fue mi compañera y amiga en los inicios, gracias a la que aprendí muchísimo del trabajo en pollata. También quiero dar gracias a Irene Lasheras porque en muy poquito tiempo se ha convertido en una de las personas más especiales y que más me han ayudado en mis momentos de incertidumbre y dudas durante la época de escritura que siempre es muy ardua; gracias por sacarme una sonrisa.

Quiero agradecer también al resto de personas que han pasado por mi grupo y siempre me han ayudado y tratado genial como son Virginia Arteta, Maria Ibañez, Maribel, Gonzalo Hinojal… y no me olvido de mis chicas de Navarra Biomed las “Spice girls” asi como Miren, Valle, Iñaki, David Guerrero… y todos los que me han ayudado durante todos estos años.

Agradecer a todos los colaboradores que he tenido durante mi tesis, en especial a Diana Llópiz, Marta Ruiz, Leire Silva y Pablo Sarobe, con los cuales tuve el placer de trabajar directamente y de los cuales aprendí muchísimas cosas, me enseñaron siempre con mucho cariño y paciencia. También a Claudia Wellbrock, Imanol Arrozarena, Ruth Vera y Cristian Smerdou por el respaldo y el material experimental cedido.

También quiero agradecer a la plataforma de proteómica liderada por los Drs. Joaquín Fernandez y Enrique Santamaria toda la ayuda desempeñada en la elaboración de la proteómica y a Xabier Perez de Morentin el análisis bioinformatico de los datos.

Agradecer al Gobierno de Navarra por confiar en mi proyecto y adjudicarme la beca predoctoral que me ha permitido desempeñar mi trabajo estos años y a la UPNA por admitirme en su programa de doctorado. Asi mismo a Navarra Biomed por proporcionarme los medios para poder desarrollar los experimentos. Quiero dar especial mención a mi tutor en la UPNA Ignacio Encío por su ayuda, consejo y seguimiento durante el curso de la tesis.

Por último agradecer a mis padres, mis hermanos, mi chico Aitor, mis txipo y mis queridas biólogas Uxue, Vicky y Naza por el apoyo en mis días bajos y por estar tan orgullosos de mi trabajo aun sin entenderlo…por darme esa confianza y cariño siempre y por soportar mi mal humor en épocas didiciles ¡¡ Nada de esto habría sido posible sin vosotros!!

ABSTRACT

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Tumors effectively escape from the immune attack by inhibiting anti-tumor activities of T cells. This is achieved by at least two mechanisms, although there are several others of importance. First, the establishment of inhibitory interactions between T and tumor cells through programmed death-1 (PD-1) binding to its ligand (PD-L1), among others. Second, by inducing the expansion of potent immunosuppressive myeloid-derived suppressor cells (MDSCs). In this Ph.D. thesis, I have studied these two key immunosuppressive barriers for cancer therapy.

PD-L1 is one of the most important immunoregulatory molecules expressed on the cell surface of many cell types including cancer cells. Apart from binding to the inhibitory receptor PD-1 on T cells, it suppresses immune responses by the delivery of intrinsic intracellular signaling pathways that enhance cancer cell survival, regulates stress responses and in this thesis we discover that it confers resistance towards pro-apoptotic stimuli such as interferons. The systemic administration of antibodies that block PD-L1/PD-1 interactions to cancer patients is demonstrating unprecedented clinical success, but much is yet to be known on the mechanisms of action. The first aim of this thesis was to firsltly identify sequence motifs within the intracytoplasmic domain of PD-L1 mediating protection from IFN-β-induced apoptosis. Two motifs implicated in the delivery of signals that inhibit IFN-β signal transduction pathways were identified. Moreover, it was found that human cancers acquire somatic mutations within these motifs that enhance the anti-interferon activities of PD-L1, favoring cancer cell growth *in vitro*. Overall, the results presented in this Ph.D. thesis uncovers a mode of action of PD-L1 in cancer cells as the first line of defense against IFN cytotoxicity.

Myeloid derived suppressor cells (MDSCs) differentiate from bone marrow precursors and expand in cancer-bearing hosts. MDSCs infiltrate tumors where they exert pro-carcinogenic activities. Identification of specific molecular pathways in MDSCs could help the development of novel anti- neoplastic treatments. The second aim of this thesis consisted in the in-depth molecular study of *ex vivo* generated murine MDSCs that resemble melanoma tumor-infiltrating subsets by high-throughput quantitative proteomics. The neoplastic MDSC proteome was compared to those of non-neoplastic MDSC controls (derived *ex vivo from* 293T conditionant medium), and conventional bone-marrow derived dendritic cells (DCs). Our analyses resulted in the most detailed interactome map of the murine MDSC to date, and uncovered the networks regulating cell lineage and cancer-induced pathways. We propose some kinases as MDSC-specific therapeutic targets. The activities of these kinases differentially regulate MDSC differentiation and activities and can be specifically inhibited in MDSCs while keeping immunogenic DCs largely unaffected.

Finally, I discuss the potential combination of therapies targeting both barriers; inhibition of PD-1-PD-L1 interactions and blockade of MDSC activities as an optimal approach for anti-cancer therapies.

RESUMEN

RESUMEN

Los tumores escapan eficazmente del ataque inmunitario al inhibir las actividades antitumorales de las células T. Esto se logra mediante al menos dos mecanismos principales. En primer lugar por el establecimiento de interacciones inhibitorias entre T y las células tumorales a través de la unión de "programed death"-1 (PD-1) y su ligando (PD-L1). En segundo lugar, al inducir la expansión de células mieloides inmunosupresoras potentes (MDSC). En esta tesis doctoral, he estudiado estas dos barreras inmunosupresoras clave para la terapia del cáncer.

PD-L1 es una de las moléculas inmunorreguladoras más importantes expresadas en la superficie celular de muchos tipos de células, incluidas las cancerosas. Además de unirse al receptor inhibidor PD-1 en las células T, suprime las respuestas inmunes a través de vías de señalización intracelulares intrínsecas que mejoran la supervivencia de las células cancerosas, regulan las respuestas al estrés y confieren resistencia frente a estímulo proapoptóticos tales como los interferones. La administración sistémica de anticuerpos que bloquean las interacciones PD-L1 / PD-1 en pacientes con cáncer está demostrando un éxito clínico sin precedentes, pero aún queda mucho por conocer sobre sus mecanismos de acción. El primer objetivo de esta tesis fue identificar qué motivos en la secuencia dentro del dominio intracitoplásmatico median la protección mediada por PD-L1 frente a la apoptosis inducida por IFN. Se identificaron dos motivos implicados en el suministro de señales que inhiben las rutas de transducción de señales de IFN. Además, se descubrió que los cánceres humanos adquieren mutaciones somáticas dentro de estos motivos que potencian las actividades anti-interferón de PD-L1, favoreciendo el crecimiento de células cancerosas *in vitro*. En general, los resultados presentados en esta tesis doctoral descubren un modo de acción de PD-L1 en células cancerosas como primera línea de defensa contra la citotoxicidad de IFN.

Las MDSC se diferencian de los precursores de la médula ósea y se expanden en los enfermos de cáncer. Las MDSC se infiltran en tumores donde ejercen actividades procarcinógenicas. La identificación de vías moleculares específicas en las MDSC podría ayudar al desarrollo de nuevos tratamientos antineoplásicos. El segundo objetivo de esta sección de la tesis consistió en el estudio molecular en profundidad de MDSCs murinas generadas *ex vivo* que se asemejan a aquellas infiltrantes de tumores de melanoma mediante proteómica cuantitativa de alto rendimiento. Se comparó el proteoma de MDSC neoplásicas con los controles de MDSC no neoplásicos (generados ex vivo a partir de medio codicionante de 293T) y las células dendríticas (DC) convencionales derivadas de médula ósea. Nuestros análisis dieron como resultado el mapa del interactoma más detallado del MDSC murinas hasta la fecha, y se descubrieron las redes que regulan el linaje celular y las vías inducidas por el cáncer. Proponemos algunas quinasas como dianas terapéuticas específicas de MDSC. Las actividades de estas quinasas regulan la diferenciación y las actividades de las MDSC, y se pueden inhibir específicamente en las MDSC mientras que las DC inmunogénicas no se ven afectadas en gran medida.

Finalmente, discuto la potencial combinación de terapias dirigidas a ambas barreras; inhibición de las interacciones PD-1/PD-L1 y el bloqueo de las actividades de las MDSC como un enfoque óptimo para las terapias contra el cáncer.

INTRODUCTION

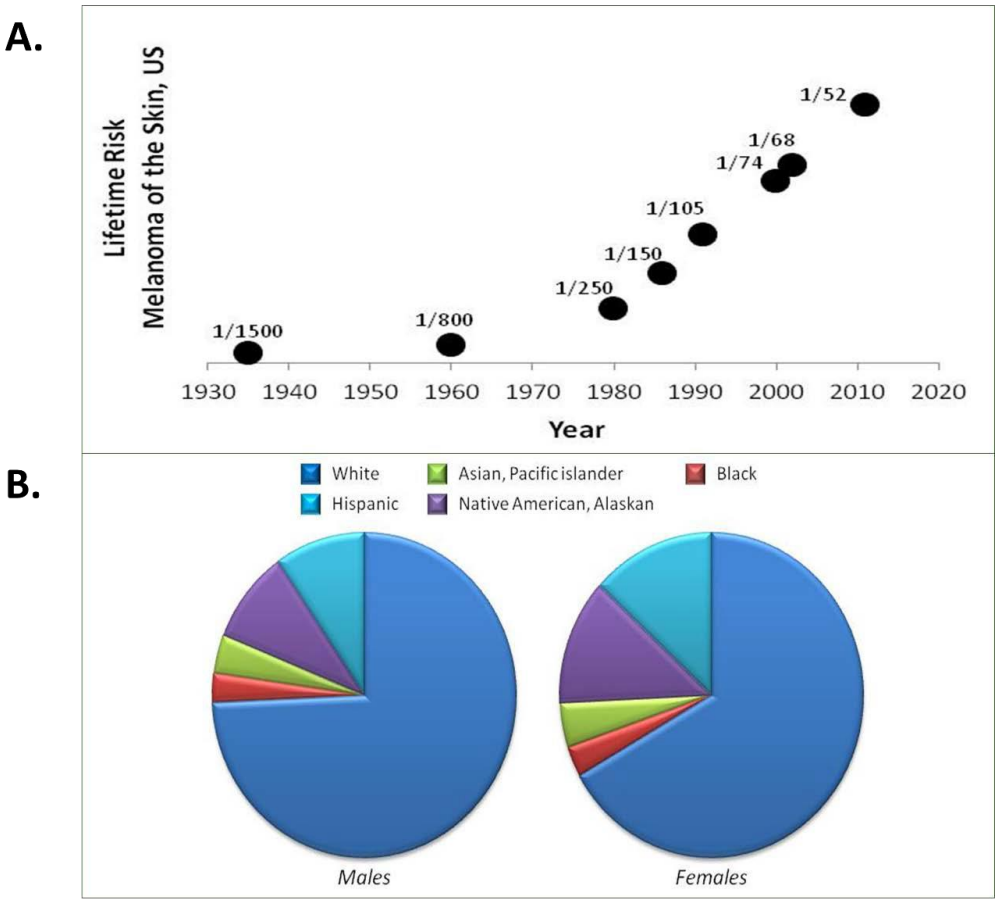
INTRODUCTION

1. CANCER AND MELANOMA

Cancer is one of the major leading causes of death as nearly 1 in 6 deaths is associated with neoplasms. Indeed, the number of new cases is expected to increase by about 70% over the next 2 decades (www.who.int/en/). As an illustrative example, the total annual cost of cancer in 2010 was estimated by the World Health Organization to be on the order of 1.16 trillion US$.

Cancer is a generic term for a large group of heterogeneous diseases that have in common the uncontrolled growth and spread of transformed mutant cells that arise through a multistage process. The malignant transformation may be started by external agents and/or inherited genetic factors that cause deleterious/dysregulating mutations in genes controlling the cell cycle, proliferation, survival pathways and apoptosis. These mutant cells may originate masses (solid tumors), or uncontrolled proliferation of circulating cells in the case of some haematological neoplasms. Cancer cells are characterized in many instances by genetic instability, which accelerates the acquisition of further mutations that may favour tumor invasion, angiogenesis, and metastasis. In time, the uncontrolled spread of cancer cells will originate secondary tumors that will interfere with the physiological function of the invaded organs, leading to disease and eventually to death.

This Ph.D. thesis will focus on malignant melanoma as an experimental model. Melanoma is a cancer of the skin that arises from melanocytic nevi as a result of mutations that confer transformed melanocytes with high invasive capacities. Although not the most frequent of skin cancers, melanoma is one of the fastest and most aggressive. In fact, the incidence rate of melanoma has doubled worldwide since 1973 particularly in regions with a high proportion of fair-skinned population. There were an estimated 14.1 million cancer cases worldwide in 2012, and this number is expected to increase to 24 million by 2035 (https://www.wcrf.org/) (Figure 1).



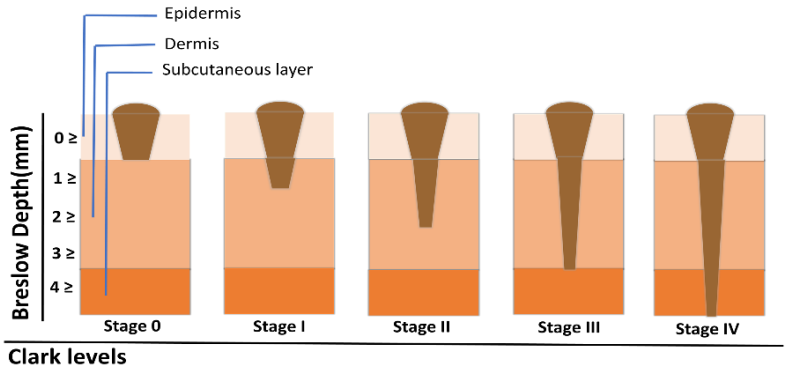
John A. D'Orazio *et-al*. Melanoma Epidemiology, Genetics and Risk Factors, Recent Advances in the Biology, Therapy and Management of Melanoma, 2013.

Figure 1: Incidence of malignant melanoma*. (A)** *Graph representing the increase in incidence of skin melanoma, US. Data are reported as lifetime risk and taken from NCI SEER reports.* ***(B) *Pie graphs representing the incidence of melanoma by race and gender in the US. Incidence rates based on NCI SEER data. Irrespective of gender, fair-skinned people have a higher incidence.*

Melanoma progression is staged following different systems (Figure 2A). The Breslow system grades melanoma lesions from I to IV according to thickness in mm (1-4 mm) (Figure 2A); The Clark system is the most extensively used and classifies melanomas according to tissue invasion (Figure 2A). It defines melanoma stages from 0 to IV according to the location of the primary tumor and whether it has spread or not. Stage zero corresponds to melanomas present only in the outer layer of the skin. Stage I when the primary melanoma lesion is thin and present in the epidermis. Stage II corresponds to melanomas that have

extended into the dermis and stage III when cancer cells have spread through the lymphatic system. All the stages are also subdivided depending on the thickness of the neoplastic lesion, the presence of ulceration and in stage III according to the number of lymph nodes with metastases. Stage IV describes melanomas that have spread to distant organs, and it is further divided into 3 groups according to the location of the metastases; in lymph nodes (first group), skin/soft tissues (second group) and lung, liver, brain, bone or gastrointestinal tract (third group). The Clark system has prognostic value. Survival rates in which the survival rates decrease significantly for each successive stage (Figure 2B). Melanomas are also staged using the TNM classification, with "T"according to thickness, "N" the number of invaded lymph nodes, and "M", presence of metastases to distant organs (Santos et al. 1984).

A.



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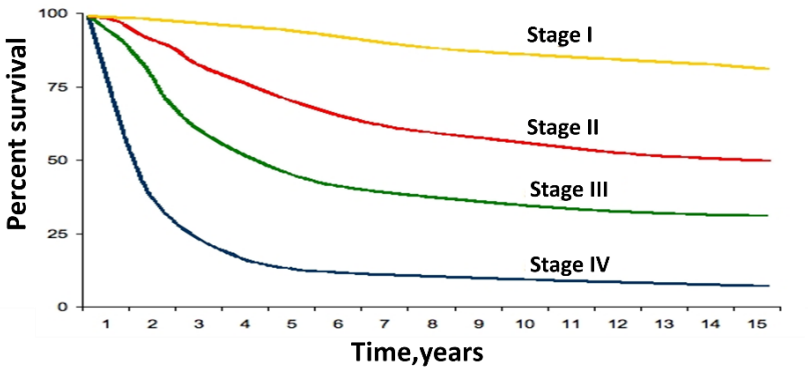
B.



https://mcreyscope.com/2014/01/17/understanding-how-melanoma-is-staged/

Figure 2: Melanoma staging and prognosis. (A) Breslow and Clark systems. *In vertical, Breslow classification system from I to IV according to thickness and depth in the skin, with 0 describing superficial lesions in the epidermis, to 4 for lesions affecting the subcutaneous layer. Clark classification is shown horizontally, according to tissue invasion: from type I (intradermal) to type V, when lesions invade the subcutaneous tissue. Stage III is used for melanomas spreading through the lymphatic system and Stage IV when the disease has metastasized to other parts of the body.* ***(B) Survival rates of malignant melanoma according to staging of disease.*** *The chart represents the overall survival rates as a function of the melanoma staging (I through IV). Survival may also be predicted by subclasses within each stage.*

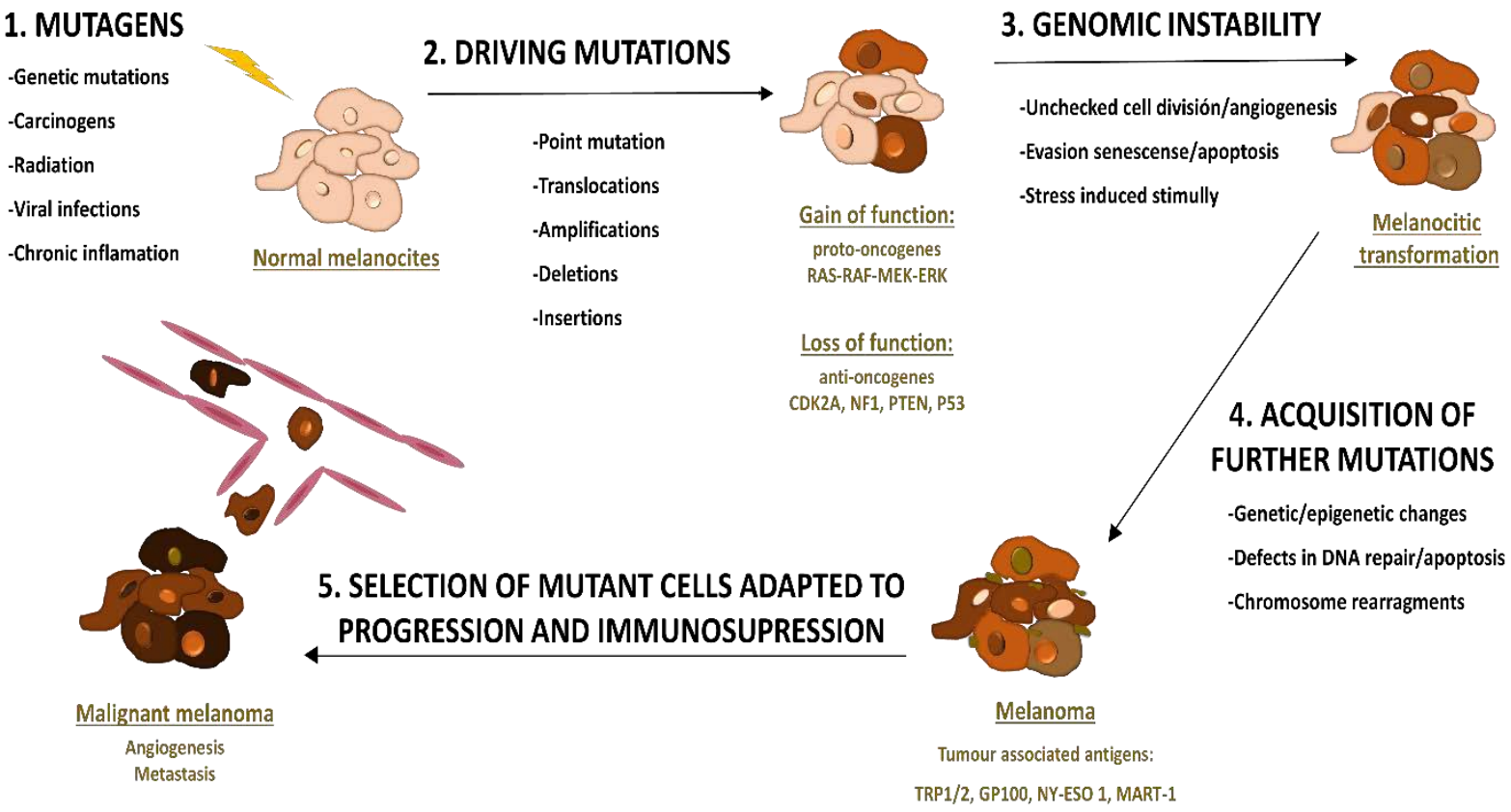
2. ONCOGENESIS IN MELANOMA

Oncogenesis is the process by which malignant transformation occurs in a normal cell, and it is driven by the accumulation of gain-of-function mutations in genes that regulate proliferation and survival (proto-oncogenes) and inactivating mutations in genes that regulate anti-proliferative or apoptotic pathways (anti-oncogenes or tumour suppressor genes) (Polsky *et al.* 2003) (Figure 3). These changes can occur through point mutations, translocations, gene amplification, deletions and insertions (www.who.int/en/). There are mutations in proto- and anti-oncogenes that appear with high frequency. Some of these changes are required for melanocytic transformation (driver mutations). Without any doubt, the most prevalent pro-oncogenic mutations in melanoma occur in the GTPase RAS signal transduction pathway. This is a core pathway in response to cytokines and growth factors, and it is characterized by a RAS-RAF-MEK-ERK kinase cascade that promotes cell division, angiogenesis, and evasion from cellular senescence and apoptosis (Karasarides et al. 2004; Wellbrock et al. 2004). Gain-of-function RAS mutations occur in most human cancers (Santos et al. 1984). Other prevalent activating mutations in melanomas are present within the B-RAF gene, which encodes a serine/threonine kinase

that activates the mitogen-activated protein kinase (MAPK)/ERK-signalling pathway (Pollock et al. 2002). Of these, the B-RAF V600E represents 90% of oncogenic RAF mutations in melanomas. C-KIT (receptor for stem cell factor) mutations also take place but at a lower frequency, enhancing intrinsic tyrosine kinase activities that facilitate cell growth.

Driver mutations take place in pre-cancerous cells (Figure 3). There are some inactivating mutations in tumor-suppressor genes which contribute to oncogenic transformation. For example, mutations in CDKN2A, NF1 and PTEN genes. P53 is one of the main cellular anti-oncogenes that responds to stress-induced stimuli including genetic instability (Tímár et al. 2016), that can also be disrupted by mutations. In addition, other mutations appear at a lower frequency which may contribute to oncogenesis, tumor progression and adaptation to pro-inflammatory and hypoxic environments. Amongst these, putative oncogenes such as CDK4, Cyclin D1, C- MYC, GNAQ, CTNNB1, ALK, EGFR4, BCL2, RAC1, MAPK2K1 or tumor-suppressor genes including WT1, ARID2 or RB1 (Tímár et al. 2016; Geis et al. 2015). Additionally, genetic instability in cancer cells makes them susceptible to accumulating additional somatic mutations. Overall, the collection of somatic mutations found in tumors (termed “mutanome”) has a significant clinical relevance particularly for immunotherapies and targeted therapies (Overwijk et al. 2013; Sahin et al. 2017) (Figure 3).

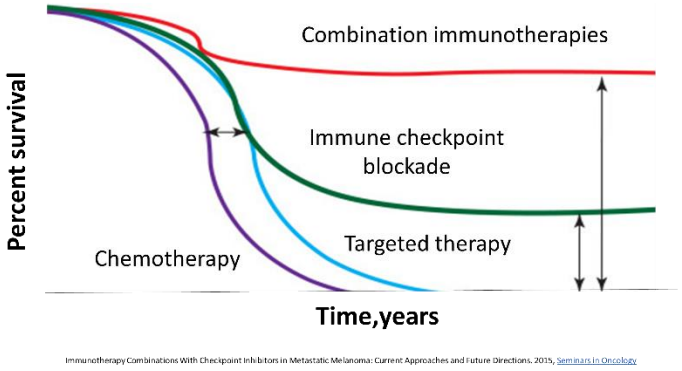
Figure 3: Oncogenesis in melanoma. *The process of melanocyte transformation into malignant melanomas is illustrated.* ***(1)*** *Main mutagens responsible for acquisition of driving mutations in normal cells. Changes in proto- or anti-oncogenes mentioned in* ***(2)*** *cause genetic instability and melanocytic transformation by the mechanisms described in* ***(3).*** *Transformed cells acquire further mutations leading to the expression of melanoma tumor-associated antigens (TAAs) as shown in* ***(4).*** *Cells are selected that are poorly immunogenic, efficacious in establishing immune suppression and surviving hypoxia. These cells may further progress by promoting angiogenesis and metastasis as shown in (5).*



3. TREATMENTS OF MELANOMA OTHER THAN IMMUNOTHERAPIES

The therapeutic options very much depend on the staging at diagnosis. The first line of treatment is the removal by surgery followed by radiotherapy and chemotherapy. During the last decade, the development of targeted therapies and immune checkpoint blockade therapies has radically changed conventional therapeutic approaches. For more than 30 years, chemotherapy has been the main therapeutic strategy for patients with advanced malignant melanoma, with dacarbazine as the standard of treatment since 1975; however, the outcomes are poor with an overall response rate (ORR) of about 20% and a median duration of 4 to 6 months (Serrone et al. 2000). Different chemotherapy agents such as temozolomide or fotemustine amongst others have failed to demonstrate superior efficacies (Middleton et al. 2000; Avril et al. 2004).

Traditional chemotherapies indiscriminately affect quickly dividing and are characterized by high collateral damage and limited long-term efficacy. Targeted therapies rebased on small molecules or antibodies that preferentially inhibit kinases mutated in malignant cells. Therefore, these agents are more selective (Figure 4).



Immunotherapy Combinations With Checkpoint Inhibitors in Metastatic Melanoma: Current Approaches and Future Directions. 2015, Seminars in Oncology

IMMUNE CHECKPOINT INHIBITORS	
PD1	Nivolumab Pembrolizumab
PDL1	Atezolizumab Durvalumab Avelumab
CTLA-4	Ipilimumab
MELANOMA	Nivolumab Pembrolizumab Ipilimumab + Nivolumab

Figure 4. Efficacies of melanoma treatment types. *The chart represents models of Kaplan-Meier survival plots for each type of treatment. Classical chemotherapy has a poor overall response rate and limited long-term efficacy. Targeted therapies are more selective, exhibiting a delay in progression and slightly better survival. Immune checkpoint blockade therapies achieve durable long-term responses. Combination therapies of immune checkpoint inhibitors approved in metastasic melanoma. Are demonstrating significantly increased responses. The right table contains examples of the type of therapies represented in the survival plot.*

B-RAF mutated at codon V600 was one of the first mutant kinases to be targeted with selective tyrosine kinase inhibitors (Ott et al. 2013). Two of them (vemurafenib and dabrafenib) have been approved by the FDA and EMA for the treatment of B-RAF^{V600E} and B-RAF^{V600K} melanomas (Kudchadkar et al. 2012). The MAP kinase MEK has also attracted interest as a target, with trametinib as the only inhibitor accepted for clinical use to date (Flaherty et al. 2012). Targeted therapies achieve fast responses after administration, but their duration is short-lived due to acquired resistances (Ott et al. 2013; Weber et al. 2015; Johnson et al. 2014) (Figure 4). Other inhibitors such as imatinib and nilotinib targeting mutated C-KIT can also be used for the treatment of melanoma (Gato-Canas et al. 2016).

4. IMMUNOTHERAPIES

Cancer immunotherapy is based on the stimulation of the immune system to selectively identify and kill tumor cells, leading to long-term responses and immunological memory.

Cancer immunotherapies englobe different therapeutic strategies that stimulate the natural capacities of the immune system to recognize and eradicate tumor cells. Especially desired is the activation of adaptive immune responses to achieve immunological memory and long-term control of tumors (Figure 4).

Cancer immunotherapies can be classified into six major strategies, which can also be combined: cytokine therapies, tumor vaccines, adoptive transfer of immune cells, immune checkpoint modulators, and depletion of immunosuppressive cells.

4.1. Tumor-associated antigens

Cancer cells express a collection of mutated self-proteins, neoantigens, oncofetal proteins or increased levels of some tissue-specific proteins that confer them with a degree of immunogenicity (quasi-antigens). These antigens are called tumor- associated antigens (TAAs) (Gato-Canas et al. 2016). The acquired immunogenicity confers the immune system the potential to identify and destroy transformed cells (Escors et al. 2014).

Melanoma is one of the most immunogenic cancers. In the 1990s, several immunogenic TAAs were identified both in murine and human melanomas, such as overexpressed endogenous tyrosinase-related proteins 1 and 2 (TRP-1, TRP-2) (Cohen et al. 1990; Tsukamoto et al. 1992; Wang et al. 1996). Immunological tolerance towards them could be broken to drive effective anti-melanoma responses (Wang et al. 1998; Parkhurst et al. 1998; Castelli et al. 1999; Noppen et al. 2000; Sun et al. 2000; Liu et al. 2009; Osen et al. 2010; Sierro et al. 2011; Overwijk et al. 1999). Indeed, TRP- 2 targeted immune responses can lead to melanoma regression (Khong et al. 2002; Bronte et al. 2000).

Some epitopes of the gp100 protein were recognized by human cytotoxic T cells (Vennegoor et al. 1988; Bakker et al. 1994; Adema et al. 1994), and have been used in preclinical studies and in human vaccines (Tarhini et al. 2012). Similarly, several CD8 and CD4 T cell epitopes were identified for NY-ESO-1 (cancer-testis antigen 1B) (Eikawa et al. 2013; Mizote et al. 2010; Robson et al. 2010; Escors et al. 2008; Jager et al. 2000; Odunsi et al. 2012; Gnjatic et al. 2006; Campos-Perez et al. 2013). T cells modified to express NY-ESO-1-specific TCRs induced tumor regression in metastatic synovial cell sarcoma and melanoma in human patients (Robbins et al. 2011). Similarly, genetically engineered autologous CD8 T cells expressing MART-1-specific TCRs demonstrated their capacities to achieve melanoma regression and long-term therapeutic effects in animal models and human patients (Abdel-Wahab et al. 2005; Kawakami et al. 1994; Bobisse et al. 2009; Khong et al. 2002; Overwijk et al. 1999). A large collection of TAAs is known nowadays, and the study and exploitation of the cancer cell mutanome remains an active area of research (Bakker et al. 1994; Cheever et al. 2009).

4.2. Oncoimmunology of melanoma

Oncoimmunology or cancer immunology can be defined as the study of the relationship between the immune system and cancer. A significant number of tumors including melanoma are certainly immunogenic and subjected to the "immunological cycle" of anti-tumor responses (Figure 5).

The initial phase of recognition is most likely mediated by innate immune responses, possibly mediated by NK cells and macrophages that induce local inflammation through production of IFNs and other pro-inflammatory cytokines and chemokines that in turn attract other immune cells. This initial direct cytotoxic attack over cancer cells (Miller et al. 2009; Ichim et al. 2005) causes the release of tumor-associated antigens (TAAs) (Figure 5). This phase is critical for the initiation of adaptive immune responses against cancer cells, and most likely mediated by recruited dendritic cells (DCs), the main professional antigen presenting cells (APCs) regulating innate and adaptive immunity

(Matzinger et al. 1994). Upon arrival, DCs take up necrotic cancer cells, process TAAs into immunogenic peptides, mature, and migrate to tumor-draining lymph nodes where they present cancer-derived antigens complexed to class I and class II major histocompatibility molecules (MHCs) to CD8 and CD4 T cells, respectively (Lipscomb et al. 2002; Goold et al. 2011) (Figure 5 and Figure 6). Activation of T helper 1 (Th1) CD4 T cells is critical for anti-cancer immunity (Kennedy et al. 2008). Th1 cells secrete IFN, IL2, and IL12 needed for differentiation, clonal expansion, and survival of antigen-specific cytotoxic CD8 T cells. Activated CD8 T cells are recruited to the inflamed tumor site where they exert their cytotoxic activities and may also further differentiate into CD8 memory cells (Curtsinger et al. 2003; Knutson et al. 2005) (Figure 5).

Both CD4 Th1 and CD8 T cells can directly exert cytotoxic activities through the production of IFN-γ, TNF-α, and secretion of perforin and granzyme-containing granules. Cytotoxic T cells express ligands of the TNF superfamily such as FasL on their cell surface, which after ligation with death receptors induce the death of targeted cells (Cullen et al. 2008). Secreted IFN-γ within the tumor environment up-regulates the expression of MHC-I, MHC-II and co-stimulatory molecules on tumor and myeloid cells, enhancing cancer cell recognition and elimination (Miller et al. 2009; Diehl et al. 2002) (Figure 5 and Figure 6). Hence, the immune system exerts a strong selective pressure by which the most immunogenic cancer cells are eliminated leaving less immunogenic cancer cell variants. This process is called cancer immunoediting. The surviving cancer cells exhibit various selected genetic mutations and epigenetic alterations that often result in reduced expression of MHC molecules on the cell surface (Zitvogel et al. 2006; Dunn et al. 2004), production of immunosuppressive cytokines that skew the immune response to a tolerogenic path (Zitvogel et al. 2006) or become insensitive to IFNs (Zaretsky et al. 2016). The growth of solid tumors favours a hypoxic, nutrient-poor microenvironment that further suppresses immune responses (Chang et al. 2015). Therefore, although many cancer patients still contain circulating TAA-specific CD4 and CD8 T cells, these have been inactivated through mechanisms of peripheral tolerance and by the tolerogenic actions of cancer cells (Bakker et al. 1994).

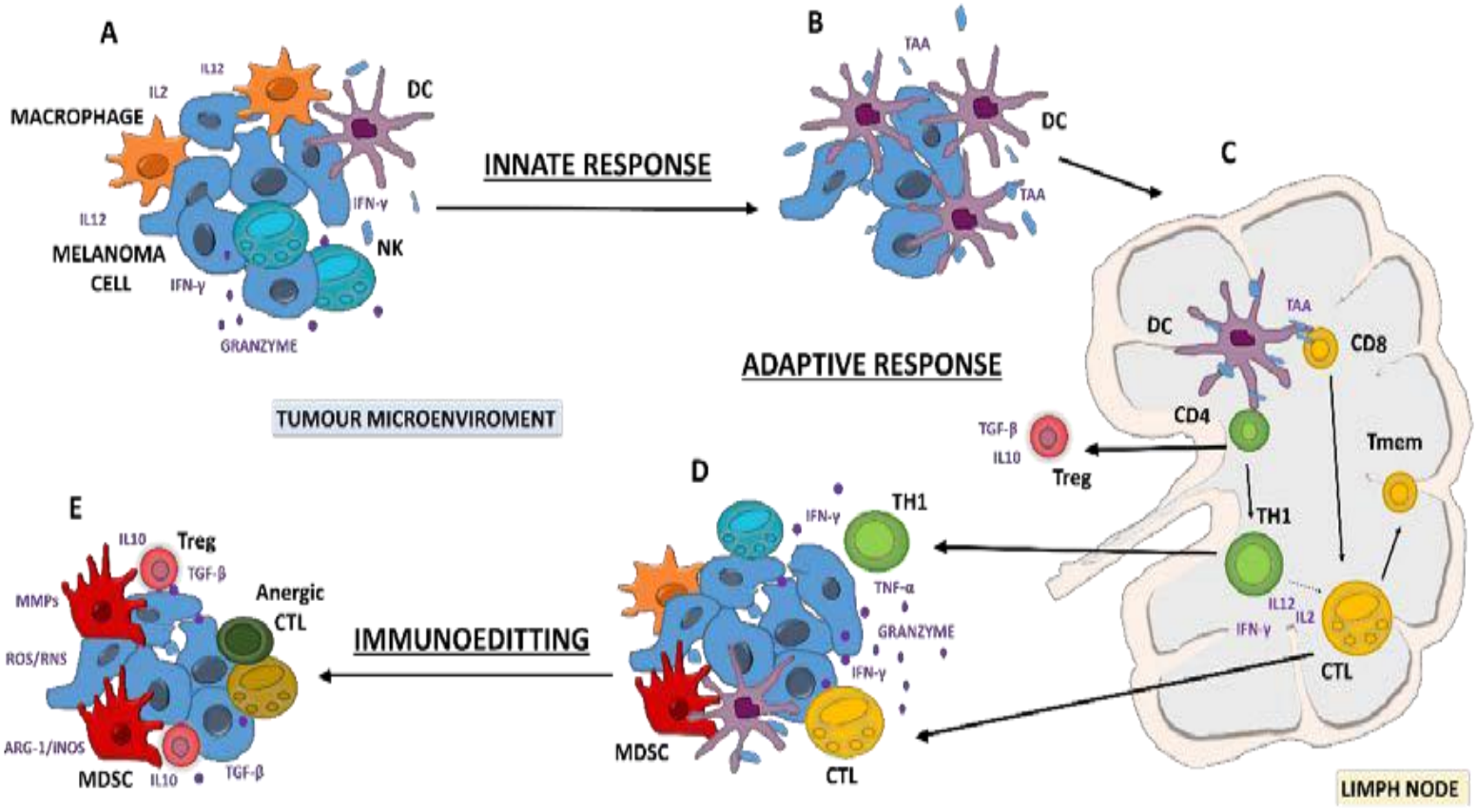


Figure 5. Innate and adaptive immune responses to nascent tumors. ***(A)*** *Tissue remodelling from growing tumors induces inflammation, which then attracts innate immune cells, such as NK cells and macrophages. NK cells cause tumor cell death through IFN-γ and granzyme-containing granules. Macrophages secrete cytokines such as IFN or IL12 which potentiate inflammation and enhance further infiltration of immune cells including DCs.*

(B) *Attack of the innate immune system leads to release of TAAs by necrotic cancer cell death. Tumor-infiltrating DCs and macrophages phagocytose TAAs, process them into antigenic peptides and present them to T cells on their surface, complexed to MHC molecules. Simultaneous recognition of danger signals released by cancer cell death leads to DC maturation by up-regulating the surface expression of co-stimulatory molecules.*

(C) *Matured DCs home to the tumor-draining lymph nodes where they present TAAs to T cells in an activatory co-stimulatory context. Cytokine secretion into the immunological synapse leads to T cell polarization and acquisition of effector functions. CD4 T cells polarise towards T helper (Th1) differentiation, while CD8 T cells towards cytotoxic T cells (CTL or cytotoxic T lymphocytes). Some CTLs further differentiate to T memory cells.*

(D) *Th1 can license CTLs and both effector T cells infiltrate tumors, where they recognize and eliminate cancer cells expressing cognate TAAs by direct cytotoxicity.*

(E) *Surviving cancer cell variants may arise that contain mutations or epigenetic alterations that reduce the expression of MHC molecules or become insensitive to IFNs. Moreover, if present, MDSCs and Tregs can inactivate T cells, NKs and DCs. Tumor growth favours a hypoxic, nutrient-poor microenvironment that further suppresses immune responses.*

4.2.1. The three-signal model of antigen presentation

Antigen presentation and T cell activation are highly regulated processes to prevent autoimmune disorders or excessive inflammation. DCs are considered to be the main professional APCs. When DCs encounter pathogenic or tumor-associated antigens, they undergo a phenotypic maturation change while homing towards secondary lymphoid organs (Goold et al. 2011; Lipscomb et al. 2002; Breckpot et al. 2010; Gallucci et al. 1999; Rescigno et al. 1998; Ardeshna et al. 2000; Fong et al. 2000). Then, DCs present antigen to CD4 and CD8 T cells, depending on whether the antigens are complexed to MHC II or MHC I molecules, respectively. MHC I molecules are complexed to peptides derived from intracellularly- expressed antigens such as TAAs or viral antigens. These antigens are degraded by the proteasome and loaded onto MHC I in the endoplasmic reticulum (ER) before transport to the cell membrane (Fong et al. 2000). MHC I molecules are recognized by CD8 T cells, which can acquire CTL effector functions upon antigen encounter. MHC II molecules are complexed to antigens that are usually (but not always) phagocytosed by APCs and degraded in endosomes, thus representing peptides from extracellular pathogens (Figure 6). Peptide-MHC II complexes are recognized by CD4 T cells, and depending on the various cytokine signals present during antigen presentation, they promote T cell polarization into various subtypes. Th1 and Th17 cells are potent stimulators of CTL responses among others like antibody ones; Th2 cells aid frequently in the initiation of humoral (B cell) immune responses; and Tregs in general terms suppress immune responses, dampens inflammation, and may promote other immunological responses like IgAs ones (Cong et al. 2009; Liechtenstein et al. 2012). An immunological synapse is formed between APCs and T cells upon TCR binding to peptide-MHC complexes. This close cell-to-cell interaction allows the APC to deliver all needed signals to the T cell to regulate its activation, proliferation, and differentiation (Boisvert et al. 2004; Rothoeft et al. 2006; Fooksman et al. 2010; Huppa et al. 2010). These T cell-regulating signals can be roughly categorized into three types (Figure 6).

Signal 1 is initiated by the specific binding of the TCR to the peptide-MHC (p- MHC) complex, but this is not usually sufficient for T cell activation. Quite the contrary, TCR triggering alone may lead to T cell anergy, characterized by limited expansion and unresponsiveness upon further antigen reencounter (Chiang et al. 2000; Bachmaier et al. 2000). Further interactions have to be provided to activate T cells, which are termed co-stimulatory signals (or signal 2). These signals are delivered by binding of DC surface ligands with the corresponding receptors on the T cell surface. Positive co-stimulation leads to T cell proliferation and acquisition of effector capacities, and are mediated by interactions such as CD80/CD86 on DCs with CD28/CD27 on T cells (Nurieva et al. 2006).

However, negative co-stimulation can also take place, for example by CD80/CD86 binding to the T cell inhibitory receptor CTLA-4. These signals generally initiate T cell anergy or Treg differentiation (Fooksman et al. 2010). Amongst negative co-stimulation, one of the most important interaction is mediated by PD-L1 binding to PD-1 (Karwacz et al. 2011; Latchman et al. 2004; Liang et al. 2006; Butte et al. 2007). Hence, the overall activation status of T cells will depend on the integration of positive and negative interactions. Immature and tolerogenic DCs express low levels of co-stimulatory molecules and higher levels of inhibitory molecules. Therefore, antigen presentation by tolerogenic DCs will not lead to T cell activation. This is a key regulatory step (immune checkpoint) by which undesired immune reactions are kept at bay in the absence of a danger signal. For example, pathogens and host-derived danger-signal molecules can trigger pathogen pattern recognition receptors such as toll-like receptors (TLRs) on DCs, leading to up-regulation of co-stimulatory molecules and p-MHC complexes (Arce et al. 2011; Escors et al. 2008; Nurieva et al. 2006). Tumors can also provide these danger signals inducing DC maturation and efficacious T cell activation.

T cells will also be regulated by a “third” signal provided by cytokines (cytokine priming or signal 3) (Figure 6). Depending on the context in which DCs encounter pathogenic molecules different cytokines will be secreted into the immunological synapse (Kapsenberg et al. 1999; Curtsinger et al 2003). CD4 T

cells thereby acquire distinct effector phenotypes and functions (Th1, Th2, Th17, Treg). IFN-γ, IL1-β, and IL12 secretion by DC will lead to Th1 differentiation. CD4 Th1 cells are crucial for the effective activation of anti-tumor CD8 CTL responses and effective anti-tumor responses, which also aid an antibody response towards increased IgG2a vs IgG1 in the murine system (Curtsinger et al. 2003; Macatonia et al. 1995; Schmidt et al. 2002; Curtsinger et al. 1999; Schmidt et al. 1999; Hernandez et al. 2002; Albert et al. 2001).

On the other hand, the presence of IL4 and IL10 will lead to Th2 differentiation, polarizing the immune response to an "antibody response" enriched in IgG1 vs IgG2a. IL23, TGF-β, IL17, and IL6 may induce Th17 differentiation. Th17 cells express IL17 and trigger strong pro-inflammatory reactions (McGeachy et al. 2007; Bettelli et al. 2006; Lewkowich et al. 2008; Sutton et al. 2006; Larsen et al. 2009; Ortega et al. 2009). Finally, Tregs can be differentiated in the presence of IL10 and TGF-β (Arce et al. 2011; Rutella et al. 2006; Escors et al. 2008; Saraiva et al. 2010; O'Garra et al. 2004; O'Garra et al. 2004). Hence, appropriate cytokine secretion by APCs is crucial for the acquisition of the proper effector functions. In the absence of signal 3, T cells acquire a tolerogenic phenotype unless antigen levels are sufficiently high (Ramanathan et al. 2011; Gerloni et al. 2005). Thus, signal 3 is particularly important when antigen levels are low as it amplifies the T cell response.

TLR ligation can alter cytokine priming. TLR4 induces DC maturation and IL12 secretion, leading to stimulation of anti-tumor immune responses (Apetoh et al. 2007; Bekeredjian-Ding et al. 2006; Cisco et al. 2004; Breckpot et al. 2009). In contrast, TLR2 stimulation preferentially activates ERK (extracellularly regulated protein kinase) signalling, prevents DC maturation and stimulates IL10 secretion (Re et al. 2004, Re et al. 2001; Qian et al. 2006). TLR2 stimulation will lead to other type of responses such as Th2, mucosal immunity and in some cases to immune regulation (Dillon et al. 2006; Manicassamy et al. 2009). If not provided in cis to DC, inflammatory citokines in the medium (in trans) can induce up-regulation of MHC and co-stimulatory molecules in DCs, these will stimulate T cell proliferation but is not such efficient in confering significant T cell effector functions (Kratky et al. 2011; Santini et al. 2000; Sporri et al. 2005;

Nolte et al. 2007; Hou et al. 2008).

Modulating co-stimulation and cytokine production can expand CTL responses efficacious for the treatment of cancer (Liechtenstein et al. 2014) or infectious diseases (Shimizu et al. 2011), while induction of Treg responses can be used to treat inflammatory disorders (Liechtenstein et al. 2012).

Efficacious activation of CD8 and CD4 Th1 responses would suffice for the recognition of tumors, which would be ultimately killed by secreted cytotoxic cytokines such as IFN-γ and apoptosis induced by FAS-FASL interactions and granzyme B secretion. However, PD-L1 is expressed constitutively in myeloid cells and many tumor cells, and inducible in many cell types after exposure to pro-inflammatory stimuli. In this way, many tumors can overexpress PD-L1, which contributes to the strong inhibition of anti-cancer T cell responses (Dong et al. 2002).

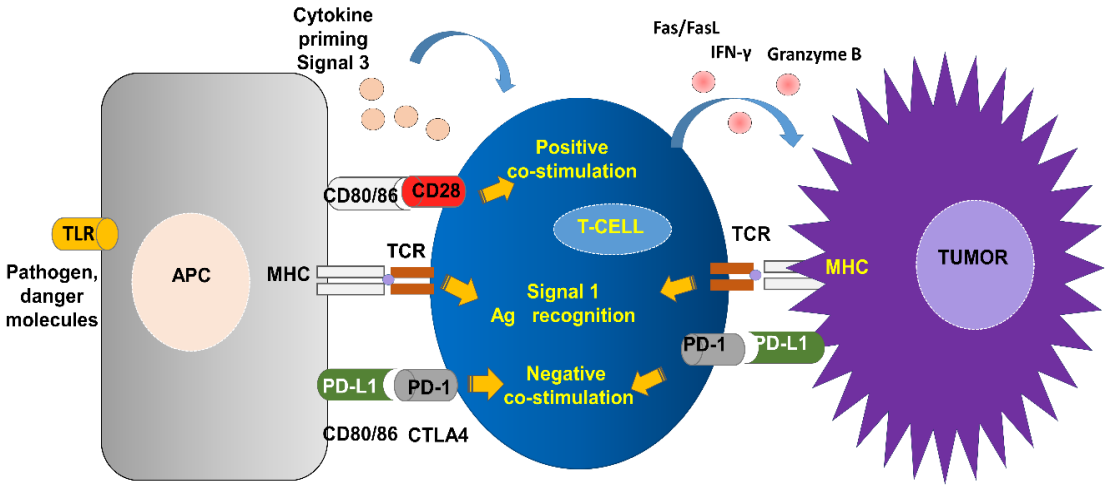


Figure 6. Three-signal model of antigen presentation in the immunological synapse. *The figure depicts antigen presentation by DCs (left) to T cells (right). Peptide-MHC complexes interact with the TCR of the T cell to initiate signal 1. Co-stimulatory (CD80/CD86) or co-inhibitory (CD80/PD-L1) ligands on DCs bind to their receptors on T cells (CD28, CTLA-4, PD-1), representing signal 2. Furthermore, cytokine secretion (Signal 3) By DCs regulates T cell differentiation. Cytokine combinations lead to differentiation of several distinct CD4 T helper*

types, or enhance CD8 proliferation and acquisition of effector activities. Activated CD8 cytotoxic T cells will then recognize antigens on tumor cells by the MHC-peptide complex and after kill them by direct cytotoxicity including secretion of IFN-γ, Granzyme B or Fas/FasL interactions. To counteract the T cell attack, tumor cells protect themselves by upregulating PD-L1.

4.3. Cytokine therapies

Cytokines are hormonal autocrine and paracrine modulators in many immune processes and can be divided into interleukins (ILs), chemokines, interferons (IFNs), tumor necrosis factors (TNFs), mesenchymal growth factors, and adipokines. Cytokines are pleiotropic molecules with multiple biological functions (Dinarello et al. 2007). Cytokines have been administered to stimulate anti-cancer immunity and several are being evaluated including IL7, IL11, IL12, IL15, IL21, IL6, TNF-α, GM-CSF, IFN-β and IFN-γ amongst others (Keilholz et al. 2002). However, their systemic administration can cause serious side effects. Some cytokines are too toxic to be used in clinical therapy, or they can even promote tumor growth *in vivo* and activate immunosuppressive mechanisms by negative feedback mechanisms (Steding et al. 2011).

4.3.1. IL2 for the treatment of melanoma

IL2 was one of the first cytokines to be approved by the FDA for treating metastatic renal cell carcinoma and metastatic melanoma (Rosenberg et al. 2014). High doses can lead to durable, complete, and curative regressions but its clinical application remains restricted by its toxicity and expansion of CD4+CD25+Foxp3+ Treg cells (Ahmadzadeh et al. 2005). Nevertheless, IL2 can be used to expand large numbers of tumor- infiltrating lymphocytes or genetically-modified T cells *in vitro* for transfer therapies that can be highly effective for melanomaand other cancer types (Rosenberg et al. 2014).

4.3.2. Type I interferon for the treatment of melanoma

Interferons are classified in three main classes: type I (IFN- α, β, ε, ω, τ and κ), type II (IFN-γ) and type III (IFN-λ1, 2, 3 and 4) (Gangaraju et al. 2009). Type I IFNs are strong antiviral agents, drivers of immune cell differentiation and inducers of cell senescence and apoptosis (Schreiber 2017). Type I IFNs comprise a single IFNβ gene and 14 IFNα genes in humans and mice (Van Pesch et al. 2004)

Type I IFNs signal through a heterodimeric receptor comprising of IFNaR1 and IFNaR2 present on the surface of most cells. IFNs activate the kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) which phosphorylate signal transducer and activator of transcription (STAT1) 1 and STAT2. In addition to canonical STAT1/STAT2 signalling, other STATs can be phosphorylated (e.g., STAT 3, 5, and 6) as well as phosphatidylinositol 3- kinase (PI3K) and mitogen-activated protein kinases (MAPK) ERK1/2 and p38. IFNs also activate some protein kinase C isoforms (PKCs) and the multifunctional adaptor protein CrkL (Schreiber et al. 2017; Terawaki et al. 2011; Hervas- Stubbs et al. 2011; Porritt et al. 2015). Together, these signalling networks converge to the activation of IFN regulatory factors (IRFs) which complexed with STATs, transactivate IFN-stimulated genes (ISGs) (Figure 7).

Type I IFNs possess anti-tumor capacities although they also promote negative feedback immunosuppressive mechanisms. For example, IFN-β exposure increases indoleamine 2,3-dioxygenase (IDO) and IL10 expression in DCs and macrophages, and upregulates expression of immunosuppressive receptors (Tsukamoto et al. 2017; Sharma et al. 2015; Terawaki et al. 2011; Rozera et al. 1999; Uehara et al. 2017; Benci et al. 2016). Multiple feedback mechanisms on the IFN receptor and signal transduction pathways have been described, including receptor endocytosis by ubiquitinylation (Schreiber et al. 2017; Bhattacharya et al. 2014; Kumar et al. 2007; Kumar et al. 2008), disruption of receptor heterodimers by USP18 or TYK2 and JAK1 dephosphorylation by suppressor of cytokine signalling (SOCS1-3) or Src homology phosphatases (SHP1-2) (Chemnitz et al. 2004). Furthermore, protein tyrosine phosphatases (PTP) and protein inhibitors of activated STAT (PIAS) dephosphorylate and /or

inhibit STATS in the nucleus. In addition to the role of IRF proteins in inducing IFN responses, IRF-2 protein is involved in IFN suppression (Porritt et al. 2015). Interference with these mechanisms exacerbates IFN-β signalling leading to inflammatory disease (Nyman et al. 2000) (Figure 7).

Type I IFNs are thought to inhibit tumor progression by a combination of cell cycle arrest and cell death (Chawla-Sarkar et al. 2001; Gong et al. 2000; Rozera et al. 1999). Hence, IFN-β induces the expression of the TNFα family member TRAIL that triggers caspase 8 and 3 dependent apoptosis in melanoma and breast cancer cells (Bernardo et al. 2013; Chawla-Sarkar et al. 2001). In cervical carcinoma, IFN-β causes proliferative arrest and accumulation of the anti- apoptotic protein cFLIP and caspase 8 (Apelbaum et al. 2013). Type I IFNs can enhance antigen presentation by up-regulating MHC-I and MHC-II, a mechanism that counteracts the frequent cancer-associated MHC-I down-modulation (Schiavoni et al. 2013; Greiner et al. 1984; Fruci et al. 2012; Wang et al. 2017). In many instances, IFN-β upregulates TAA expression (Boyer et al. 1989; Greiner et al. 1984), activates DCs and macrophages to cross-present TAAs to T cells and promotes CD8 T cell effector functions (Nguyen et al. 2002; K. Shimizu et al. 2001; Rozera et al. 1999; Kalinski et al. 1999). Moreover, IFN-β negatively regulates Treg proliferation (Pace et al. 2010; Hashimoto et al. 2014; Bacher et al. 2013; Stewart et al. 2013; M. Sharma et al. 2010) and MDSC numbers and their immunosuppressive activities.

The IFN signal transduction pathway in cancer cells is frequently inactivated by deletion of type I IFN genes, down-regulation of their receptors, inactivating mutations in JAK1 (Shin et al. 2017) and loss of STAT1 and IRF1 (Katlinskaya et al. 2016; Pietila et al. 2007; Sakaguchi et al. 2003; Bacher et al. 2013; Lee et al. 2006; Colamonici et al. 1994). Breaking free from type I IFN-mediated regulation seems to be critical for cancer progression (Shin et al. 2017; Medrano et al. 2017).

The anti-tumor properties of type I IFNs have been exploited during the last decades. Improved survival was demonstrated in combination with therapies for chronic myeloid leukaemia (CML) (Guilhot et al. 1997) and myeloma (Osterborg et al. 2017). However, results in solid tumors including

melanoma are not clear-cut (Alberts et al. 2006, Alberts et al. 2008; Nethersell et al. 1984). Other diverse strategies range from the stimulation of tumor cells to produce endogenous IFN-α/β or to deliver IFN to the tumor microenvironment (Medrano et al. 2017).

4.3.3. Type II interferon for the treatment of melanoma

IFN-γ consists of a homodimer molecule that binds to two IFN-γ receptor 1 (IFNGR1) subunits, which is followed by recruitment of IFNGR2 and its pre-associated JAK 1-2 (Parker et al. 2016) (Figure 7). STAT1 and STAT3 homo-heterodimers are preferentially phosphorylated and activated by IFN-γ signalling, and translocate to the nucleus where they bind gamma activated sequence (GAS) elements in IRG promoters (Parker et al. 2016). It was thought that its expression was limited to T cells and NK cells (Parker et al. 2016), although there is recent evidence that APCs and B cells secrete IFN-γ (Schroder et al. 2004). IFN-γ orchestrates leukocyte attraction and directs growth, maturation, and differentiation of many cell types, in addition to enhancing NK cell activity and B cell functions such as immunoglobulin (Ig) production and class switching (Schroder et al. 2004) (Figure 7).

Similar multiple layer feedback mechanisms to those of type I IFNs have also been described for type II IFNs (Porritt et al. 2015). Surprisingly, IFN-γ administration has failed for the treatment of melanoma (Franco et al. 2017), although patients harboring DNA lesions in the IFN-γ gene, as well as mice carrying tumors mutated in IFNGR1 respond poorly to immunotherapy (Gao et al. 2016). A possible explanation is that IFN-γ is involved in the effector phase of anti-tumour activities. Ipilimumab-treated patients displayed T cells with enhanced production of IFN-γ (Gao et al. 2016), so the mutational status of IFN-γ signaling genes could be a prognostic tool for selection of patients eligible for Ipilimumab therapy (Franco et al. 2017). On the other hand, IFN-γ reduces the capability of CD8+ T cells to recognize and kill melanoma cells through different mechanisms including PD-L1 up-regulation, explaining its dual role as a pro- and anti- tumor effector (Cho et al. 2011; Benci et al. 2016) (Figure 7).

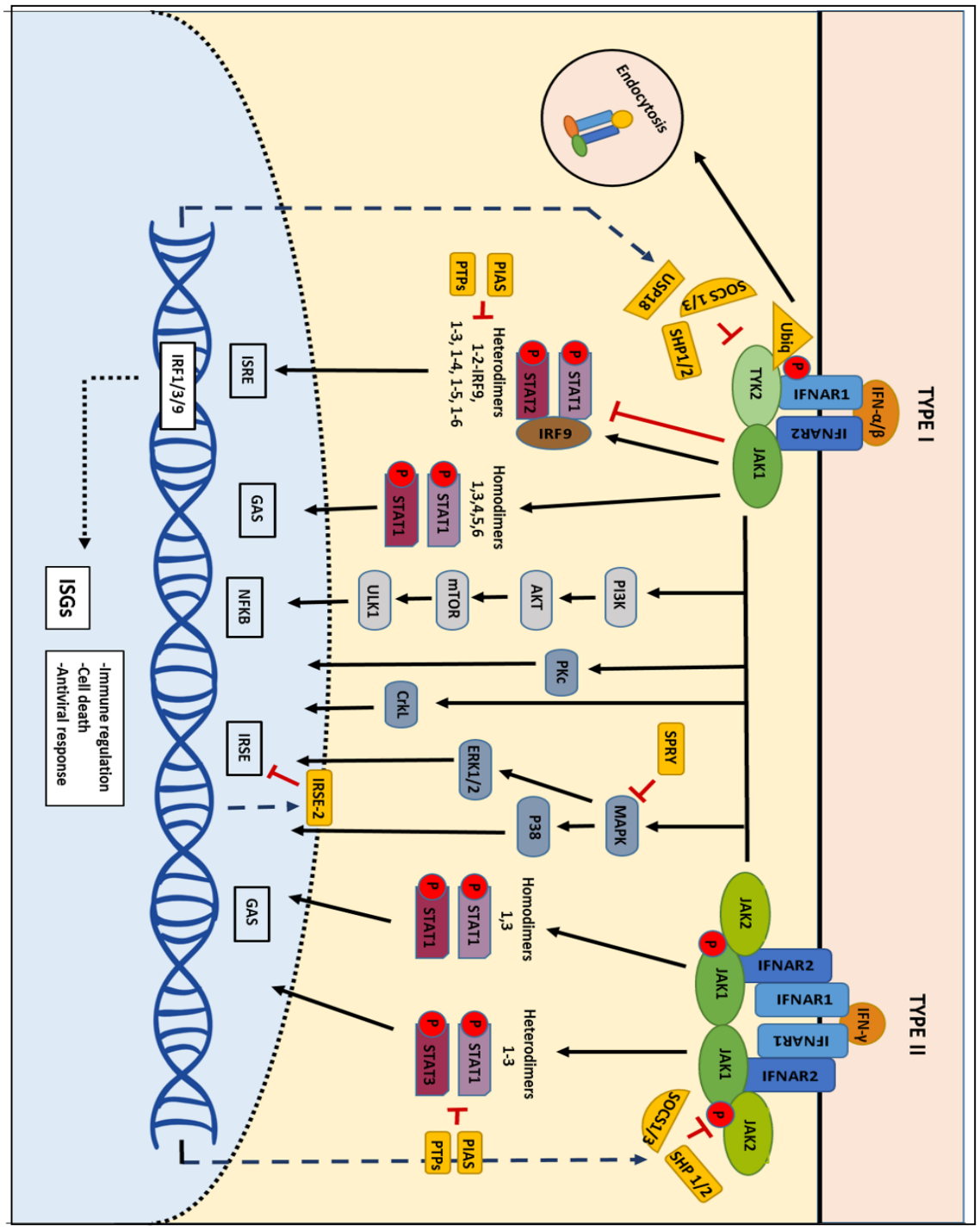

TYPE I
TYPE II
IFN-α/β
IFN-γ
IFNAR1
IFNAR2
TYK2
JAK1
JAK2
Ubiq
P
Endocytosis
USP18
SOCS 1/3
SHP1/2
PIAS
PTPs
Heterodimers
1-2-IRF9,
1-3, 1-4, 1-5, 1-6
STAT1
STAT2
IRF9
Homodimers
1,3,4,5,6
PI3K
AKT
mTOR
ULK1
PKc
CrkL
SPRY
MAPK
ERK1/2
P38
IRSE-2
Homodimers
1,3
Heterodimers
1-3
STAT3
ISRE
GAS
NFKB
IRSE
IRF1/3/9
ISGs
-Immune regulation
-Cell death
-Antiviral response

Figure 7. Type I and II IFN pathways. *Type I IFNs, including IFN-α and β, signal through a heterodimeric receptor comprising of IFNaR1 and IFNaR2 that activates the kinases Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2) which phosphorylate signal transducer and activator of transcription (Stat) 1 and Stat2. In addition to canonical Stat1/Stat2 signalling, other Stats can be phosphorylated (e.g., Stat 3, 5, and 6) as well as phosphatidylinositol3- kinase (PI3K) and mitogen-activated protein kinases (MAPK) ERK1/2 and p38. IFNs also activate certain protein kinase C (PKC) isoforms and the multifunctional adaptor protein CrkL (Delgado 2003; Ivashkiv et al. 2015). Together, these signalling networks converge to the activation of IFN regulatory factors (IRFs) which together with STATs transactivate IFN-stimulated genes (ISGs). These ISGs are usually pro-inflammatory, antiviral and anti-tumor mediators.*

There is only a single type II IFN, IFN-γ. It forms a homodimer that binds to two IFN-γ receptor 1 (IFNGR1) subunits followed by recruitment of IFNGR2 and its pre-associated Janus kinase 1 and 2(JAK) which phosphorylate signal transducer and activator of transcription (Stat) 1 and Stat3. They form homo-heterodimers, witch translocate to the nucleus where they bind gamma activated sequence (GAS) elements in IRG promoters(Delgado et al. 2003). MAPK proteins, P38 or PKc signaling pathways could be activated too (Agrawal et al. 2006). IFN-γ possess antitumour capacities among others.

Multiple layer feedback mechanisms on the receptor and on its activation and signalling have also been described in both IFN types, including endocytosis of IFN-I receptors by ubiquitinilation and disruption of receptor heterodimers by USP18124 (Kumar et al. 2007; Kumar et al. 2008). IFN I and II-TYK2 and JAK1 dephosphorylation by suppressor of cytokine signalling (SOCS1-3) (Ramanathan et al. 2010) and Src homology phosphatase (SHP1-2) (Miyamoto et al. 1988). Furthermore protein tyrosine phosphatases (PTP) and protein inhibitors of activated STAT (PIAS) dephosphorylate and /or inhibit STATS in the nucleus (Sato et al. 1998). In addition to the role of IRF proteins in inducing IFN responses, IRF-2 protein is involved in type I and II IFN suppression.

4.4. Tumor vaccines

Tumor vaccine formulations have been developed in a manner analogous to those for conventional infectious diseases. Tumor vaccines can be grouped in three types; protein/peptide vaccines, cell-based vaccines, and genetic vaccines.

Protein/peptide vaccines consist of the administration of TAAs or their various peptides containing B and T cell epitopes. To utilize peptide-based vaccines, the specific antigenic epitopes for particular MHC genotypes need to be known beforehand. Consequently, only individual-specific vaccines can be prepared for specific MHC types. These peptides are combined with strong adjuvants to overcome their intrinsic poor immunogenicity. Even so, clinical efficacy remains rather low (Guo et al. 2013; Zhang et al. 2013).

Tumor cell-based vaccines incorporate whole cancer cells in the formulation. This strategy presents several advantages. Firstly, endogenous APCs endocytose and process whole cancer cells. Hence, the whole range of TAAs can be supplied without previous knowledge on patient-specific class I and class II MHC genotypes. Tumor cell-based vaccines can be either autologous irradiated cells or allogeneic (from allogeneic tumors or human tumor cell lines). Overall, these vaccines have shown disappointing results in Phase III clinical trials (Anguille et al. 2014).

Genetic vaccines are based on delivery of DNA encoding TAAs, TAA-derived epitopes or polyantigenic fusion proteins directly to the subject or to APCs (genetic immunotherapies). The approaches for delivery of TAA DNA vary from bacterial plasmids, or by virus-based vectors. This latter methodology present an additional advantage as virus-like particles frequently possess immunostimulatory capacities (Liechtenstein et al. 2013; Dullaers et al. 2006). The use of DNA encoding multiple epitopes or mutated TAAs can further enhance anti-tumor immune responses (Guevara-Patiño et al. 2006; Liu et al. 2009). While preclinical studies showed good efficacies, their application in human therapy has been disappointing. This lack of efficacy is possibly due to the absence of adequate co-stimulation during antigen presentation and to tumor-induced immune suppression (Guo et al. 2013; Bodey et al. 2000).

4.5. Adoptive cellular immunotherapies

This strategy relies on the administration of either autologous or allogeneic cancer- specific effector immune cells or antigen-presenting cells to elicit therapeutic responses. These cells can be tumor-reactive T cells, professional APCs or natural killer (NK) cells (Gato-Canas et al. 2016).

4.5.1. T cell immunotherapies

Steven Rosenberg and colleagues in the 1980s isolated tumor-infiltrating lymphocytes (TILs) and expanded them in vitro with IL2. Then, these T cells can be re-infused back to the lymphocyte-depleted patient. Although promising results were obtained, problems in TILs isolation, expansion and lack of complete responses in melanoma were observed (Rosenberg et al. 2011).

Gene transfer technologies and T cell engineering have enabled more versatile approaches, by genetically modifying T cells to target cancer-specific antigens, via physiological TCRs or chimeric antigen receptors (CARs) (Chan et al. 1991; Kochenderfer et al. 2010; Pule et al. 2008; Kakarla et al. 2013; Zhang et al. 2014; Rosenberg et al. 2015; Milone et al. 2018; Balmer et al. 2016). TCRs are usually cloned from tumor-reactive TILs specific for TAAs with no or very limited expression in normal adult tissue (Sharma et al. 2017; Ascierto et al. 2016). This approach is MHC-restricted and ineffective for tumors with downregulated MHC. To overcome this limitation, CAR technology was developed by Eshhar and collaborators in 1993. This strategy relies on genetically engineering T cells to express single chain antibodies (scFv) targeting tumor cell surface antigens linked to intracellular signalling adaptors from the TCR signalosome. This approach has evolved with time, and it has been extensively reviewed elsewhere (P. Sharma et al. 2017). CAR T cells do not depend on MHC restriction and possess enhanced T cell functions (P. Sharma et al. 2017). CARs targeting CD19 have shown significant clinical success in B cell malignancies (Maude et al. 2014). Other approaches improve T cell effector functions independently of MHC recognition by physically linking T cells to

cancer cells, such as the expression of bispecific T cell engagers (BITEs), a recombinant molecule made of two scAbs of different specificities fused by a peptide linker. BiTEs have shown therapeutic efficacy in tumor/bearing humanized mice and in some clinical trials with patients with non-solid tumors (Horn et al. 2017; Yu et al. 2017; Dao et al. 2015).

4.5.2. Dendritic cell (DC) immunotherapies

Autologous DCs can be generated ex vivo in large numbers by retrieving monocytes by apheresis and inducing their differentiation with recombinant GM-CSF, IL4 and other cytokines (Inaba et al. 1992; Zhou et al. 1996). DCs can then be either directly loaded with TAAs or expressed from a variety of vectors. Classically, DCs are loaded with antigenic peptides, although this approach depends on the previous knowledge of epitopes for specific MHC-restriction. Moreover, the duration of antigen presentation is limited in time (Escors et al. 2013; Escors et al. 2014).

To achieve sustained antigen processing and effective presentation, DCs need to be matured by incubation with TLR agonists such as LPS and analogues, or by other means including genetic modification (Van Lint et al. 2014). This last approach has been used in our group by expressing modulators of intracellular signalling pathways associated to TLR ligands. For example, lentivector expression of NF-κB and p38 activators such as NIK or KSHV Vflip, or the constitutively active MKK6 EE mutant leads to DCs maturation and enhance their immunogenicity (Rowe et al. 2009; Enslen et al. 1998; Zhang et al. 2013; Arce et al. 2012; Liechtenstein et al. 2013). Expression of dominant negative mutants such as MEK1 ΔNES AA that interferes with ERK phosphorylation also matures DCs (Burnet et al. 1970). Expression of immunostimulatory cytokines such as IL12 is a very effective approach as well (Esslinger et al. 2002; Goyvaerts et al. 2015). Direct immunization with lentivectors targeted to DCs *in vivo* has worked well in pre-clinical models (Yang et al. 2008).

Inhibitory signals in DCs can also be interfered. Thus, PD-L1 silencing in DCs inhibits TCR down-modulation in T cells and strengthens their association to APCs and cancer cells (Liechtenstein et al. 2014; Fife et al. 2009; Karwacz et al. 2011, Karwacz et al. 2012) hyperactivating and enhancing T cell multifunctionality (Liechtenstein et al. 2014; Karwacz et al. 2011; Pen et al. 2014).

Autologous DC and CTL cell transfer therapies in their various forms have demonstrated some clinical success and are susceptible of combination. Indeed, the combined use of autologous tumor-lysate loaded DCs with TIL transfer leads to clinical responses in advanced melanoma patients (Poschke et al. 2014).

5. IMMUNOLOGICAL BARRIERS IN MELANOMA

5.1. Failure of classical immunotherapies

Anti-cancer immunotherapy has been classically developed following conventional rationales based on the experience with infectious diseases. Despite encouraging results from extensive pre-clinical work, translation into the clinic has been characterized more for failures until recently. The key difference between classical vaccination approaches and cancer immunotherapy is the distinct nature of tumors and their relationship with the immune system. Tumor cells form complex tissues that recruit other cell types as a result of an initial inflammatory response (Gato-Canas et al. 2016). Tumor cells respond to the initial immune attack by triying to counteract antitumor inflammation. Tumor-secreted factors suppress anti-tumor effector cells and skew infiltrating immune cells to a suppressive phenotype (Hanahan et al. 2011).

Furthermore, cytokines and factors produced by growing tumors alter myelopoiesis in the bone marrow (BM), leading to the differentiation of immunosuppressive myeloid cells which are released to the systemic circulation (Rabinovich et al. 2007; Gabrilovich et al. 1996). In addition, inflammation up-regulates immune checkpoint molecules such as PD-L1, that inhibits cytotoxic T

cells by engaging with PD-1 expressed on the surface of antigen-experienced T cells. Hence, the expansion of MDSCs and the up-regulation of PD-L1 represent two major immunological barriers for anti-cancer immunotherapy. Indeed, the recent development of immune checkpoint inhibitors has demonstrated that it is possible to achieve truly efficacious cancer immunotherapies. In this Ph.D. thesis, I have studied these two major barriers to identify novel targets and ways to improve cancer immunotherapies, while at the same time gaining knowledge on their biological mechanisms of action.

5.2. Myeloid-derived suppressor cells (MDSC)

Cancer-related natural suppressor cells were first described in 1989 in tumor-bearing mice as immature cells of myeloid origin that accumulated in tumors and stimulated their vascularization and immune evasion (Talmadge et al. 2013). Nowadays, these cancer-induced immunosuppressive myeloid cells are termed myeloid-derived suppressor cells (MDSCs) by most investigators. MDSCs are comprised of myeloid progenitors and precursors at various stages of differentiation (Condamine et al. 2011). Their phenotypic characterization remains rather controversial due to cell heterogeneity, investigator-dependent phenotypic marker profiles, and tumor-dependent variability. Indeed, MDSCs are phenotypically similar to their immunogenic counterparts, such as monocytes and neutrophils. Hence, their immunosuppressive activity still remains as their key characteristic. Moreover, MDSC-like cells can be found in stress conditions exerting physiological functions such as prevention of tissue damage and stimulation of wound healing caused by inflammation (Youn et al. 2010; Bronte et al. 1998).

MDSCs are classified in two subtypes. MDSCs with a monocytic-like phenotype are called monocytic MDSCs (m-MDSCs) and are characterized in mice as $CD11b^{+}$ $Ly6G^{low/-}$ $Ly\text{-}6C^{high}$ (Bronte et al. 2000; Youn et al. 2008). MDSCs exhibiting a granulocytic-like morphology are called granulocytic MDSCs (g-MDSCs) and are characterized in mice as $CD11b^{+}$ $Ly6G^{high}$ $Ly\text{-}6C^{low/-}$ (Bronte et al. 2000; Youn et al. 2008). There is evidence from the Gravilovich

group and our own group that murine g-MDSC arise from m-MDSC after a maturation process, possibly regulated by HIF1-α-dependent epigenetic inhibition of retinoblastoma protein (Corzo et al. 2010; Liechtenstein et al. 2014). In humans, their phenotype is rather controversial, but they are mostly described as expressing CD33, CD11b and low or no HLA-DR (Almand et al. 2001). $CD11b^{+}$ $CD33^{+}$ $HLA\text{-}DR^{-/low}$ $CD15^{-}$ $CD14^{+/low}$ correspond to m-MDSCs (Youn et al. 2008; Filipazzi et al. 2007), while $CD11b^{+}$ $CD33^{+}$ $HLA\text{-}DR^{-/low}$ $CD15^{+}$ $CD14^{-}$ $CD66b^{+}$ correspond to g-MDSCs (Slavin et al. 1979; Ko et al. 2009; Youn et al. 2008). Several research groups propose additional markers to describe human MDSC (Talmadge et al. 2013; Elliott et al. 2017).

MDSCs show high phenotypical and functional plasticity, which further complicates their classification, isolation, and study. Many variables including growth factors, cytokines, chemokines, hypoxia, and glucose levels influence their differentiation, accumulation, and function (Gabrilovich et al. 2012). The exact combination of signals and mechanisms influencing MDSC accumulation, function, and activation remain a subject of current investigation (Gabrilovich et al. 2012; Talmadge et al. 2013).

Nonetheless, MDSC accumulation correlates with cancer progression (Solito et al. 2011; Almand et al. 2000) and metastasis (Steding et al. 2011; Diaz-Montero et al. 2009). There is an inverse correlation between MDSC numbers and T cell numbers in tumor-bearing mice (Joice et al. 2014). MDSC depletion restores NK and T cell function and numbers in murine models and patients (Li et al. 2009; Thaci et al. 2014b; Srivastava et al. 2012; Thaci et al. 2014a). MDSCs induce NK cell anergy (Li et al. 2009), and there is evidence demonstrating inhibition of effector T cells by both antigen-specific and non-specific mechanisms. MDSC-dependent antigen-specific T cell suppression is probably mediated during antigen presentation through negative co-stimulation and by immunosuppressive cytokines, leading to T cell anergy, apoptosis or Treg differentiation (Figure 6). This mechanisms is similar to that of tumor-infiltrating tolerogenic DCs. Antigen non-specific inhibition relies on secretion of immunosuppressive cytokines such as IL10 and TGF-β, depletion of essential

aminoacids such as L-arginine by arginase-1 and/or iNOS (Liechtenstein et al. 2014) activity or tryptophan by IDO (Gabrilovich et al. 2009), and secretion of reactive oxygen and nitrogen species (ROS, RNS and NO) by NOX2 or iNOS respectively (Nagaraj et al. 2007; Corzo et al. 2009). These mechanisms can also induce Tregs (Gabrilovich et al. 2009; Escors et al. 2010; Nagaraj et al. 2007). Extensive interaction exists between MDSCs and tumour cells. MDSCs help tumour cells escape the immune system and aid in progression, angiogenesis, and metastasis by secreting cytokines, chemokines, and matrix metalloproteinases (MMPs) (Liu et al. 2012) (Figure 5). Tumour cells on the other hand positively enhance MDSC differentiation from the BM.

Nonetheless, counteracting their activities strongly enhances anti-cancer treatments (Sevko et al. 2013). Early observations in cancer patients demonstrated that the concentration of peripheral blood MDSCs were positively correlated with tumor burden and clinical stage; surgical removal of tumors decreased circulating MDSCs numbers (Diaz-Montero et al. 2009; Zhang et al. 2013; Almand et al. 2001). Many of the current studies demonstrating the efficacies of MDSC-targeted therapies are in murine models or early clinical trials. Treatment strategies can be categorized as, some examples are showed (Table 1):

1. Inhibiting MDSC development and expansion: stem cell factor blockade by sorafenib (Sevko and Umansky 2013) ; multi kinase inhibitors like sunitinib (Ko et al. 2010); JAK2/STAT3 blockade by curcumin or docetaxel (Lin et al. 2010); VEGF inhibition (Fricke et al. 2007); migration blockade by or CSF-1, (Priceman et al. 2010) or MMP9 (Gnant et al. 2015; Diel et al. 1998; Markowitz et al. 2014; Guan et al. 2015); CCR2 (Wang-Gillam et al. 2016), CCR5 (Yang et al. 2018) and IL8 Inhibitors (Alfaro et al. 2016) and vemurafenib (Curtin et al. 2005).

2. Differentiating MDSCs into more mature cells: ATRA, IL12 (Repka et al. 2003; Ansell et al. 2002; Pen et al. 2013) and PI3K inhibitors (Galloway et al. 2016; Davis et al. 2017).

3. Inhibiting MDSC function: via NO inhibition by phosphodiesterase (Diaz-Montero et al. 2009) /nitroaspirin (Molon et al. 2011), or ARG-1 inhibition

by L-NAME (Reisser et al. 2002) /COX2 inhibitors (Fujita et al. 2011).

4. <u>MDSC destruction:</u> cytotoxic agents like 5-fluorouracil (Vincent et al. 2010), doxorubicin (Diaz-Montero et al. 2009), gemcitabine (Suzuki et al. 2005), cisplatin (Elias et al. 2015), HSP90 inhibitors (Rao et al. 2012) or anti IL13 (Kalinski et al. 2017).

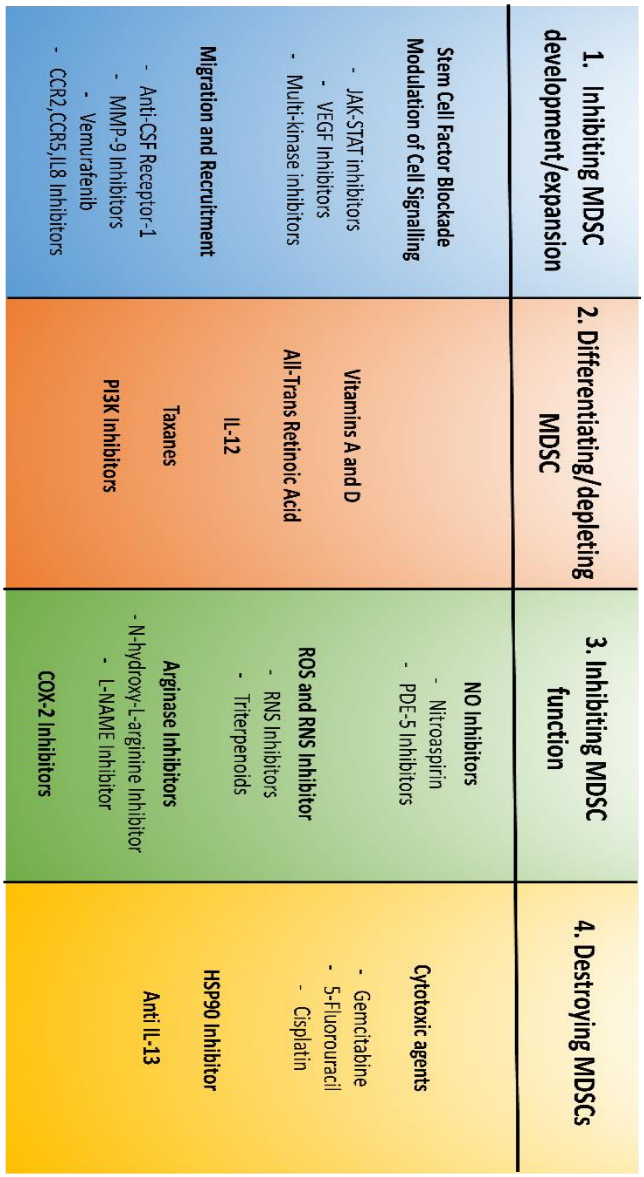


Table 1. Current treatment strategies of MDSC inhibition categorized by general mechanisms of action. *CSF: colony stimulating factor, COX-2: cyclooxigenase-2, HSP: heat shock protein, iNOS: inducible nitric oxide synthase, IL: interleukin, JAK: janus kinases, L-NAME: N-nitro-L-arginine methyl ester, MMP-9: metalloproteinase-9, MDSC: myeloid derived suppressor cell, PDE-5: phosphodiesterase-5, PGE: prostaglandin E, RNS: reactive nitrogen species, ROS: reactive oxygen species, STAT: signal transducer and activator of transcription, VEGF: vascular endotelial growth factor, CCR2/5: C-C chemokine receptor type 2/5.*

However, MDSC differentiation and functions are still poorly understood. This is due to the difficulty of isolating them from the tumor, which requires very large numbers of mice (Maenhout et al. 2014; Escors et al. 2013; Condamine et al. 2014; Thaci et al. 2014b; Youn et al. 2008; Schouppe et al. 2013; Corzo et al. 2010). Isolation from the spleen (Corzo et al. 2010; Maenhout et al. 2014) yields MDSCs that do not faithfully resemble tumor-infiltrating subsets. Large batches of intra-tumor MDSCs purified by standard procedures are usually contaminated with other myeloid cells, do not proliferate well *ex vivo*, lack plasticity of differentiation and are prone to apoptosis (Youn et al. 2008; Escors et al. 2013; Condamine et al. 2014).

Therefore, the molecular study of MDSCs is certainly a challenge. Our group devised a highly efficient, rapid and economic method to produce very large numbers of MDSCs *ex vivo* that resembled melanoma-infiltrating subsets without inducing tumors in mice (Liechtenstein et al. 2014) (Figure 8). This system allowed the study of MDSC biology in controlled conditions at a very low cost.

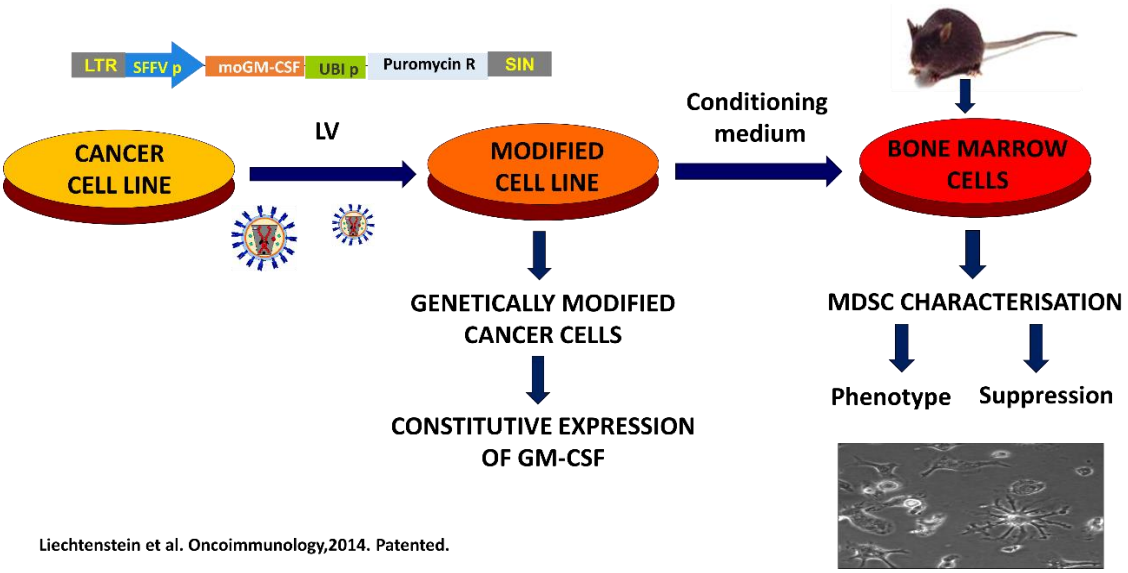


Figure 8. Simulation of tumor environment for the ex vivo differentiation of MDSCs from BM cultures. *Schematic representation of the generation of MDSCs. Scheme of the lentivector construct co-expressing murine GM-CSF and puromycin resistance genes. Cancer cell lines are transduced with the lentivector LV-GMCSF-puroR. Conditioning medium (CM) is collected from GM-CSF-expressing cells to simulate myelopoiesis within a tumor environment. BM cells from healthy C57/Bl6 mice were cultured in the presence of CM for minimum 5 days. LTR, long terminal repeat; SFFV, spleen focus-forming virus promoter; moGM-CSF, mouse GM-CSF gene; Puromycin R, puromycin resistance gene; UBI p, ubiquitin promoter; SIN, self-inactivating LTR.*

In a previous publication that was the basis of this PhD thesis, we initially compared these MDSCs to no-neoplastic counterparts and to conventional DC using quantitative proteomics and systems biology approaches (Liechtenstein et al. 2014). Differentially activated/deactivated pathways caused by cell type differences and by the melanoma tumor environment were identified (Liechtenstein et al. 2014). MDSCs increased the expression of trafficking receptors to sites of inflammation and tumors like c-type lectins, adhesion or TLR-associated molecules. MDSCs showed enhanced expression of proteins involved in endocytosis and vesicle trafficking, that are linked to the activity

of intracellular signaling pathways known to be active in MDSCs (SRC, Ras, Stat3, NF-Kb, MAPK…). An increased expression of nucleous proteins involved in transcription,splicing and translation was observed, which indicates that MDSCs were in an active metabolic state, that are furthermore linked to NOS/ROS production and responses to hypoxia. In order to obtain energy (as aerobic ATP production in down modulated) lipid metabolism is increased by ApoB receptor, Perilipin 3 or mitochondrial proteins. All those processes generates highly toxic metabolites, thus, detoxification enzymes and ROS scravenger proteins such as P450 reductase or Sod2 are upregulated. Differences between tumor enviroment pointed to an adaptation to oxidative stress. Neoplasic MDSC upregulate antoxidant enzymes like Sod1 and peroxiredoxin 6. Furthermore they increase aminoacid synthesis by D3-phosphoglycerate dehydrogenase. This study provided more than 60 novel potential MDSC-specific therapeutic targets and confirmed known targets such as P405R, STAT3 (Emeagi et al. 2013), Sod2 (Hartmann et al. 2013) and S100 proteins (Qin et al. 2014) (Figure 9).

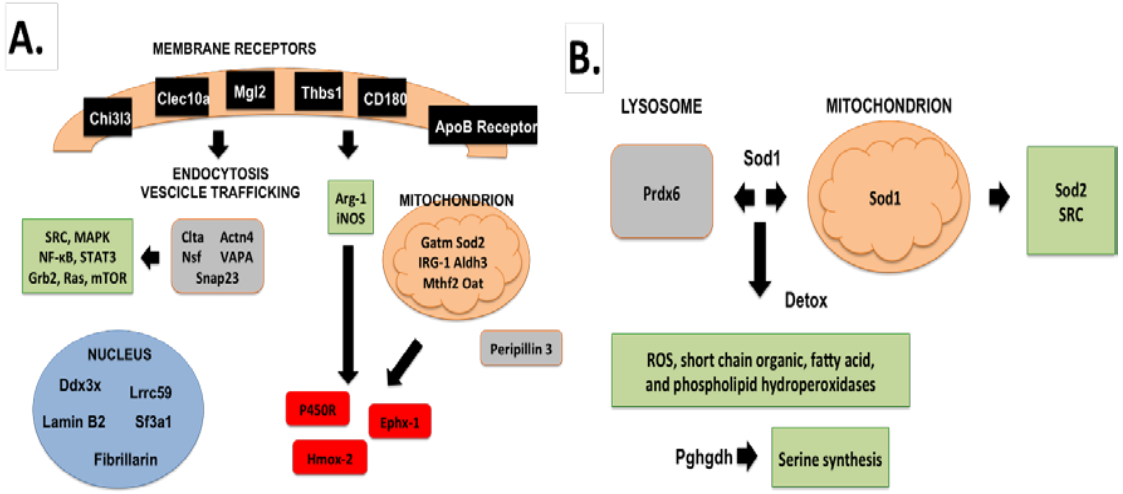


Figure 9: Comparative quantitative proteomics between ex vivo B16-MDSCs and conventional immature DCs using Protein Pilot ***(A)*** *Schematic diagram integrating the biological relationships and pathways inferred from the up-regulated proteins in B16- MDSCs using String 9.1, DAVID and Panther programs. All significantly increased proteins are indicated, grouped according to cell location. Arrows indicate direct pathways between the indicated protein groups. In dark blue, detoxifying enzymes. Proteins within green boxes indicate pathways which are predicted to be activated from biological interactions of the up-regulated proteins.* ***(B)*** *Same as a, but representing differences caused by the tumor environment as highlighted after comparing non-neoplastic 293T- MDSCs with melanoma-specific B16-MDSCs.*

To derive human MDSCs *in vitro*, the starting material is frequently peripheral blood mononuclear cells (PBMCs), as the isolation of bone marrow precursors is an invasive procedure and not practical for these studies. So the differentiation efficiency is much lower compared to murine models, as these cells are fairly differentiated to start with.

In addition, MDSCs isolated from patients are still poorly characterized it is challenging to compare ex vivo differentiated MDSCs with their natural counterparts. Therefore, the detailed differentiation and characterization of human MDSCs populations is still at very early stages. We are working on that issue and maybe soon we are able to obtain human MDSC in an easily, cheap and efficiently way. Once, this system is ameliorated, human MDSCs could be generated by patient-specific tumor cells and high troughtput analyses could be done in order to describe new potential therapeutic targets.

5.3. Programmed death-1 ligand 1 (PD-L1)/programmed death-1 (PD-1) interactions

T lymphocyte activity is regulated by immune checkpoint interactions. Growing tumors harness these inhibitory signals to stop T cell cytotoxicity. My Ph.D. thesis will focus on PD-1/PD-L1 interaction, a classical research model in our group.

5.3.1. PD-1 and PD-L1 structure, expression and signal transduction

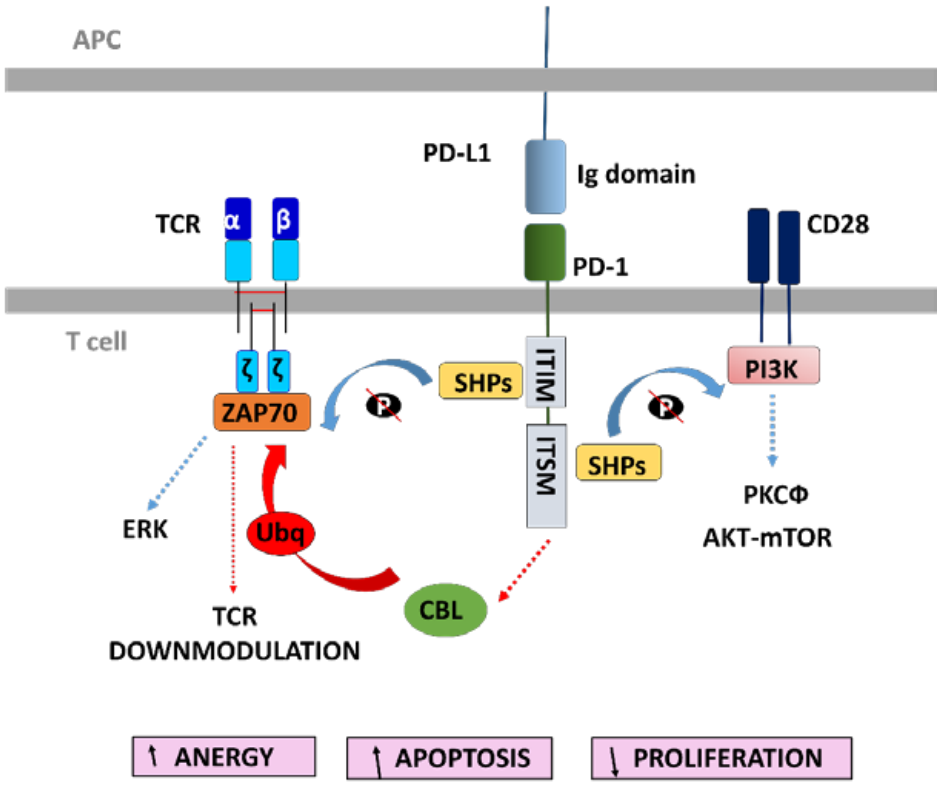
Programmed cell death (PD-1) is a member of the CD28 superfamily expressed on T cells after activation (Michael et al. 2017). PD-1 is also expressed at low levels on double-negative (CD4-CD8) T cells in the thymus, Tregs, activated natural killer T cells and in B cells. There are reports on its expression by monocytes and immature Langerhans´cells (Michael et al. 2017), and also by some cancer cells including human and murine melanoma (Kleffel et al. 2015). This latter claim is rather controversial. PD-1 is a type I transmembrane protein consisting on an extracellular N- terminal immunoglobulin variable-like region (IgV), a stalk that separates the IgV domain from the plasma membrane, a transmembrane domain, and a cytoplasmic tail containing tyrosine-based signaling motifs. These motifs consist of an immunoreceptor tyrosine-based inhibition motif (ITIM), and an immunoreceptor tyrosine-based switch motif (ITSM) (Michael et al. 2017), (Figure10A). These motifs regulate PD-1 signal inhibitory functions. More specifically, PD-1 recruits SHP phosphatases to its ITIM and ITSM motifs, thereby inducing dephosphorylation of TCR-associated kinases such as ZAP70 and PI3K. Consequently, downstream intracellular pathways are terminated such as AKT, ERK, and PKCϴ. In addition, PD-1 on antigen-activated T cells participates in the internalization of the TCR which restrains T cell activities during the exponential phase of T cell expansion ((Michael et al. 2017; Burnet et al. 1970). This mechanism involves the transcriptional up-regulation of E3 ubiquitin ligases of the CBL family, consequent ubiquitination and down modulation of the TCR facilitating T cell disengagement from APCs (Figure 10A).

PD-L1 is a type I transmembrane protein which belongs to the B7 family of molecules that regulate antigen presentation to T cells. PD-L1 is expressed constitutively by professional APCs and on a wide variety of non-hematopoietic cell types, including vascular endothelial cells, pancreatic islet cells, and in cells from immune-privileged sites such as the placenta, testes, and eye. PD-L1 expression can be up-regulated by proinflammatory cytokines including type I and type II interferons, tumor necrosis factor (TNF-α), and VEGF2 or induced in some PD-L1-negative cell types (Karwacz et al. 2012; Arasanz et al. 2017). It is likely that PD-L1 up-regulation at inflammation sites represents a natural negative feedback mechanism to restrain T cell activities and minimize collateral damage. Many studies have shown that PD-L1 expression is transcriptionally regulated by distinct stimuli depending on the cell type, physiological and pathological conditions. For example, the PDL1 expression is regulated by Sox2 in hepatocellular carcinoma (Zhong et al. 2017), STAT3 in human glioma (Kumar et al. 2014) and STAT1 in multiple myeloma (Liu et al. 2007). The variety of mechanisms that regulate PD-L1 expression is a reflection of roles that this molecule is playing in physiological conditions depending on location and cell type.

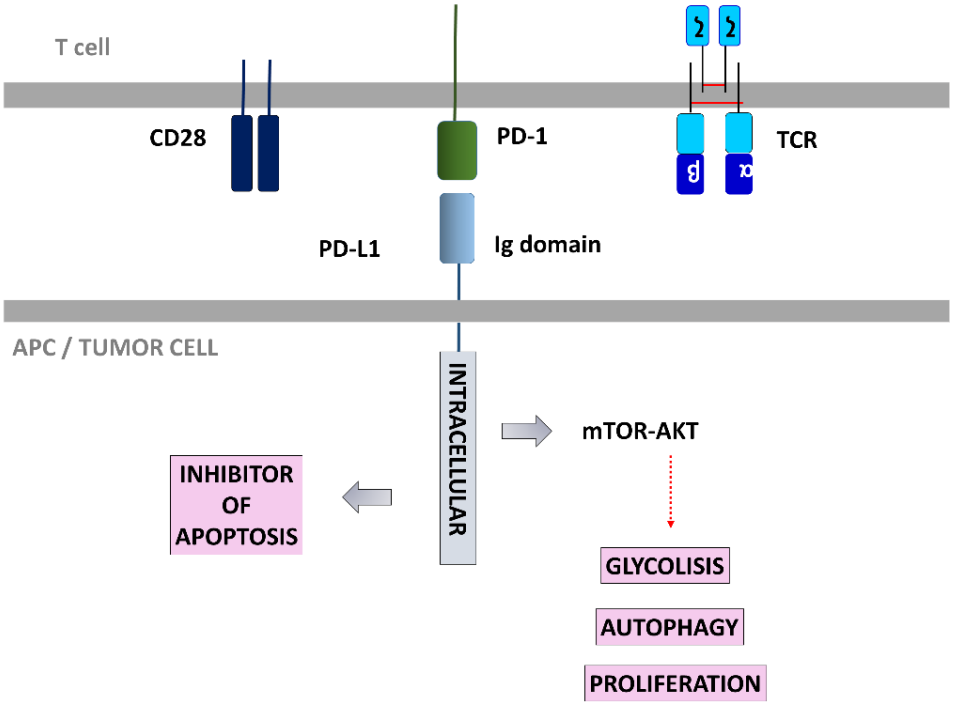
PD-L1 belongs to the immunoglobulin superfamily, and hence it presents an immunoglobulin-like extracellular part, followed by a transmembrane domain and a short intracytoplasmic domain (Figure 10B). The extracellular domain is composed of an Ig variable (V) distal region and an Ig constant (C) proximal region. The variable region shows a standard Ig-like domain which includes complementarity determining-like regions (CDRs). PD-L1 binds to PD-1 in a 1:1 stoichiometry through its V-domain in analogy to antigen recognition by antibodies and TCRs (Zak et al. 2015). Interestingly, and in contrast to PD-1, there are very scarce studies on intracellular signal transduction events induced by PD-L1. Indeed, no obvious sequence motifs in the intracytoplasmic domain have been either predicted or identified up until now (Gato-Cañas et al. 2017; Azuma et al. 2008) (Figure 10B).

In 2008 Azuma *et al.* provided evidence that PD-L1 possessed intrinsic capacities to transmit signals to cancer cells that protected them from T cell-mediated killing independently from its direct inhibitory action towards T cells through PD-1 binding (Azuma et al. 2008). Firstly they tested the susceptibility of cancer cell lines to T cell-mediated killing in the presence or absence of anti-PD-L1 antibodies using cancer cells that expressed a PDL1 molecule in which its intracellular domain was replaced by GFP (Azuma et al. 2008). Later, by using T cells expressing signal-null PD-1 to prevent its signaling in co-culture studies with cancer cells, the authors demonstrated that the protective action of PD-L1´s "molecular shield" was absolutely dependent on the intracellular domain of PD-L1, whether it engaged PD-1 or not. Nevertheless, Azuma and cols could not determine the exact molecular pathways by which PD-L1 was exerting its protective activities, nor its motifs regulating signal transduction (Azuma et al. 2008). Chang *et al.* 2015 demonstrated that tumors derived from sarcoma cells consume large quantities of glucose, depleting the tumor microenvironment of glucose (Geng et al. 2008; Palmer et al. 2015). Treatment of cancer cells with a PD-L1 blocking antibody or silencing PD-L1 with shRNA inhibited the AKT/mTOR signaling axis, leading to reduced translation of mRNAs encoding glycolytic enzymes (Palmer et al. 2015). These results strongly suggested that PD-L1 molecules were regulating the metabolism of cancer cells by controlling intracellular pathways such as AKT/mTOR. Shortly after, it was again corroborated that PD-L1 delivered intracellular signals in the absence of T cells which regulated the mTOR pathway in murine B16 melanoma and ID8agg ovarian cancer cells (Clark et al. 2016). However, this study did not directly address whether PD-L1 was exerting these effects through signaling or not.

A.



B.



Karwacz et al. 2011. EMBO Mol Med
Zuazo Ibarra et al. 2017. Annals of trasl Med
Ostrand-Rosenberg y cols. 2014. J Immunol,

Figure 10. PD-1-PD-L1 structure. ***(A) PD-1-dependent T cell inhibitory mechanisms****. In the figure, the two main inhibitory mechanisms exerted by PD-1 ligation are depicted. The TCR complex including the co-stimulatory molecule CD28 is associated closely to PD-1, which is up-regulated following antigen presentation. SHP phosphatases associate with the phosphorylated ITIM and ITSM motifs in the cytoplasmic domain of PD-1, as shown in the figure. These proteins de-phosphorylate and inhibit kinases mediating TCR signal transduction such as ZAP70 and PI3K (blue arrows). Downstream intracellular pathways are terminated as exemplified in the figure with AKT, ERK, and PKCϴ. This mechanism is probably activated after the recognition of target cancer cells to limit T cell effector activities. The second major mechanism plays a key regulatory role during antigen presentation to and activation of naïve T cells. It involves the transcriptional up-regulation of CBL E3 ubiquitin ligases as shown, that trigger TCR endocytosis by its ubiquitination. Hence, TCRs are removed from the T cell surface, facilitating their disengagement from APCs (red arrow). ITM, immunoreceptor tyrosine-based activation motif; ITSM, immunoreceptor tyrosine-based switch motif. α and β, TCR alpha and beta chains; CBL, casitas B-lineage lymphoma protein.* ***(B) PD-L1 intracellular signaling.*** *The figure represents the main mechanisms by with PD-L1 intrinsically transmits signals to tumor cells or APCs before publication of the data from this PhD thesis. PD-L1 signals inhibit apoptosis and increase mTOR-AKT signaling leading to elevation of glucose consumption, inhibition of autophagy inhibition and increased proliferation.*

5.3.2. PD-L1/PD-1 interactions within the tumor environment

A significant number of tumors constitutively express PD-L1 or can up-regulate PD-L1 in response to an inflammatory environment (Thomas Condamine and Gabrilovich 2011) leading to inhibition of T cell functions and cytotoxicity (Fife et al. 2009; Katarzyna Karwacz et al. 2011; Herrmann et al. 2015; Gato-Cañas et al. 2017). Indeed, oncogenic activation of pathways such as AKT-mTOR, EGFR, MEK-ERK signaling axis and possibly the MAPK p38 pathway contribute to PD-L1 up-regulation (Lastwika et al. 2016; Minchom et al. 2017; Ota et al. 2015; Noh et al. 2015). Genomic amplifications containing the gene encoding PD-L1 are also frequently selected in cancer cells, and likely linked to increased PD-L1 levels (Straub et al. 2016). PD-L1 expression is also enhanced by hypoxia (Noman et al. 2014; Ruf et al. 2016; Chang et al. 2016) and regulated through epigenetic mechanisms. For example, microRNA 513 down-modulates PD-L1 mRNA translation in human cholangiocytes (Gong et al. 2010) and microRNA 152 in gastric carcinoma (Wang et al. 2017). In some cases, the structure of the 3´UTR of its mRNA is disrupted in several cancer types, which constitutively increases PD-L1 expression levels in cancer cells (Kataoka et al. 2016). Therefore, there is abundant experimental evidence on the correlation between high PD-L1 expression and tumor progression and aggressiveness.

PD-L1 tumor expression strongly correlates with poor prognosis (Gato-Cañas et al. 2017) although not in all tumor types. Overall, the experimental evidence points to PD-L1 intrinsic signal transduction as a means to interfere with pro-apoptotic stimuli and enhance cancer cell proliferation. Furthermore, in 2008 Takeshi *et al.* proposed PD-L1 as a universal anti-apoptotic receptor (Azuma et al. 2008). The authors of this study demonstrated that PD-L1 expression makes cancer cells refractory to Fas-induced apoptosis, resistant to the protein kinase inhibitor Staurosporine and to T cell cytotoxicity following binding to PD-1 (Azuma et al. 2008).

5.3.3. PD-L1/PD-1 blockade in human anti-cancer therapy

Monoclonal antibodies (mAbs) that block immune checkpoints can restore the anti-tumor activities of cytotoxic T cells (Ito et al. 2015).

The first clinically efficacious activities for anti-PD-L1 and anti-PD-1 antibodies were demonstrated in 2012 by Topalian *et al.* (Brahmer et al. 2010, Brahmer et al. 2012) since then anti PD-1 or anti PD-L1 antitibodies have been approved for an increasing number of cancers such as melanoma, lung cancer, and gastric adenocarcinoma, just to mention a few.

Antibodies approved for human clinical use include nivolumab and pembrolizumab anti–PD-1 agents (Ansell et al. 2015; Robert et al. 2014), and atezolizumab, durvalumab, avelumab as anti–PD-L1 (Gato-Canas et al. 2016). There are still ongoing many phase II or III clinical trials in numerous cancers.

The first to show significant therapeutic efficacies in human melanoma was ipilimumab, a CTLA-4-specific antibody that blocks its interaction with CD80 (Peggs et al. 2006; Quezada et al. 2006; Herrmann et al. 2014; Walker et al. 2011; Pardoll et al. 2012; Hodi et al. 2008; Postow et al. 2015; Weber et al. 2013; Tomasini et al. 2012). Focussing on melanoma treatment aproved by the FDA and in clinical use, Nivolumab has demonstrated highly durable tumor regressions (>1 year) with good safety profiles in metastatic melanoma (Topalian et al. 2012; Ansell et al. 2015; Topalian et al. 2014). Pembrolizumab has an optimized Fc region that minimizes antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) (Moreno et al. 2015) with durable responses for advanced melanoma (Hamid et al. 2013). Both have superior clinical effects and a better safety profile than chemotherapy and ipilimumab monotherapy (Robert et al. 2014; Weber et al. 2015). Both antibodies are approved as first-line agents in melanoma patients(Force et al. 2017; Barnhart et al. 2015), and pembrolizumab recently in lung cancer in monotherapy and in combination with platinum chemotherapy (Gandhi et al. 2018). Furthermore, last year, the FDA approved pembrolizumab for all type of solid tumors with microsatellite instability that have progressed with previous treatments (https://www.fda.gov/). Nivolumab is being used in

combination therapy with ipilimumab increasing survival rates (Wolchok et al. 2013) in metastasic melanoma and renal cell carcinoma. (Figure 4).

5.3.4. Resistance to PD-L1/PD-1 blockade

Two major challenges remain for an optimal management of anti-cancer therapies. First, a significant number of patients who are refractory to the initial application of the treatment (intrinsic resistance). Secondly, tumor progression from cancer cells that have acquired resistance by several means after an initial phase of objective responses. Possibly, the major goal in oncology is the identification of patients who will benefit from therapies and the identification of the mechanisms leading to acquired resistance. Some mechanisms of adaptive resistance to PD-L1/PD-1 blockade therapy have been described. For example, upregulation of alternative immune checkpoints such as TIM-3, to compensate for the PD-1 blockade in T cells (Koyama et al. 2016; Kim et al. 2016; Shaked et al. 2016). The loss of beta2-microglobulin (ß2m) through inactivating mutations leading to the elimination of MHC I surface expression (Restifo et al. 1996). A role for ß2m in acquired resistance to PD-1 blockade in melanoma was recentlysupported by a study reporting a ß2m truncating mutation in relapsing lesions (Zaretsky et al. 2016).

Evaluation of the cancer secretome by proteomics is helping in the identification of resistance to immune checkpoint blockade (Skalnikova et al. 2017; Choi et al. 2012). Recently, several candidate biomarkers have been identified that correlate with clinical benefit, including the apelin receptor (APLNR) (Patel et al. 2017). Other immune-related genes have been demonstrated to be required for efficacious immunotherapies including antigen presentation (PD-L1, CD47) and IFN gamma signalling molecules (PTPN2, STAT1, JAK1, IFNGR2, IFNGR1, or JAK2) (Manguso et al. 2017).

An intact interferon signal transduction pathway in cancer cells seems to be required for the efficacy of PD-L1/PD-1 blockade therapy. Inactivating mutations in JAK1/2 genes were linked to the failure of PD-L1 up-regulation in cancer cells, becoming refractory to therapy (Zaretsky et al. 2016; Shin et al. 2017). The authors of these studies hypothesized that lack of adaptive PD-L1

expression was also the cause of primary resistance to PD-1 blockade (Shin et al. 2017).

6. LENTIVECTOR-BASED GENETIC IMMUNOTHERAPIES

In this Ph.D. thesis, lentiviral vectors (lentivectors) are used for genetic immunotherapy and as gene-modifying tools. Lentivectors have been extensively reviewed elsewhere (Escors et al. 2013; Trono et al. 2000; Escors et al. 2010), and here they will only briefly explained for understanding the context of the experiments in which they have been used.

6.1. Lentivector structure and production

Most lentivectors are derived from the HIV-1 genome devoid of virulence and replication genes, leaving space for insertion of promoter sequences and genes of interest. These vectors have been improved to achieve a high degree of biosafety (He and Falo 2007). Most of them are self-inactivating lentivectors, in which most of the U3 promoter region is eliminated following integration into the target DNA (Zufferey et al.1998), and thereby reducing the likelihood of producing replication-competent viruses (Figure 11).

Lentivectors are usually assembled in 293T cells and secreted to the cell culture supernatants following co-transfection of three plasmids: the transfer vector plasmid, the packaging plasmid, and the envelope plasmid. The transfer vector will produce an RNA encoding the whole vector that will be packaged by the structural and non-structural proteins encoded by the packaging plasmid. The envelope plasmid will produce a membrane glycoprotein that will cover the lentivector particle conferring the desired specificity for different cell receptors (Figure 11).

The packaging plasmid used in this thesis (p8.91) encodes the HIV-1 gag-pol, rev, and tat genes under the control of the cytomegalovirus early promoter (CMV), but lack accessory genes (vif, vpr, vpu, nef) (Zufferey et al. 1997) (Figure 11).

The envelope plasmid used in this thesis is the pMDG and encodes the vesicular stomatitis virus G protein (VSV-G) under the control of the CMV promoter. VSV-G pseudotypes the lentivector particle conferring the vector a wide species and cell type tropism (Akkina et al. 1996; Naldini et al. 1996; Yee et al. 1994) (Figure 11).

The transfer lentivector plasmid used in this thesis is the pSIN vector as shown in Figure 11 (Escors et al. 2008). Summarizing, this is a second generation, self-inactivating lentivector that contains the spleen focus-forming virus promoter (SFFV) followed by the gene of interest. This vector was used as the basis for constructing the pDUAL and the pHIV-SIREN lentivector plasmid series (Figure 11). Briefly, the pDUAL lentivector contains the SFFV and the human ubiquitin promoters to co-express two genes. A pDUAL version containing a PD-L1-targeted microRNA was designed by Liechtenstein et at (Liechtenstein et al. 2014). The pHIV-SIREN plasmids contain the U6 promoter to express shRNAs, and the phosphoglycerate kinase promoter (PGKp) to express selection genes (antibiotic resistances) (Gato-Cañas et al. 2017) (Figure 11).

An “all-in-one” CRISP/CAS9-sgPD-L1 lentivector was generously donated by Prof. Karine Breckpot from the Free University of Brussels (Belgium) to disrupt the PD-L1 gene (Figure 11D).

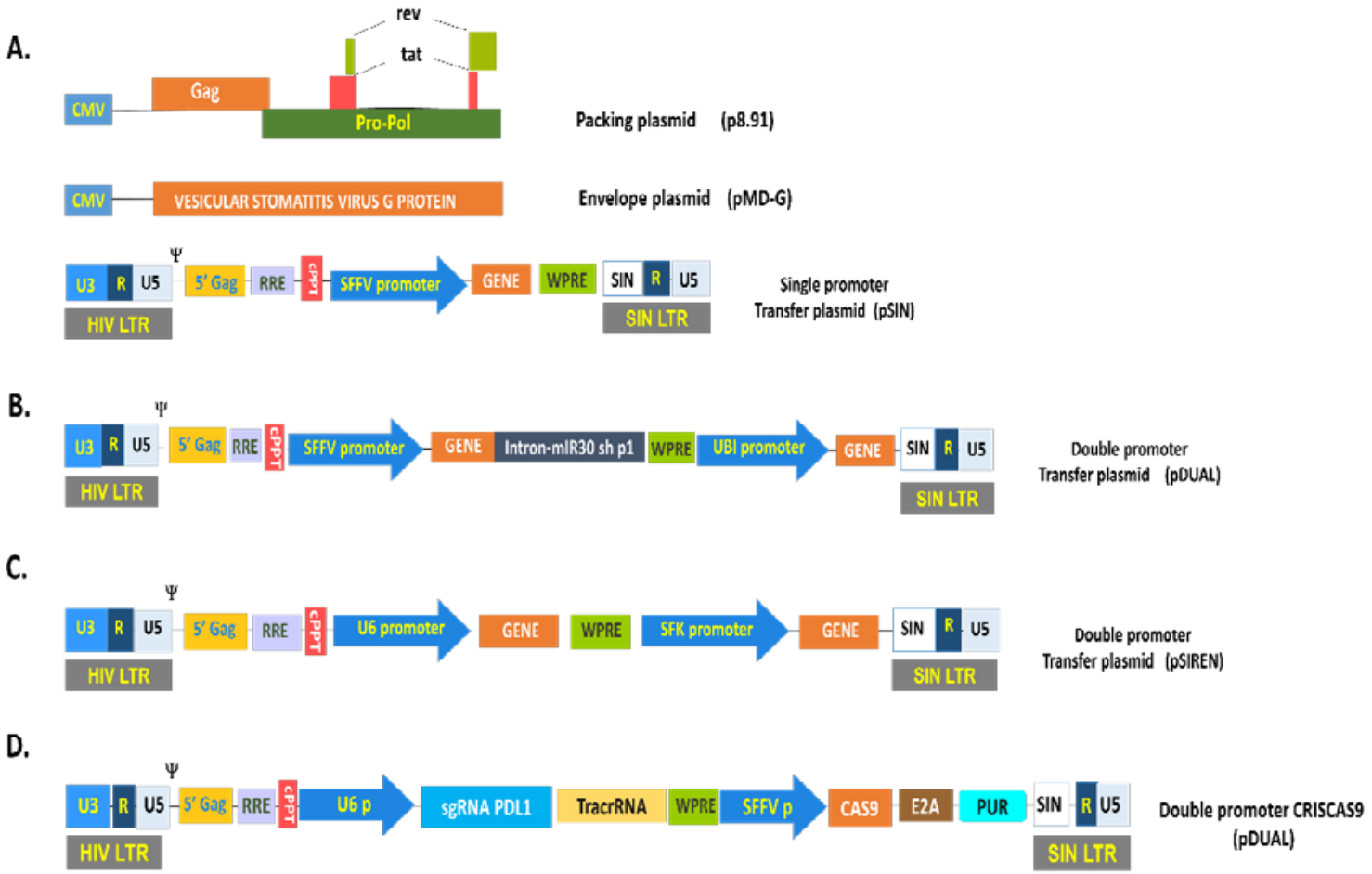


***Figure 11. Lentivector structure and production*. (A)** *The three lentivector production plasmids are shown. The packaging plasmid contains the structural and enzymatic genes from HIV required for reverse transcription, integration, and assembly, such as Gag-Pol- Rev-Tat. The envelope plasmid encodes VSV-G in this case. The transfer vector plasmid lacks all the genes that are provided in trans by the packaging plasmid. In this figure the transfer vector is the we use a pSIN vector that contains long-terminal repeats or LTRs (U3- R-U5), including a self-inactivating 3' end LTR (SIN-R-U5) and an expression cassette. This cassette is made of the spleen focus-forming virus (SFFV) promoter controlling the expression of the gene of interest. Ψ, Rev response element (RRE), central polypurine flap (cPPT) and woodchuck post-transcriptional response elements (WPRE) are shown.*

***(B)** pDUAL lentivector contains two promoters to co-express two different genes, the SFFV and the human ubiquitin promoter. A version was generated to include a PD-L1-targeted microRNA.*

***(C)** The pHIV-SIREN plasmid contains the U6 promoter to express shRNAs and the SFK promoter* encoding the selection antibiotic or other gene of interest.

***(D)** "All-in-one" lentivector plasmids to eliminate expression of PD-L1 by disrupting the gene using CRISPR-Cas9 technology. The plasmid was generously donated by Prof. Karine Breckpot.*

6.2. Lentivectors for genetic cancer immunotherapy

Subcutaneous lentivector injection transduces human and mouse APCs (Esslinger, Romero, and MacDonald 2002) and displays significant T cell adjuvant activities by providing TLR ligands to APCs (Breckpot et al. 2007; Breckpot et al. 2010; Esslinger et al. 2003; Rossetti et al. 2011). Our research group has been working during the last years to use them as cancer vaccines.

Lentivectors have a good biosafety profile for their application in human therapy. For example, integration-deficient lentivectors remain in the nucleus as episomes (Hu, Dai, and Wang 2010; Yanez-Munoz et al. 2006) to prevent genotoxicity in human therapy (Hu et al. 2010; Yanez-Munoz et al. 2006). There is now extensive experience in production of clinical grade lentivector batches with high biosafety profiles (Levine et al. 2006). Hence, lentivectors have been used for the treatment of HIV and in human gene therapy clinical trials (X-linked adrenoleukodystrophy, β-thalassaemia, and advanced leukaemia) without major concerns (Cartier et al. 2009; Levine et al. 2006; Tebas et al. 2013; Cavazzana-Calvo et al. 2010; Porter et al. 2011; Kalos et al. 2011).

Lentivectors are used in two ways for genetic cancer immunotherapies. First, they can modify T cells to express TCRs of interest or chimeric antigen receptors (CARs). Complete remissions in 90% of relapsed and refractory acute lymphoblastic leukemia (ALL) patients were achieved using lentivector-modified T cells (Maude et al. 2014). Secondly, lentivectors induce immune responses by direct administration as they transduce preferentially conventional DCs leading to transgene expression, processing and antigen presentation in draining lymph nodes (Goyvaerts et al. 2015). Hence, lentivector transduction of DCs (Bukrinsky et al. 1993; Naldini et al. 1996) does not affect their viability or antigen presenting capabilities (Breckpot et al. 2003; He et al. 2005; He et al. 2006; Gruber et al. 2000; Zarei et al. 2002; Dyall et al. 2001; Karwacz et al. 2012). Direct transfer of transduced DCs achieves prolonged *in vivo* antigen presentation in murine models, increasing the potency and duration of CTL responses (He et al. 2006).

Escors *et al* demonstrated that DCs could be effectively transduced and matured using lentivectors that expressed MAPK kinase and IFN-pathway activators, or ERK inhibitors, thereby boosting anti-lymphoma immunity in murine models (Escors et al. 2008).

The co-expression of some of these DC molecular activators with a PD-L1-targeted microRNA using lentivectors further enhanced their immunogenicity (Karwacz et al. 2011). A new generation of lentivector vaccines was also engineered by Lienchtestein *et al.* co-expressing melanoma antigens, the PD-L1 microRNA, and various T cell-polarizing cytokines (Liechtenstein et al. 2014). The combination of IL12 expression with PD-L1 silencing counteracted MDSC suppressive activities and significantly enhanced anti-melanoma immunity in prophylactic and therapeutic vaccination strategies (Liechtenstein et al. 2014). The group of Prof. Breckpot developed a fusion gene of IFNβ with the ectodomain of TGFBR-II (a TGF-β trap), called Fβ2. Its expression from a mRNA vaccine reduced tumor cell proliferation, enhanced DC antigen presenting capabilities and reduced MDSC suppressive activities (Van der Jeught et al. 2014).

Considering previous results from our group, in this Ph.D. thesis I designed lentivector plasmids to study PD-L1 biology and related signal transduction pathways, engineering vaccines with superior efficacies against melanoma, and modify MDSCs.

7. Ph.D. AIMS

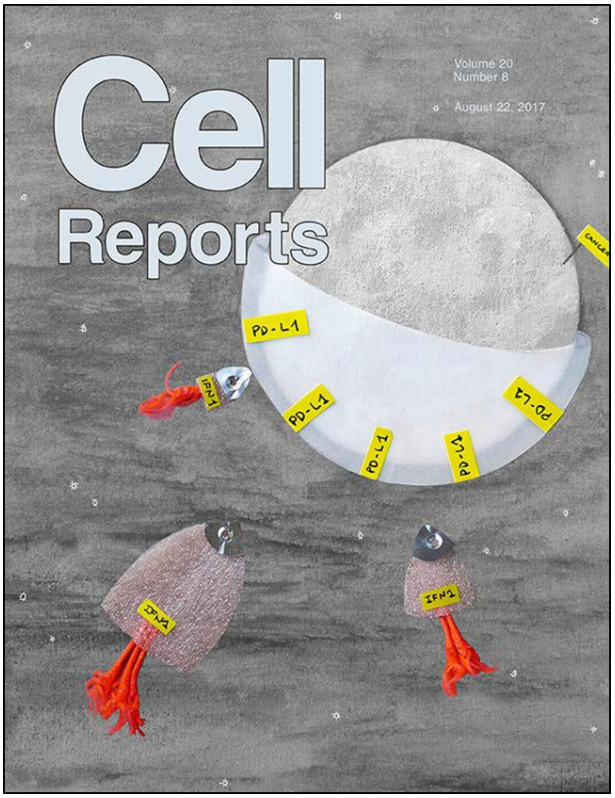
The main goals of this Ph.D. thesis were the study of two major immunological barriers for melanoma immunotherapy and the identification of novel therapeutic targets. In the first part, I characterize and identificate PD-L1 intracellular signaling motifs and their anti-interferon functions. In the second part, I describe MDSCs proteome and specific signaling pathways involved in its suppressive function. The specific aims of the Ph.D. are:

1. Identification of the mechanisms of melanoma resistance to interferon cytotoxicity mediated by PD-L1 intracellular signalling.

2. Quantitative study of the proteome of melanoma-specific MDSC for the identification of potential therapeutic targets.

CHAPTER 1

PDL1 Signals through conserved sequence motifs to overcome interferon-mediated cytotoxicity



PDL1 SIGNALS THROUGH CONSERVED SEQUENCE MOTIFS TO OVERCOME INTERFERON-MEDIATED CYTOTOXICITY

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KEYWORDS

PDL1, PD-L1, B7-H1, CD274, PD1, interferon, signal transduction, immunotherapy

SUMMARY

PDL1 blockade produces remarkable clinical responses, thought to occur by T cell reactivation through prevention of PDL1-PD1 T-cell inhibitory interactions. Here we find that PDL1 cell-intrinsic signaling protects cancer cells from interferon (IFN) cytotoxicity and accelerates tumor progression. PDL1 inhibited IFN signal transduction through a conserved class of sequence-motifs that mediate crosstalk with IFN-signaling. Abrogation of PDL1 expression or antibody-mediated PDL1 blockade strongly sensitized cancer cells to IFN cytotoxicity through a STAT3/caspase-7 dependent pathway. Moreover, somatic mutations found in human carcinomas within these PDL1 sequence-motifs disrupted motif-regulation resulting in PDL1 molecules with enhanced protective activities from type I and type II IFN cytotoxicity. Overall, our results reveal a mode of action of PDL1 in cancer cells as a first line of defence against IFN cytotoxicity.

INTRODUCTION

Programmed death 1 ligand 1 (PDL1) is a member of the B7 family of co-stimulatory/co-inhibitory molecules expressed by a wide variety of cell types including tumors (Sharpe et al., 2007). PDL1 is a transmembrane protein consisting of an immunoglobulin-like extracellular part, followed by a transmembrane domain and a short intracytoplasmic domain. Programmed death 1 (PD1) is its prototypical receptor, which is expressed mainly by effector immune cells such as B and T cells (Freeman et al., 2000; Zak et al., 2015). However, PDL1 can also bind CD80 (Butte et al., 2007). The physiological role of PDL1 is to maintain peripheral tolerance and contribute to antigen presentation to T cells by dendritic cells (Karwacz et al., 2011; Sharpe et al., 2007). In neoplastic conditions, PDL1 tumor expression strongly correlates with increased progression and poor prognosis, being an indicator of resistance to conventional treatments such as chemotherapy and radiotherapy.

It is widely accepted that PDL1 protects cancer cells by engaging with PD1 expressed on the surface of activated cytotoxic T cells (Fife et al., 2009). This engagement is strongly inhibitory to T cells leading to decreased effector

activities. As a consequence, PDL1-engaged PD1 in T cells interferes with the T cell receptor (TCR) signalosome stopping T cell cytotoxic activities and production of cytokines such as interferons (IFNs) (Fife et al., 2009; Karwacz et al., 2011). Recent evidence suggests that PDL1 can activate intrinsic signals in the absence of PD1 that enhance cell proliferation and survival through the inhibition of autophagy and mTOR activation (Clark et al., 2016; Chang et al., 2015). However, in contrast to PD1, there is still very little evidence for specific signal transduction events induced by PDL1. No obvious sequence motifs in the intracytoplasmic domain with signal transduction capacities have been either predicted or identified so far (Azuma et al., 2008).

PDL1/PD1 blockade therapy has achieved unprecedented therapeutic clinical success for a variety of cancers including melanoma. PDL1/PD1 blocking antibodies cause the recovery of T cell anti-tumor cytotoxicity and production of IFNs that inhibit tumor cell growth and survival. Therefore, a significant number of treated patients experience long-lasting anti-tumor responses (Hodi et al., 2016). However, a large number of patients are still intrinsically resistant to anti-PDL1/PD1 therapy, or exhibit tumor progression after a period of therapeutic responses. Recently, it has been shown that inactivating mutations in *JAK1*, *JAK2* and β2-microglobulin genes in cancer cells are responsible for primary and acquired resistance to anti-PD1 therapy in a cohort of cancer patients (Shin et al., 2017; Zaretsky et al., 2016). As IFN signals are potent transcriptional transactivators of PDL1, these mutations inhibited PDL1 upregulation in cancer cells. The authors proposed that the loss of PDL1 up-regulation abrogated the antitumor efficacy of PDL1/PD1 blockade in these patients (Sharma et al., 2017).

Here we demonstrate that PDL1 expression represents a direct line of defense for cancer cells by transducing signals that counteract IFN signal transduction within cancer cells. Moreover, we demonstrate that the PDL1 intracytoplasmic domain is essential for its protective functions through the activity of regulatory non-classical signal transduction motifs.

RESULTS

IFNβ expression coupled to PDL1 silencing is lethal to melanoma cells

To engineer an immunogenic cell-based cancer vaccine, we attempted to generate a B16F10 melanoma cell line with silenced PDL1 that would secrete IFNβ. To achieve this, we cloned the IFNβ gene into a lentivector expressing a PDL1-targeted microRNA (μPDL1) and a puromycin selection gene previously described (Karwacz et al., 2011; Liechtenstein et al., 2014a) **(Figure 1A)**. Strikingly, although puromycin-resistant cells could be obtained, these cells died within one or two weeks of culture. To identify the component conferring lethality, the IFNβ gene was expressed with or without μPDL1 (Figure 1A). Interestingly, it was possible to generate B16 cell lines expressing high levels of secreted IFNβ (6 ng/ml) only in the absence of μPDL1. As these cells had very high surface PDL1 protein expression (Figure 1B), this indicated that PDL1 upregulation could be an adaptation to survival from sustained IFNβ signaling. These results suggested that cancer cells may utilize PDL1 expression to negatively regulate IFN signal transduction. To confirm that toxicity associated to PDL1 silencing was mediated through enhanced IFNβ signaling, B16 cells with a silenced type I IFN receptor (B16-IFNAR1^{KD}) were generated using lentivector delivery of shRNA. Additionally, B16 cells overexpressing a PDL1 mutant with reduced complementarity to the μPDL1 (B16-PDL1) were also generated to strengthen PDL1 signaling **(Figure 1C)**. B16-IFNAR1^{KD} cells proliferated well whether they expressed IFNβ or IFNβ-μPDL1 confirming that lethality was conferred by IFN signal transduction **(Figure 1C)**. Importantly, PDL1 overexpression in B16 cells overcame the inhibitory effects of IFNβ and the lethality of the IFNβ-μPDL1 combination **(Figure 1C)**. Type I IFN receptor is a homodimer of IFNAR1 and IFNAR2 molecules, of which IFNAR1 is essential for signal transduction (Ragimbeau et al., 2003). As B16-PDL1 cells showed levels of surface IFNAR1 and IFNAR2 expression comparable to unmodified B16 cells **(Figure 1C and not shown)**, we concluded that PDL1 interfered with IFNβ-signal transduction rather than causing IFNAR down-modulation.

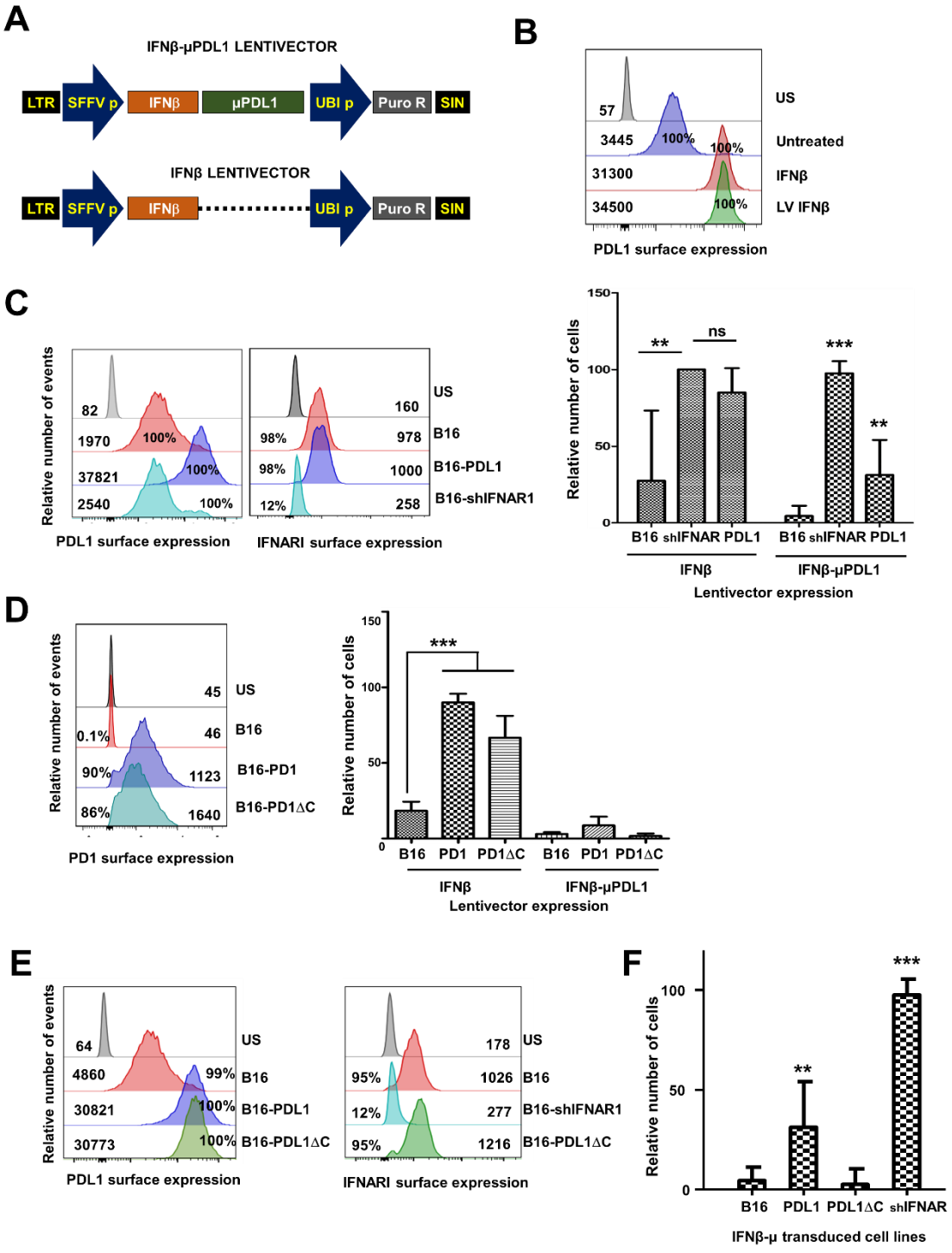


Figure 1. PDL1 protects melanoma cells from IFNβ toxicity. (A) *Lentivector expression vectors used in the studies. LTR, long terminal repeats; SFFV, spleen focus forming virus promoter; μPDL1, microRNA targeting murine PDL1; UBIp, ubiquitin promoter; PuroR, gene conferring puromycin resistance.* ***(B)*** *Expression of surface PDL1 in B16 cells treated with recombinant IFNβ or transduced with the IFNβ-expressing lentivector. Data shown as flow cytometry histogram plots.*

Numbers and percentages indicate mean fluorescent intensities and percentage of positive cells compared to the unstained (US) control. ***(C)*** *Left, flow cytometry histograms for PDL1 or IFNAR1 surface expression (as indicated) in the B16 cell lines as shown on the right of the histograms. Numbers and percentages indicate mean fluorescent intensities and percentage of positive cells. Right, Bar graphs representing the mean relative number of cells with error bars (standard error of the means) after two weeks of puromycin selection following transduction of the indicated B16 cell lines (bottom of the graphs) with lentivectors co-expressing IFNβ-PuroR or IFNβ-μPDL1-PuroR. Data obtained from 10 independent experiments.* ***(D)*** *Left, flow cytometry histograms for PD1 surface expression in the indicated B16 cell lines on the right of the plots. Numbers and percentages indicate mean fluorescent intensities and percentage of positive cells. PD1ΔC, indicates a PD1 protein lacking the intracytoplasmic signaling domain. On the right, bar graphs representing the mean relative number of cells with error bars (SEM) after two weeks of puromycin selection following transduction of the indicated B16 cell lines (bottom of the graphs) with lentivectors co-expressing IFNβ-PuroR or IFNβ-μPDL1-PuroR. Data was obtained from 4 independent experiments.* ***(E)*** *Flow cytometry histograms for surface expression of PDL1 (left) or IFNAR1 (right) on the indicated cell lines on the right of the plots. Numbers and percentages indicate mean fluorescent intensities and percentage of positive cells. PDL1ΔC, PDL1 protein without the intracytoplasmic region.* ***(F)*** *Bar graphs representing the mean relative number of cells with error bars (standard error of the means) after two weeks of puromycin selection following transduction of the indicated B16 cell lines (bottom of the graphs) with lentivectors co-expressing IFNβ-μPDL1-PuroR. Relevant statistical comparisons are indicated within the graphs from three independent experiments. ** and ***, indicate very significant (P<0.01) and highly significant (P<0.001) differences.*

PDL1 signal transduction counteracts IFNβ toxicity

To test whether the engagement of PDL1 with its receptor PD1 would deliver a protective signal against IFNβ cytotoxicity, B16 cell lines were generated that constitutively expressed high levels of PD1 or a mutant with a deletion of its intracytoplasmic domain (PD1ΔC) incapable of signal transduction **(Figure 1D)**. Both B16-PD1 and B16-PD1ΔC cells significantly overcame the inhibitory effects of IFNβ expression, for which only the PD1 extracellular part was required but not its intracytoplasmic signaling domain. As expected, expression of IFNβ together with PDL1 silencing was still lethal to B16-PD1 and B16-PD1ΔC cells **(Figure 1D)**. These results demonstrated the requirement of PDL1 to transmit a survival signal that is nevertheless potentiated by PD1 engagement.

To assess whether PDL1 possessed intrinsic signal transduction capacities that protected against IFNβ toxicity, B16 cells overexpressing a deletion mutant lacking the intracytoplasmic domain (B16-PDL1ΔC) were generated. This PDL1 mutant did not have the target sequence for µPDL1 and was efficiently expressed on the cell surface. Again, PDL1ΔC overexpression did not alter the surface expression of IFNAR1 **(Figure 1E and not shown)**. Unlike the wild-type version, the overexpression of PDL1ΔC did not overcome the lethality conferred by the co-expression of IFNβ with µPDL1 **(Figure 1E)**. Considering these data, we concluded that PDL1 counteracts IFNβ cytotoxicity by signal transduction through its intracytoplasmic domain.

Conserved motifs within the intracytoplasmic domain of PDL1 regulate protection from IFNβ cytotoxicity

We then thoroughly analyzed the PDL1 intracytoplasmic domain using several bioinformatics tools. PDL1 presents a strongly amphiphilic intracytoplasmic domain without any obvious signaling domains **(Figure S1)**. After extensive searches using different databases and algorithms, only MotifFinder produced a positive hit with a domain present in a bacterial and eukaryotic DNA-dependent RNA polymerase β subunit **(Figure S1)**. No sequences related to signal transduction were found, suggesting that PDL1 is using non-conventional signaling motifs.

Therefore, to identify PDL1 signal transduction functional domains we reasoned that these would be phylogenetically conserved. The intracytoplasmic region of 10 mammalian PDL1 molecules were aligned including the corresponding sequence from salmon as a divergent control **(Figure 2A)**. Three conserved sequences were identified that we termed "RMLDVEKC", "DTSSK" and "QFEET" motifs. PDL1 undergoes ubiquitination which leads to its destabilization (Lim et al., 2016), and we found that lysines 271 and 280 within RMLDVE**K**C and DTSS**K** motifs were putative targets for this post-translational modification according to the application of a random forest algorithm (Radivojac et al., 2010) **(Figure 2A)**. To test the functionality of these domains, B16 cells lacking PDL1 (B16-PDL1^{KO}) were generated using CRISPR/Cas9 **(supplemental figure 2)**. Then, a PDL1 gene was constructed to prevent its cleavage by Cas9 through mutation of the CRISPR/Cas9 target site while conserving the wild-type aminoacid sequence (PDL1wt). PDL1wt and mutants with deletions of each motif were expressed in B16-PDL1^{KO} cells **(Figures 2A, S2)**. Two additional PDL1 mutants with conservative lysine-to-arginine substitutions were generated to eliminate the possibility of PDL1 undergoing ubiquitination within the intracytoplasmic domain **(Figure 2A)**. All PDL1 mutants were efficiently transported and expressed on the cell surface, as assessed by flow cytometry following surface staining with PDL1-specific antibodies **(Figure S2)**. Then, the inhibitory activities of recombinant IFNβ over these B16 modified cell lines were tested by real-time monitoring of cell growth/viability (ACEA RTCA). An IFNβ concentration of 10 ng/ml was chosen as it caused at least 50% growth inhibition to B16-PDL1wt cells as assessed by RTCA. CRISPR/Cas9 abrogation of PDL1 (B16-PDL1^{KO}) strongly sensitized B16 cells to recombinant IFNβ, and even causing cell death **(Figure 2B and 2C)** in agreement with our initial observations based on IFNβ expression with a lentivector, and also discarding potential off-target effects of µPDL1 **(Figure 1)**. PDL1 with a deleted QFEET motif retained its protective capacities. In contrast, removal of the RMLDVEKC motif completely abrogated the anti-IFNβ activities **(Figure 2B)**. Interestingly, deletion of the DTSSK motif as well as lysine-to-arginine mutations within the RMLDVEKC or DTSSK motifs significantly enhanced resistance to IFNβ **(Figure 2B).** These results strongly suggested that DTSSK was a regulatory motif

that modulates anti-IFN activities. To confirm this, the conserved D, S, and K residues in the DTSSK motif were mutated to alanines and the resulting PDL1 molecule was expressed well on the surface of B16-PDL1^{KO} cells (B16-DA cells). The alanine replacement of these residues showed an enhancement of the protective functions of PDL1 against IFNβ, strongly reinforcing the evidence that DTSSK was an inhibitory motif of PDL1 protective functions **(Figure 2C)**.

Overall, we concluded that the RMLDVEKC motif was essential for PDL1 protection against IFNβ, while the DTSSK motif and the lysines 271 and 280 acted as negative regulators.

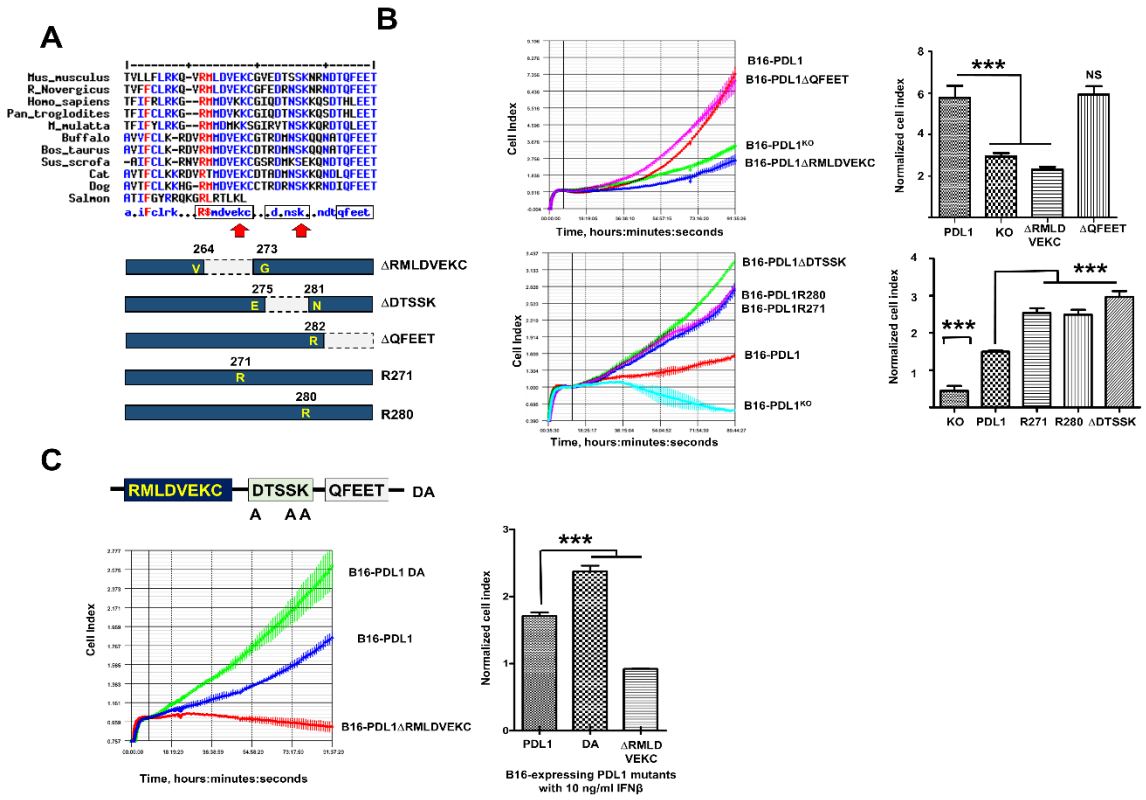


Figure 2. PDL1 protection from IFNβ cytotoxicity is regulated by conserved sequence motifs. (A) *Alignment of the intracytoplasmic domain from PDL1 molecules of the indicated species. In red and blue, highly conserved residues. The consensus sequence is shown on the bottom, with the three conserved motifs within open boxes. Lysines predicted to be capable of undergoing ubiquitination are indicated with arrows. Below the alignment, schemes indicating deletions or arginine substitutions are shown.* ***(B)*** *On the left, real time monitoring cell growth*

graphs from B16-PDL1^{KO} cells reconstituted with the deletion mutants shown in (A), as indicated. Data is shown as means from two independent cultures. On the right, bar graphs plotting the cell index during the last 20 hours for each mutant as indicated, using means from duplicates as data for the analyses. ***(C)*** *As in (B), but using B16 cells expressing a PDL1 mutant with DSK residues mutated to alanines. Relevant statistical comparisons are indicated within the graph. ***, highly significant differences (P<0.001).*

STAT3-caspase 7 is the main effector pathway conferring IFNβ lethality to PDL1 silencing

Type I IFNs exert their activities by engaging with its receptor IFNAR1/IFNAR2 on the cell surface. The main signal cascade depends on the recruitment of JAK1 and TYK2 that phosphorylates STAT1, STAT2 and STAT3, which associate into STAT1/STAT2 heterodimers or STAT1/STAT1 and STAT3/STAT3 homodimers. In addition, type I IFNs cause caspase-dependent apoptosis although the exact mechanisms are yet unclear. To identify the downstream effectors leading to exacerbated toxicity by lack of PDL1, B16 cell lines were generated with a selection of key components of the IFN signal transduction pathway silenced **(Figure 3A).**

These cell lines were tested in the IFNβ survival assay as described above **(Figure 1).** As expected IFNAR1 and JAK1 silencing abrogated IFNβ toxicity. Interestingly, silencing of *STAT3* and caspase 7 (*CASP7*) also reduced IFNβ inhibitory effects **(Figure 3B)**. Moreover, lethality of the IFNβ-μPDL1 combination was only averted by silencing *IFNAR1*, *JAK1*, *STAT3* and *CASP7* **(Figure 3C)**. To find out whether the absence of PDL1 signals enhanced IFNβ signal transduction, the expression of STATs was assessed in B16 and CRISPR/Cas9 PDL1 knockout B16 cells after IFNβ treatment for 24 h **(Figure 3D)**. STAT1 and STAT2 were upregulated to the same extent in B16 and PDL1^{KO} cells. In contrast, STAT3 levels increased only in PDL1^{KO} cells in response to IFNβ. Then, we performed a time-course assay of STAT3 phosphorylation after IFNβ stimulation. Interestingly, STAT3 Y705 phosphorylation was stronger and occurred faster in PDL1^{KO} cells, while STAT3 S727 phosphorylation remained unchanged in B16 and in B16-

PDL1^{KO} cells **(Figure 3E)**. These results indicated that PDL1 signals were inhibiting STAT3 Y705 phosphorylation and prevented STAT3 up-regulation.

Survival assays with caspase-silenced B16 cell lines suggested that interference with PDL1 expression was causing cell death in response to IFNβ through CASP7. Our results also indicated that cell death was largely caused by apoptosis rather than caspase-independent necroptosis, although the participation of this last mechanism cannot be completely ruled out. To confirm these results RTCA was performed with caspase-silenced B16 cells or caspase-silenced B16-PDL1^{KO} cell lines in response to recombinant IFNβ **(Figure 3F)**. While *CASP3* silencing did not abrogate toxicity to recombinant IFNβ, *CASP7* silencing inhibited IFNβ inhibitory actions. The same results were also observed in B16-PDL1^{KO} cells although in this assay *CASP9* silencing counteracted toxicity as well. Then, the expression and processing of effector caspases in B16 cells after treatment with recombinant IFNβ was compared to B16-PDL1^{KO} cells. Overall, basal expression of CASP3, 7 and 9 were increased in B16-PDL1^{KO} cells, especially after IFNβ treatment **(Figure 3G)**. In agreement with the shRNA data, the processing of CASP7 was strongly enhanced in B16-PDL1^{KO} following IFNβ treatment **(Figure 3F).** Overall, these data suggested that the effector pathway for IFNβ cytotoxicity caused by PDL1 silencing was mainly mediated by a reinforced STAT3-caspase 7 pathway.

Antibody-mediated PDL1 blockade abrogates the protective functions of PDL1 in murine and human cancer cell lines

To test whether direct blockade of PDL1 could sensitize B16 and other murine and human cancer cells to IFNβ, the growth and viability of murine B16, CT26 colorectal and 4T1 breast cancer cells were monitored by RTCA in the presence of increasing concentrations of a PDL1-blocking antibody or an isotype control **(Figures 4A, 4B, 4C).** PDL1 antibody blockade sensitized all three murine cancer cell types to recombinant IFNβ. The same results were obtained with human *B-RAF* mutated melanoma HTB72 cells. Taken together these results confirmed that the anti-IFNβ mechanism regulated by PDL1 is conserved in murine and human cancer cells.

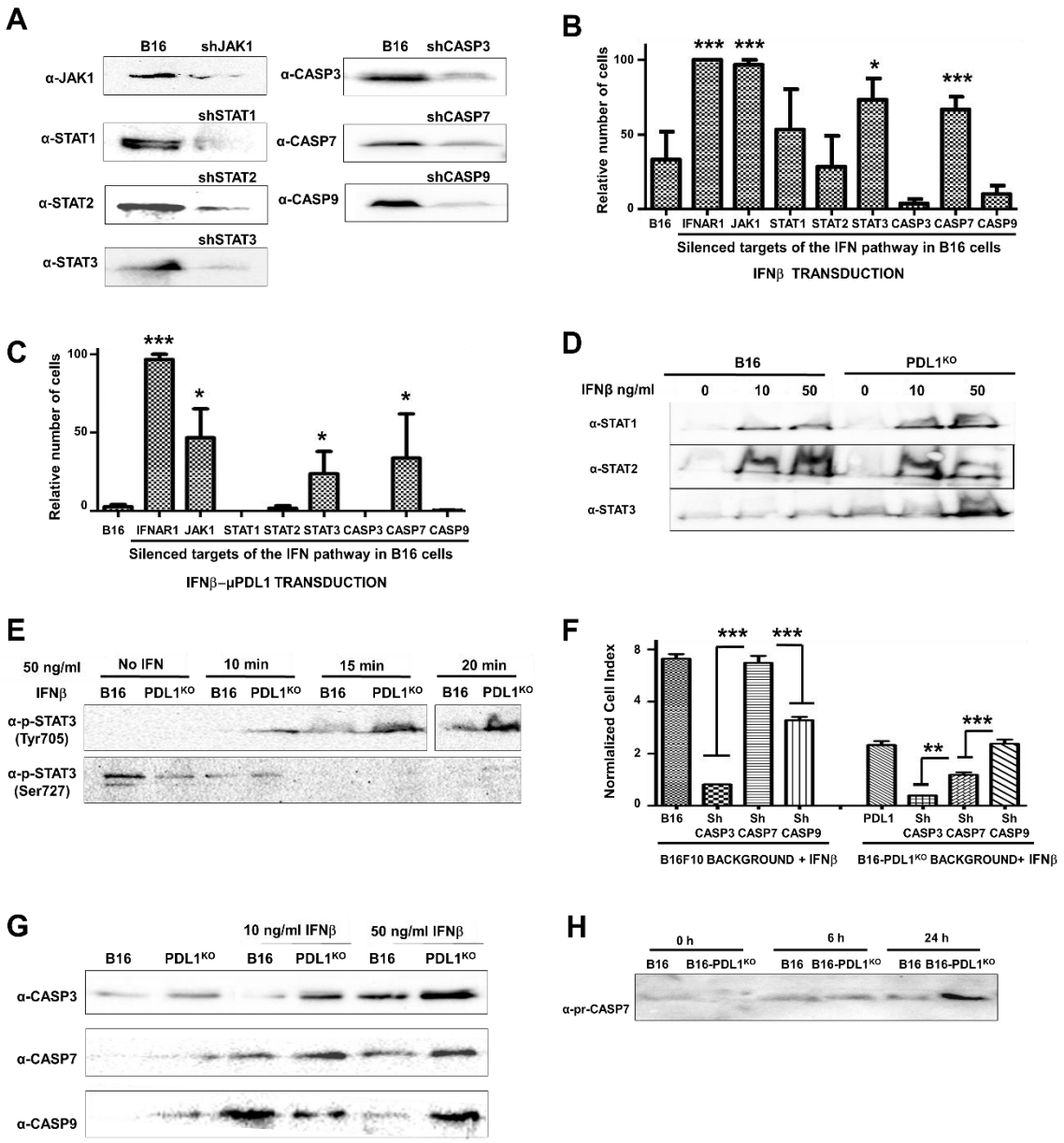


Figure 3. IFNß enhanced cytotoxicity by PDL1 silencing depends on a STAT3-caspase 7 pathway. (A) *Western blots of the specific signal transduction molecules as shown on the left in B16 cell lines constitutively expressing shRNAs indicated on top.* ***(B)*** *Bar graphs representing the mean relative number of cells with error bars (standard error of the means) after two weeks of puromycin selection following transduction of the indicated B16 cell lines (with the indicated silenced genes as shown) with lentivectors co-expressing IFNß-PuroR.* ***(C)*** *Same as in (B) but transductions were performed with lentivectors co-expressing IFNß-μPDL1-PuroR.* ***(D)*** *Detection by western blotting of the indicated STAT molecules*

in B16 or B16-PDL1^{KO} cells untreated or treated with IFNβ as shown on top. ***(E)*** *Western blot of STAT3 Y705 phosphorylation (top) at the indicated time points in B16 and B16-PDL1^{KO} cells after IFNβ stimulation, as indicated. Western blot (bottom) of STAT3 S727 phosphorylation in B16 or B16-PDL1^{KO} cells after IFNβ stimulation, as indicated.* ***(F)*** *Bar graphs representing RTCA cell index of the indicated B16 cell lines with silenced caspases either in an unmodified or PDL1^{KO} background as shown in the graphs in the presence of 10 ng/ml of recombinant IFNβ.* ***(G)*** *As in (D) but detection of the indicated caspases.* ***(H)*** *Western blot of processed caspase 7 on a time course of B16 or B16-PDL1^{KO} cells treated with 10 ng/ml of recombinant IFNβ as shown on top. Relevant statistical comparisons are indicated within the graphs. *, **, ***, indicate significant (P<0.05), very (P<0.01) and highly significant differences (P<0.001), respectively.*

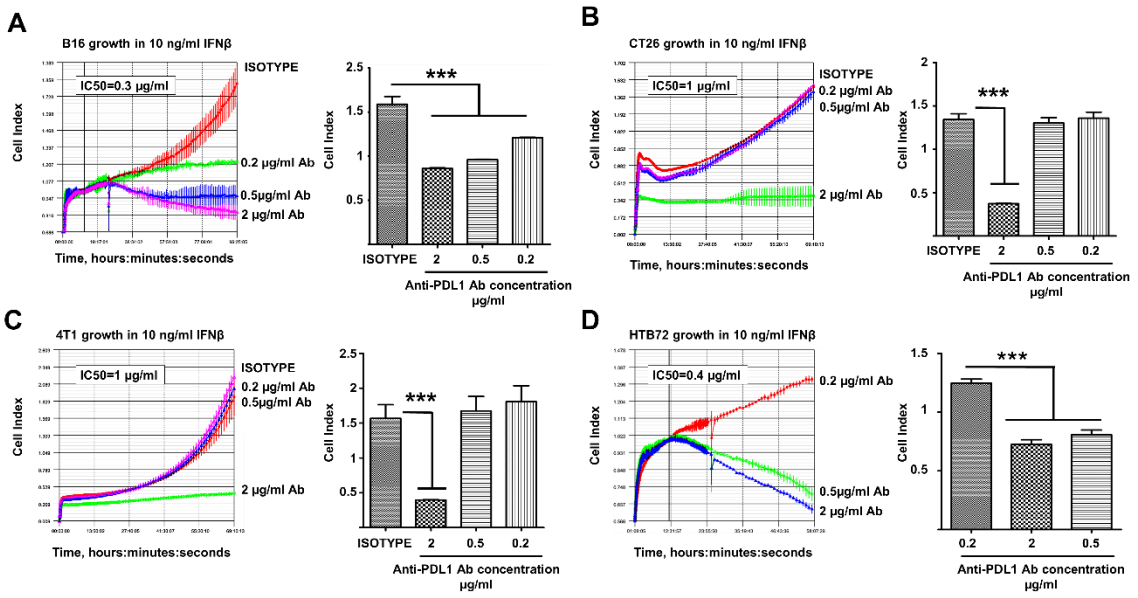
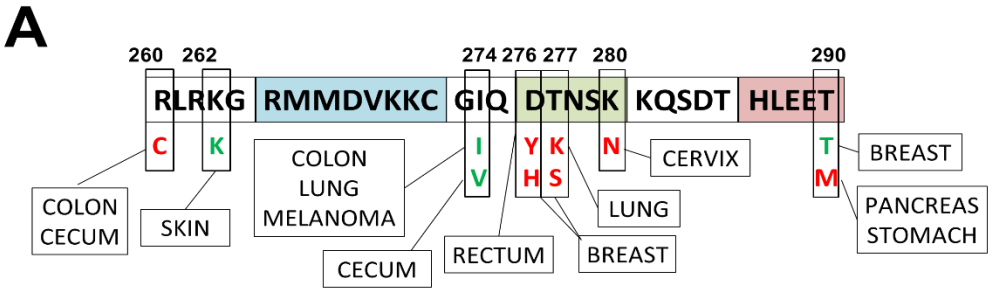


Figure 4. PDL1 blockade sensitizes murine and human cancer cells to IFNβ. (A) *Left, RTCA graph of murine B16 melanoma cells in the presence of recombinant IFNβ and the indicated concentrations of anti-PDL1 antibody. Right, same data as a bar graph with means and standard deviations with error bars (n=4).* ***(B)*** *Same as (A) with murine CT26 colorectal cancer cells.* ***(C)*** *Same as (A) with murine 4T1 breast cancer cells.* ***(D)*** *Same as (A) with human HTB72 melanoma cells. Calculated IC50s are shown within the graphs. ***, indicates highly significant differences (P<0.001).*

Somatic mutations in human cancers targeting the DTSSK domain strongly potentiate global anti-IFN activities of PDL1

Our data strongly suggested that cancer cells rely on PDL1 signal transduction to counteract IFN toxicity. As this could be relevant for human immunotherapy, we studied the somatic mutations within the intracytoplasmic domain of PDL1 in human neoplastic malignancies. The COSMIC, ICGC, Intogen and TCGA catalogues of somatic mutations in cancer were consulted and several mutations leading to aminoacid changes were identified in carcinomas including skin and lung cancers. Interestingly, the majority of these (5 out of 7) directly affected the human homologue of the DTSSK motif **(Figure 5A)**. To test the effects of these mutations over PDL1 protective functions, the two most disruptive mutations (D276H and K280N) were introduced into the equivalent murine PDL1 gene and B16-PDL1^{KO} cells were transduced to express each mutant.

Then, RTCA was used to monitor the growth/survival of the B16 cell lines in the presence of recombinant IFNβ **(Figure 5B),** IFNα **(Figure 5C)** and IFNγ **(Figure 5D)**. Consistent with our previous results, these mutations within the DTSSK motif strongly enhanced resistance to cytotoxicity mediated by type I and type II IFNs, while B16-PDL1^{KO} cells were highly sensitive to IFNs α, β and γ. These results confirmed the regulatory role of the DTSSK motif by selection of variants in human carcinomas that disrupt its inhibitory activity leading to hyperactive PDL1 proteins. Moreover, these mutations extended wide protection also from IFNs α and γ. Finally, the inhibitory activity of DTSSK was not exclusively dependent on lysine 280 as the D276H mutation also enhanced protective activity of PDL1.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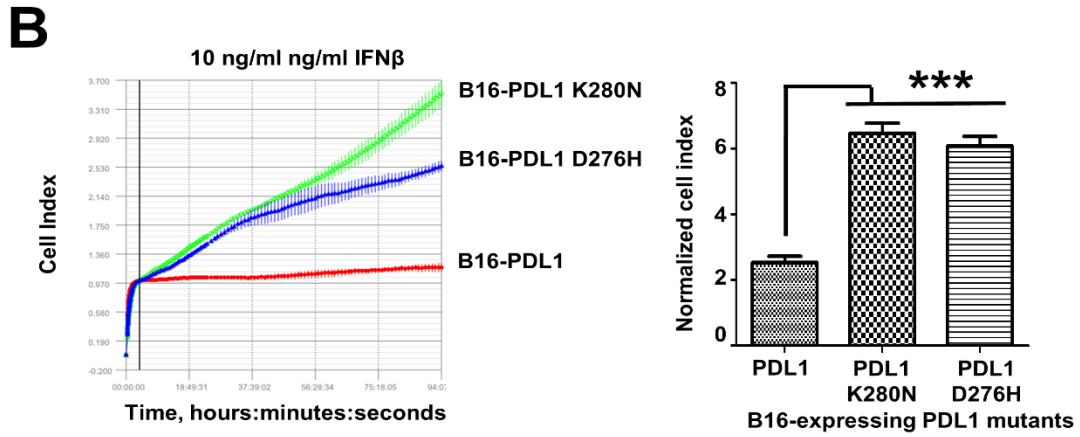
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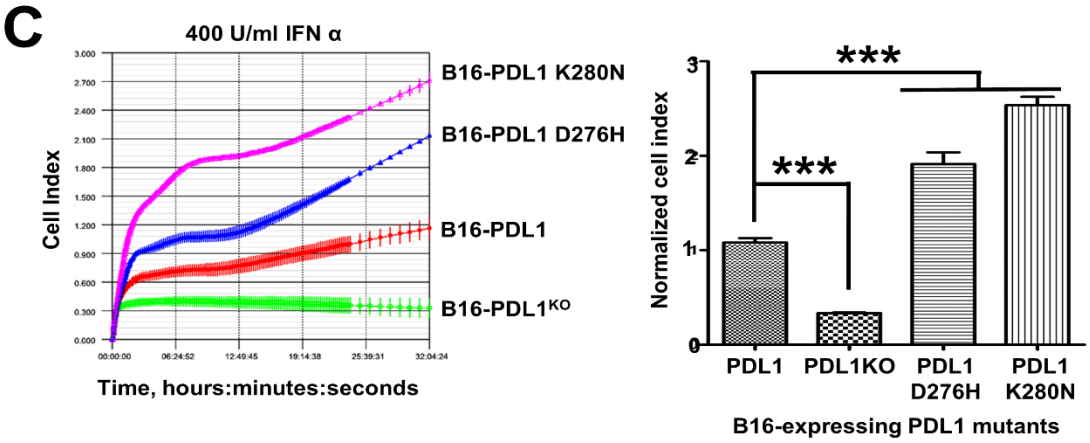
B16-expressing PDL1 mutants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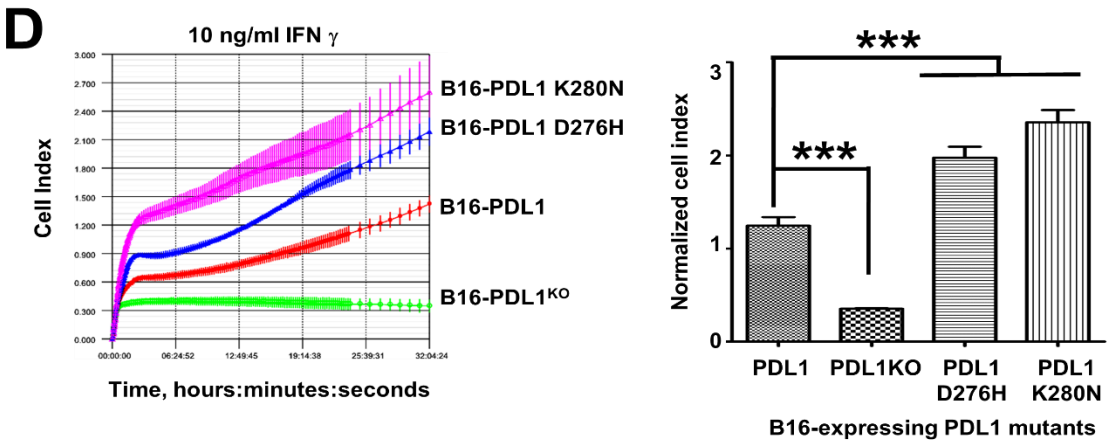
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PDL1 K280N
B16-expressing PDL1 mutants

Figure 5. Somatic mutations in the human DTSSK homologue motif leads to hyperactive PDL1 molecules that protect cells from type I and type II IFNs. (A) *Schematics on the distribution of somatic mutations found within the intracytoplasmic domain of human PDL1. In blue, green and red are shown the homologous human RMLDVEKC, DTSSK and QFEET motifs. Mutations are shown below, with conservative changes in green and non-conservative changes in red. The specific carcinomas for which mutations were described are indicated within boxes. Numbers represent aminoacid positions in the murine and human PDL1 molecule.* ***(B)*** *The two most disruptive mutations were introduced in the DTSSK murine motif and the resulting PDL1 molecules expressed in B16-PDL1^{KO} cells. On the left, RTCA plot of the indicated B16 cell lines expressing the PDL1 mutants compared to PDL1wt in the presence of 10 ng/ml of recombinant IFNβ. On the right, the same data as bar graphs representing the mean of the normalized cell index from duplicate cultures together with standard deviations as error bars.* ***(C)*** *As in (B) but with IFNα. PDL1KO indicates B16 cells in which PDL1 was disrupted with CRISPR/Cas9.* ***(D)*** *As in (B) but with IFNγ. Relevant statistical comparisons are indicated within the graph. ***, indicates very highly significant differences (P<0.001).*

PDL1 signal transduction in cancer cells is required for *in vivo* protection against IFNβ

Our data collectively suggested that PDL1 intrinsic signaling within cancer cells would confer resistance to IFNs *in vivo* independently of its inhibitory role over T cells. To prove this, we studied *in vivo* tumor growth from B16 cells in which PDL1 expression or signaling were altered, followed by intra-tumor administration of IFNβ expressed by a lentivector or a GFP-expressing control. First, groups of mice were subcutaneously inoculated with B16, B16-PDL1 and B16 cells harboring a published PDL1-targeted shRNA which reduced the basal expression of PDL1 (Broos et al., 2017) **(Figure 6A)**. Then, lentivectors expressing either GFP (control) or IFNβ-GFP were injected into tumors 7 and 14 days later. Intra-tumor expression of IFNβ-GFP delayed B16 melanoma tumor growth **(Figure 6A)**. PDL1 overexpression completely abrogated this sensitivity to IFNβ-

GFP. In contrast, tumors arising from B16-shPDL1 were highly sensitive to LV-IFNβ-GFP, resulting in a significant delay in tumor growth and increased survival **(Figure 6A)**. We repeated the experiment using B16-PDL1^{KO}cells. As expected, tumors arising from B16-PDL1^{KO} cells were highly responsive to intra-tumor lentivector delivery of IFNβ-GFP, leading to a highly significant increase in survival **(Figure 6B)**. In fact, the lack of PDL1 was sufficient to delay tumor progression even in the absence of intratumor expression of IFNβ **(Figure 6B)**.

These results showed a higher sensitivity in these B16 tumors to IFNβ when PDL1 expression was interfered with. However, inhibition of PDL1 expression in cancer cells could still enhance the cytotoxicity of T cells through reduced PDL1-PD1 engagement. Therefore, we carried out the same experiments but including a group in which B16-PDL1ΔC cells were inoculated. PDL1 in these cells can still engage PD1 on the surface of T cells, but with impaired signal transduction within cancer cells **(Figure 1)**. Then, tumors from B16, B16-PDL1 and B16-PDL1ΔC were inoculated with lentivectors expressing IFNβ-GFP. As expected, PDL1 overexpression in B16 cells very significantly counteracted the IFNβ inhibitory effects and accelerated tumor progression **(Figure 6C).** In contrast, expression of PDL1ΔC did not confer resistance to intra-tumor delivery of IFNβ. As this mutant can still engage PD1 on T cells, these results indicate that PDL1 signal transduction contributes significantly to protection of cancer cells from type I IFNs. Overall, these results showed that independently from its role in inhibiting cytotoxic T cells, PDL1 provides a first protective barrier to cancer cells by interfering with IFN signal transduction.

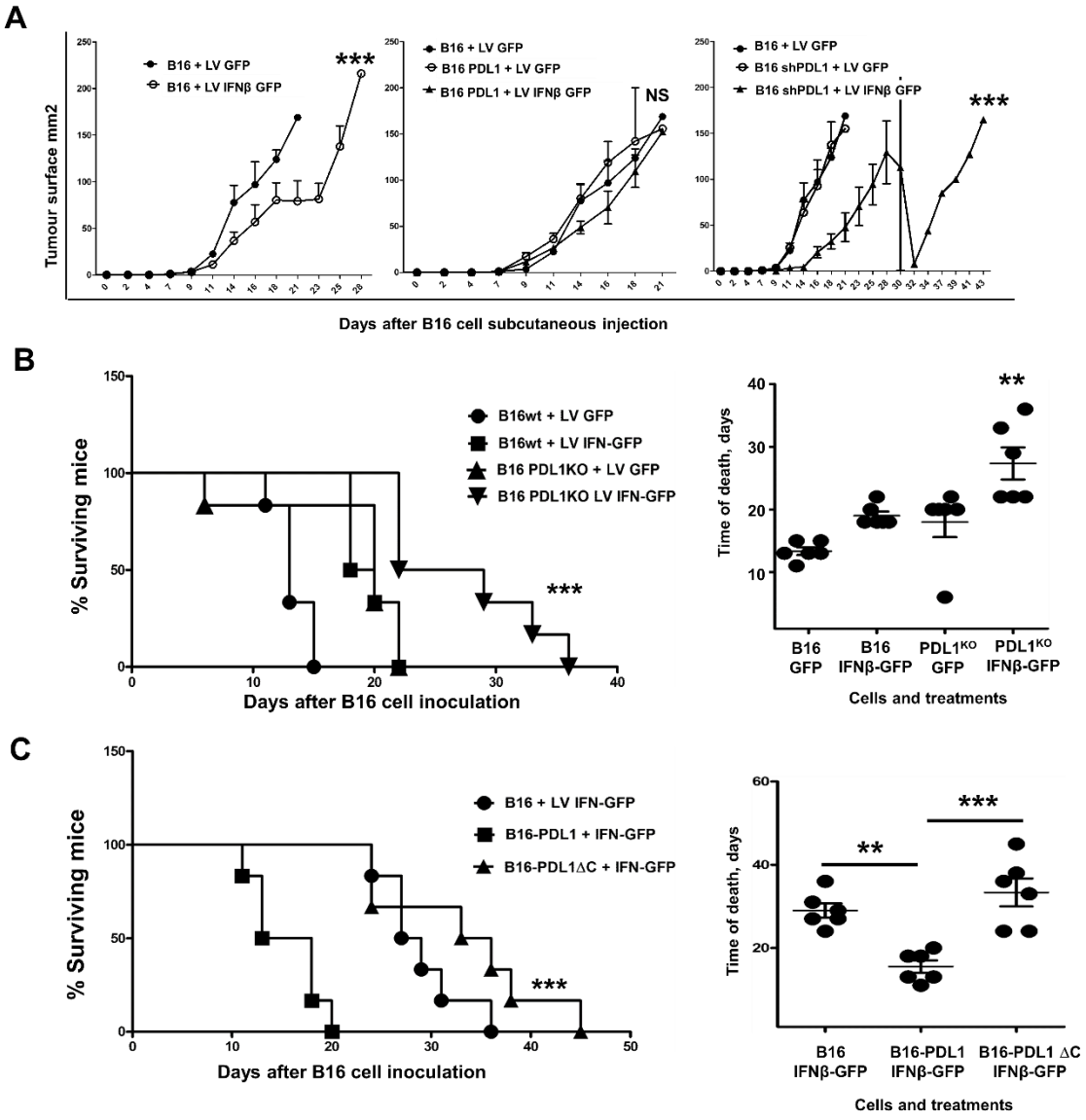

Figure 6. PDL1 intrinsic signaling in cancer cell protects tumors from IFNβ in vivo. (A) *On the left, tumor growth graphs from injected B16 cells followed by intratumor injection with the indicated lentivectors, represented as means of tumor surfaces from groups of 6 mice with standard deviations as error bars. The middle and right graphs as in the left graph but using the indicated B16 cell lines overexpressing PDL1 (B16-PDL1) or B16 cell lines expressing a PDL1-targeted shRNA (B16-shPDL1).* ***(B)*** *The growth graph as in (A) but using B16 wild-type or B16 PDL1 knock-out cells. The graph on the right represents the time of death of each mice from the data shown on the left graph. The cell lines and the injected lentivectors are shown on the bottom of the graph.* ***(C)*** *Same as in (B) but using B16 wild-type, B16-PDL1 or B16-PDL1ΔC cells which express a PDL1 mutant*

*lacking the intracytoplasmic domain. **, *** indicate very (P<0.05) and highly (P<0.001) significant differences, respectively.*

DISCUSSION

IFNs are known to exert anti-tumor effects which include caspase-dependent apoptosis (Apelbaum et al., 2013), cell growth arrest (Vannucchi et al., 2000) and cell senescence (Katlinskaya et al., 2016; Yu et al., 2015). IFNs play a critical role in anti-cancer immune responses, and contribute to the efficacy of conventional treatments and immunotherapies. There is ample evidence on the role that IFNs play in tumor repression, immune editing and progression (Zitvogel et al., 2015). Many treatments including chemotherapy and targeted therapies need an intact IFN signal transduction pathway in cancer cells to exert their anti-tumor effects. However, type I IFNs in progressing tumors also drive immune editing (Smyth, 2005). In fact, acquisition of inactivating mutations affecting IFN signaling could be considered a core mechanism for tumor escape and progression. Recently, it has been shown that inactivating *JAK1*, *JAK2* and β2-microglobulin mutations in cancer cells are responsible for primary and acquired resistance to anti-PD1 treatment in a cohort of patients (Shin et al., 2017; Zaretsky et al., 2016). The authors of these studies proposed that switching off IFN signal transduction prevented the adaptive PDL1 expression in cancer cells, becoming functionally PDL1-negative and refractory to PDL1/PD1 blockade. However, PDL1 itself can transmit signals without engaging PD1 or CD80 through mTOR (Clark et al., 2016; Chang et al., 2015). Here we demonstrate that PDL1 signal transduction in murine and human cancer cells does in fact represent a barrier of protection against IFN cytotoxicity by inhibitory crosstalk with the type I IFN signal transduction pathway. It is important to emphasize that basal PDL1 expression reduces IFN cytotoxicity but does not completely abrogate it. Therefore, cancer cells respond to IFNs by up-regulating the surface expression of PDL1 possibly as a negative feedback mechanism to regulate IFN signaling. It has to be taken into account that IFNs are potent transcriptional activators of PDL1 (Zaretsky et al., 2016).

PDL1 possesses a short intracytoplasmic region without any obvious known domains regulating signal transduction. However, it has been shown by others and us that there is PDL1 intrinsic signaling regulating cell growth, survival and protection against apoptotic signals (Azuma et al., 2008; Clark et al., 2016; Chang et al., 2015; Liechtenstein et al., 2014a). We have identified functional regulatory sequence motifs within the intracytoplasmic domain responsible for PDL1 protection against IFN. The conserved RMLDVEKC motif is required to counteract IFNβ toxicity, while the DTSSK motif and arginines 271 and 280 act as negative regulators of PDL1 functions. These sequences constitute non-classical signal transduction motifs as they do not resemble any known signal transduction consensus. Only the sequence EKCGVEDTSSKNR shows high similarity to a domain in DNA-directed RNA polymerase subunit β which interestingly includes the DTSSK motif **(supplemental figure 1)**. However, the relevance of this observation is unclear.

Our findings also show that PD1 expression protects cancer cells from IFNβ toxicity by engaging PDL1. Hence, both PDL1 and PD1 blockade would sensitize cancer cells to IFN-mediated cytotoxicity. Furthermore, we found that antibody-mediated PDL1 blockade is sufficient to sensitize cancer cells to IFNs. Therefore, any adaptation of cancer cells to either inhibit the IFN signaling pathway (Shin et al., 2017; Zaretsky et al., 2016) or potentiate PDL1 activities will favor their escape from the immune attack. Accordingly, a variety of human carcinomas select somatic mutations which affect residues within the inhibitory DTSSK motif, thereby increasing the anti-IFN activities of PDL1. These cancer cells with hyperactive PDL1 mutants are very likely selected in human malignancies as a result of immune editing.

Summarizing, our data demonstrates that PDL1 signal transduction through conserved signaling motifs represents a protective barrier of cancer cells against IFN cytotoxicity, which would be reinforced by its inhibitory properties to T cells when engaged with PD1. A therapeutic approach such as PDL1/PD1 blockade would cause rapid cancer cell death due to further sensitization to IFN. This situation strongly favors the survival of variants resistant to IFN signal

transduction. Therefore, cancer cells with mutated JAK1 or JAK2 kinases are intrinsically resistant to IFN toxicity and do not show adaptive PDL1 upregulation (Shin et al., 2017; Zaretsky et al., 2016).

EXPERIMENTAL PROCEDURES

Cells and mice

Human embryonic kidney (HEK) 293T cells were purchased from the American Type Cell Culture Collection (ATCC). Murine melanoma B16F10 cells were grown as described (Liechtenstein et al., 2014a). Murine CT26 colorectal and 4T1 breast cancer cells were grown in DMEM. Human HTB72 cells were grown in RPMI. C57BL/6 female mice were purchased from The Jackson Laboratories. Approval for animal studies was obtained from the Animal Ethics Committee of the University of Navarra (Pamplona, Navarra, Spain), and from the Government of Navarra. When indicated, recombinant IFNβ was added to the cell cultures at the appropriate concentrations. Cell growth/survival was monitored in real time using xCELLigence real-time cell analysis system (RTCA, ACEA Biosciences) by seeding between 3000 to 10000 cells as required, in the presence or absence of recombinant IFNβ. Inhibitory concentration 50 (IC50) was calculated by RTCA for B16 cell lines with increasing concentrations of recombinant IFNβ. Experiments of antibody-mediated PDL1 blockade were carried out with anti-PD-L1 MAb (clone 10B5) (Dong et al., 2002). For human PDL1 blockade, the in-house phage-display engineered humanized IgG1 recombinant antibody Plimilumab was used.

Plasmids

The FB2 fusokine transgene is described in (Van der Jeught et al., 2014) and consists on a fusion gene between the murine IFN-β and the ectodomain of the TGFβ receptor II. The FB2 transgene was cloned into the pDUAL-p1-PuroR vector by standard cloning techniques. This vector contains a PDL1-targeted microRNA and it as described in (Liechtenstein et al., 2014a). The IFN-β coding sequence was amplified by PCR and cloned into pDUAL-μPDL1-PuroR and pDUAL-GFP (Liechtenstein et al., 2014a). Likewise, the TGFβRII was amplified by PCR

introducing the IFN-β signal peptide for secretion at the 5' end, and cloned into pDUAL-p1-PuroR. When required, the same transgenes were expressed without the PDL1-targeted microRNA. The pHIV-SIREN lentivectors (Lanna et al., 2014; Liechtenstein et al., 2014b) were used to express short-hairpin RNAs targeting the indicated genes (supplemental Table 1) together with blasticidin resistance. The PDL1 transgene was ordered from Geneart and includes 7 silent mutations (c**c**aa**a**ga**tctt**ta**t**g, mutations in bold and underlined) and 6 silent mutations (**a**gaaa**c**ga**c**ac**g**ca**g**tt**t**) at the amino and carboxy termini to prevent its silencing by either μPDL1 or PDL1-targeted CRISP/CAS9. PDL1ΔC encodes a carboxy-terminal deleted PDL1 gene and was generated by PCR using oligos FW (ggatccgccaccatgaggatatttgctggc) and RS (cggccgcttattgttttctcaagaagaggaggaccg). PDL1-deletion and single-point substitution mutants were generated by overlap-extension PCR as described (Escors et al., 2001) using the PDL1 gene as a template and the indicated oligonucleotides (supplemental Table 2). The murine PD1 transgene was ordered from Genart and cloned into pDUAL-BlastR which expresses blasticidin resistance under the control of the ubiquitin promoter. A carboxy terminus-deleted version was also generated by PCR using the FW (gggggggatccgccaccatgtgggtccggcaggtacc) and RS (gcggccgcttatgagcagaagacagctagggcccaggc) oligos, followed by cloning into pDUAL-BlastR.

The mouse CD274 (PDL1) sgRNA CRISPR/Cas9 'All-in-One' lentiviral transfer vector was used to knock-out PDL1 as described (Broos et al., 2017).

Lentivector production, cell transduction and generation of B16 knock-down stable cell lines

Lentivector production and titration were carried out as described (Karwacz et al., 2011; Liechtenstein et al. 2014a; Selden et al., 2007). Transduction of the indicated cell lines was carried out with a multiplicity of transduction of 10, and transduced cells were selected with the appropriate concentration of either puromycin (GIBCO) or blasticidin (GIBCO). Transduced cells are then analyzed for the expression of the target of interest either by flow cytometry or western blot.

Western blotting

Western blots were performed as described (Escors et al., 2008). Polyclonal anti-caspase 3, 7, 9 and anti-processed caspase 3, 7 and 9, and anti-phosphorylated STAT3 molecules were purchased from Cell Signalling. Mouse anti-JAK1, STAT1, STAT2 and STAT3 antibodies were purchased from Cell Signaling, and anti-GADPH from Calbiochem. Peroxidase-conjugated polyclonal anti-mouse and anti-rabbit antibodies were purchased from DAKO and Cell Signaling.

Cell staining and flow cytometry

Surface and intracellular staining were performed as described previously (Escors et al., 2008) using the indicated antibodies. PE-Cy7-conjugated streptavidin, APC-conjugated streptatividin, PE-conjugated anti-IFNARI were purchased from Biolegend. PE-and FITC-conjugated streptavidin from Invitrogen. Biotin-conjugated anti-PDL1 was purchased from eBioscience. APC-conjugated anti-PD1 from Miltenyi Biotec.

B16-IFNβ cell survival assays

The goal of this assay is to quantify viable growing B16 cell lines that constitutively express IFNβ following lentivector transduction with IFNβ-PuroR or IFNβ-µPDL1-PuroR followed by selection with puromycin. For this assay 100000 of the indicated transduced or non-transduced cells were plated in 6-well culture plates in triplicate. Cells were then transduced at a multiplicity of transduction of 10. One well was left as a non-transduced control. The next day, puromycin was added at 1 µg/ml and surviving cells allowed to grow for two weeks. Surviving cells were quantified and represented as a percentage compared to the growth of non-transduced, non-treated cells.

IFN treatment of PDL1 mutants and real-time living cell monitoring (RTCA)

The appropriate cell types were seeded at a density of 5000 cells per well on two L8 cell culture chambers for xCELLingence RTCA monitoring system (ACEA biosciences). Cells were grown in DMEM or RPMI medium with recombinant murine or human IFNβ (10ng/ml, eBioscience) as required. Murine IFNα (400 units/ml) and IFNγ (10 ng/ml) were purchased from (Peprotech). Growth and survival of cell lines were monitored by RTCA for a minimum of 3 days.

Vaccination and tumor experiments

Experiments were usually performed with six C57BL/6 mice per group. Mice were subcutaneously inoculated with 10^6 of the indicated B16 cell lines. Tumor size was monitored every 2 days. When required, tumors were injected with 10^6 lentivector transducing particles expressing IFNβ-GFP or with GFP only as a control. Mice were sacrificed when tumor surface was above 150 mm^2.

In silico sequence analyses

PDL1 protein sequences from mouse, human, pig, cow, buffalo, cat, dog and salmon were aligned using the multialign tool (http://multalin.toulouse.inra.fr/multalin/) (Corpet, 1988). Prediction of ubiquitination sites was performed with UbPred tool (http://www.ubpred.org/) (Radivojac et al., 2010). The search for conserved protein domains was performed with MotifFinder (http://www.genome.jp/tools-bin/search_motif_lib). The data on somatic mutations in PDL1 from human cancers was obtained from the COSMIC (http://cancer.sanger.ac.uk/cosmic) and TCGA (https://cancergenome.nih.gov/) databases. The following mutations were found: R260C (colon and cecum carcinoma; COSU3769). , R262K (Daud et al.,2016) (basal cell carcinoma, COSP39263), I274I (lung, cecum carcinoma and melanoma,COSP29675,COSU540,COSU17), I274V (colon carcinoma, COSU144), D276H (breast invasive carcinoma, TCGA-AR-A0TX-01; COSU414), D276Y (rectum carcinoma), T277K (George et al., 2015) (small cell lung cancer; COSP40339), T277S (lung carcinoma,COSP40399) , K280N (cervical squamous cell carcinoma;

TCGA-FU-A3HZ-01; COSU415), T290M (pancreas and stomach carcinomas, TCGA-BR-4362-01; COSU541) and T290T (breast cancer, COSU541).

Statistical analyses

GraphPadPrism was used for plotting data and statistical analyses. No data was considered an outlier. Data from B16-IFNβ survival assay was confirmed to be normally distributed, therefore analyzed by two-way ANOVAs with a random criterium (inter-experiment variability). Two pair comparisons were carried out following the ANOVA analyses using either Bonferroni or Tukey´s tests. Tumor growth and survival data were analyzed as described before (Karwacz et al., 2011). ACEA RTCA cell index data was analyzed for each sample in duplicates and plotted as means and standard deviations. The data was highly homogeneous and the data normally distributed. For statistical analyses, the cell index data collected at 75, 80, 85, 90 hours of cell growth. The data was analyzed by two-way ANOVA with time as a random criterium followed by Tukey´s pair-wise comparisons.

AUTHOR CONTRIBUTIONS

MGC designed and executed most of the experiments and analyzed the data. MZ, HA, MIV, GFH performed the experiments and analyzed the data. LL generated B16 cells lines overexpressing PD1. CS, EM, DL, RM, PS, IA and CW contributed to PDL1-antibody blockade experiments. RV contributed to the analysis of clinical data and supervision of HA and GFH. KB generated the interferon constructs. GK and DE supervised the project, planned and executed experiments, and analyzed the data. All authors contributed to the writing of the paper.

ACCESSION NUMBERS

Original flow cytometry data was deposited in Flowrepository.org with the following accession numbers: FR-FCM-ZY8H and FR-FCM-ZY8W.

ACKNOWLEDGMENTS

This project was funded by a crowdfunding project by FECYT (PRECIPITA, Spain), a FIS grant (PI14/00579) from the Instituto de Salud Carlos III, a grant from the Government of Navarre (BMED 033-2014, Spain) and a grant from Fundación Caixa (Spain) to GK. We thank SARAY foundation (Navarra, Spain) and Sandra Ibarra´s foundation for their financial support. MG is funded by a PhD fellowship granted by the Government of Navarre. MZ is funded by a fellowship granted by Universidad Publica de Navarra (UPNA), Spain. MIV is funded by a Sara Borrel Fellowship. IA is funded by a Miguel Servet Fellowship. KB received funding from Kom Op Tegen Kanker (Belgium). We specially thank Luciano Escors, Lucia Murugarren and family for their donations and overall support, as well as all the donors that contributed to the “PRECIPITA” project. DE is funded by a Miguel Servet Fellowship (CP12/03114) awarded by the Instituto de Salud Carlos III (Spain).

SUPPLEMENTAL FIGURES

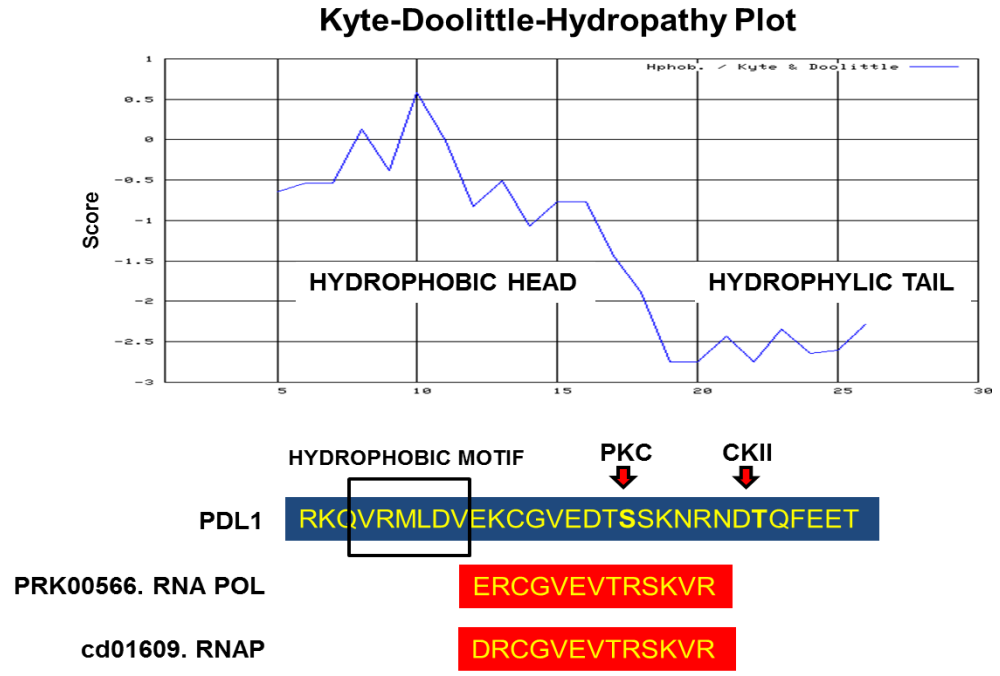

Figure S1, related to Figure 2A. Bioinformatic analysis of the PDL1 intracytoplasmic domain. *On top, Kyte-Doolittle hydrophathy plot of the intracytoplasmic domain of PDL1, with a hydrophobic head containing the VRMLDV hydrophobic motif and the hydrophilic tail as shown below the hydrophathy plot. Serine and threonine phosphorylatable residues by PKC and CKII were predicted and indicated with an arrow. Motifinder produced two similarity hits with DNA-dependent RNA polymerase beta subunits as shown within red boxes.*

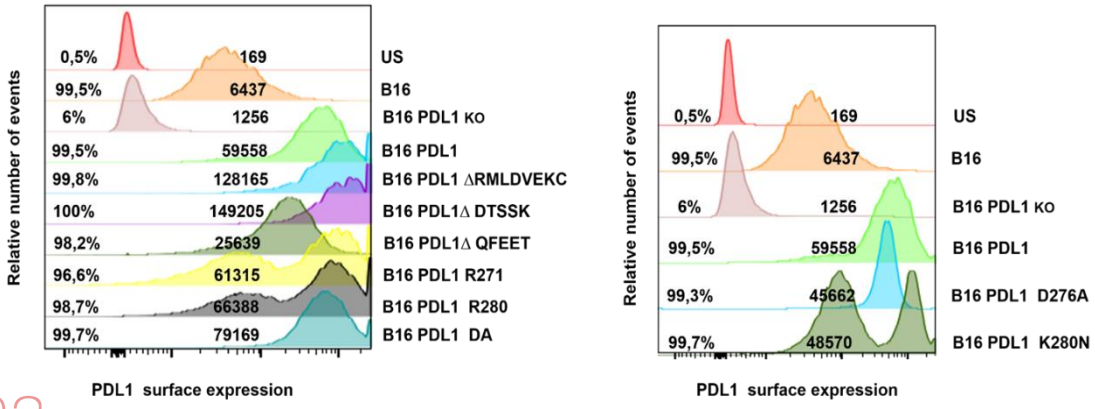

Figure S2, related to Figures 2, 5 and 6. Surface expression of PDL1 molecules in B16-PDL1^{KO} cells. *The histograms show flow cytometry data on the surface expression of the indicated B16 cell lines expressing PDL1 mutants (on the right of the graphs). Percentages and numbers represent percentages of PDL1 positive cells relative to the background fluorescence from an unstained control (US), and mean fluorescent intensities*

SUPPLEMENTAL TABLES

Supplemental Table 1, related to Figures 1, 3 and 6. shRNA sequences cloned into pHIV-SIREN vectors.

Targeted gene	shRNA sequences
IFNAR1	GAATGAGGTTGATCCGTTTATTTCAAGAGAATAAACGGATCAACCTCATTCTTTTTT
JAK1	GCCCTGAGTTACTTGGAAGATTTCAAGAGAATCTTCCAAGTAACTCAGGGCTTTTTT
STAT1	GCTGTTACTTTCCCAGATATTTTCAAGAGAAATATCTGGGAAAGTAACAGCTTTTTT
STAT2	AGTCACATGCTTCGGTATAAGTTCAAGAGACTTATACCGAAGCATGTGACTTTTTTT
STAT3	GCCTGAGTTGAATTATCAGCTTTTCAAGAGAAAGCTGATAATTCAACTCAGGTTTTTT
CASP9	GCCACTGCCTCATCATCAACAATTCAAGAGATTGTTGATGATGAGGCAGTGGTTTTTT
CASP7	GACCTGATTTACGGGAAAGATTTCAAGAGAATCTTTCCCGTAAATCAGGTCTTTTTT
CASP3	GCACATTCTCACTCGCGTTAATTCAAGAGATTAACGCGAGTGAGAATGTGCTTTTTT
PDL1	GCCGAAATGATACACAATTCTTCAAGAGAGAATTGTGTATCATTTCGGTTTTTT

Supplemental Table 2, related to Figures 1, 2, 5 and 6. Oligonucleotides used to generate PDL1 mutants.

PDL1 mutant	Oligo forward	Oligo reverse
PDL1-ΔRMLDVEKC	GGCGTTGAAGATACAAGCTCAAAA AACCG	CTTCAACGCCCACTTGTTTTCTCAAGAAGAGGAGG
PDL1-ΔDTSSK	AACCGAAATGATACACAATTCGAGG AGACG	GTATCATTTCGGTTTTCAACGCCACATTTCTCCAC
PDL1-ΔQFEET	GGATCCGCCACCATGAGGATATTTG CTGGC	GCGGCCGCTTATCGGTTTTTTGAGCTTGTATCTTCAACG
PDL1-R271	TAGATGTGGAGAGATGTGGCGTTG AAG	CTTCAACGCCACATCTCTCCACATCTA
PDL1-R280	GATACAAGCTCAAGAAACAGAAAC GACACG	CGTGTCGTTTCTGTTTCTTGAGCTTGTATC
PDL1-DA	GCGACAAGCGCGGCGAACAGAAAC GACACGCAG	CGCCGCGCTTGTCGCTTCAACGCCACATTTCTCCAC
PDL1-K280N	GAAGATACAAGCTCAAACAACAGA AACGACACGCA	TGCGTGTCGTTTCTGTTGTTTGAGCTTGTATCTTC
PDL1-D276H	GAAATGTGGCGTTGAACACACAAG CTCAAAAAC	GTTTTTTGAGCTTGTGTGTTCAACGCCACATTTC

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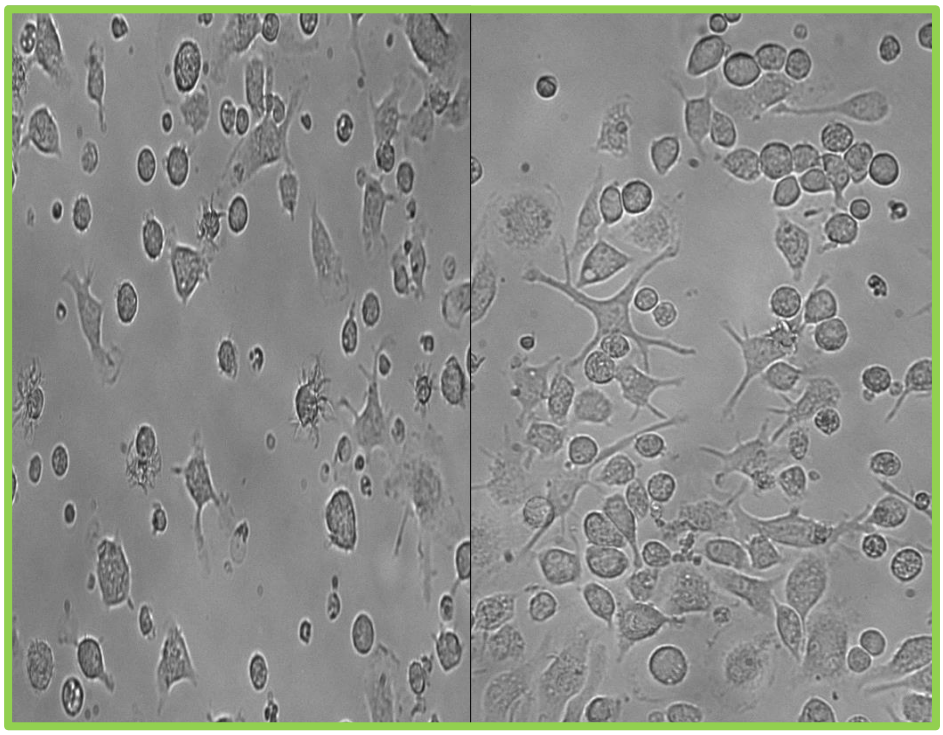
CHAPTER 2

A core of kinase-regulated interactomes defines the neoplastic MDSC lineage



A CORE OF KINASE-REGULATED INTERACTOMES DEFINES THE NEOPLASTIC MDSC LINEAGE

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Keywords: *MDSC, proteomics, interactomes, kinases, therapeutic targets*

Received: *May 25, 2015* ***Accepted:*** *July 13, 2015* ***Published:*** *July 23, 2015*

ABSTRACT

Myeloid-derived suppressor cells (MDSCs) differentiate from bone marrow precursors, expand in cancer-bearing hosts and accelerate tumor progression. MDSCs have become attractive therapeutic targets, as their elimination strongly enhances anti-neoplastic treatments. Here, immature myeloid dendritic cells (DCs), MDSCs modeling tumor-infiltrating subsets or modeling non-cancerous(NC)-MDSCs were compared by in-depth quantitative proteomics. We found that neoplastic MDSCs differentially expressed a core of kinases which controlled lineage-specific (PI3K-AKT and SRC kinases) and cancer-induced (ERK and PKC kinases) protein interaction networks (interactomes). These kinases contributed to some extent to myeloid differentiation. However, only AKT and ERK specifically drove MDSC differentiation from myeloid precursors. Interfering with AKT and ERK with selective small molecule inhibitors or shRNAs selectively hampered MDSC differentiation and viability. Thus, we provide compelling evidence that MDSCs constitute a distinct myeloid lineage distinguished by a "kinase signature" and well-defined interactomes. Our results define new opportunities for the development of anti-cancer treatments targeting these tumor-promoting immune cells.

INTRODUCTION

Anti-cancer treatments are primarily aimed at causing arrest of tumor cell growth or tumor cell death. In recent years, immunotherapy has resurfaced as anattractive therapeutic alternative [1]. However, the expansion of immunosuppressive cell types in cancer patients strongly interferes with anti-tumor immune responses. These immunosuppressive cells enhance tumor progression/metastasis and counteract classical anti-neoplastic treatments. Amongst these, myeloid-derived suppressor cells (MDSCs) are major contributors to tumor progression. MDSCs differentiate from precursors within the bone marrow (BM) in tumor-bearing hosts. MDSCs distribute systemically and infiltrate tumors, where they contribute to tumor progression through a variety of mechanisms [2, 3]. However, MDSC differentiation and functions are still

poorly understood. This is due to the difficulty of isolating them from tumor-bearing subjects, or differentiating them *in vitro* so that they faithfully model *in vivo* cell subsets [4]. Nonetheless, counteracting their activities strongly enhances anti-cancer treatments [5]. Thus, finding treatments that would specifically eliminate MDSCs could improve the efficacy of anti- cancer therapies.

While the most valuable source of MDSCs for research is the tumor itself, their isolation is still a challenge [4, 6]. Therefore, other sources such as spleen or blood are widely used. However, these MDSCs are phenotypically and functionally different from tumor- infiltrating subsets [6–9]. To overcome these difficulties, we developed an *ex vivo* differentiation system that produces MDSCs modeling tumor-infiltrating subsets (B16-MDSCs) and non-cancerous (NC) MDSCs (293T-MDSCs) [8]. These *ex vivo* MDSCs have been phenotypically and functionally validated in B16 melanoma and CT26 colorectal cancer models [8–11].

The use of high-throughput analytical techniques for the identification of cellular regulatory pathways and novel molecular targets is on the increase. Two independent studies on the proteome of blood and spleen MDSCs have been published using LC-MS/MS mass spectrometry and label-free quantification [12, 13]. Although relevant data was obtained, none of these studies included control cell types such as myeloid DCs and NC- MDSCs. Therefore, studies that have been published so far have not discriminated pathways associated to cell lineage or the tumor environment.

To overcome these issues, we carried out in-depth proteomic analyses comparing myeloid DCs, MDSCs modeling tumor-infiltrating subsets or modeling NC-MDSCs. We found a kinase signature that defined neoplastic MDSCs which could be specifically targeted to interfere with MDSC differentiation from myeloid precursors.

RESULTS

MDSC lineage-specific interactomes

iTRAQ-based quantitative proteomics were performed on MDSCs modeling melanoma-infiltrating subsets (B16-MDSCs), using immature myeloid DC proteomes as a comparative standard to identify melanoma MDSC lineage-specific interactomes. 3609 proteins were unambiguously identified with an FDR lower than1%. Differential protein quantification was performed between DCs and B16-MDSCs, and the most affected proteins with a significance level of 0.01 were used for further analyses (Fig. 1a). Expression of 58 proteins was found up-regulated in MDSCs while 46 were down- modulated (Fig. 1b and Supplementary Table 1). Ingenuity Pathway Analysis was used to reconstruct functional interactome maps with differentially expressed proteins. Three distinct interactomes resulted from the analyses, with highly detailed interaction relationships between nodes (Figs. 2, 3, 4). The top canonical pathways which separated B16-MDSCs from DCs were: (1) mitochondrial dysfunction (P = 1.5 × 10^{-7}); (2) leukocyte extravasation signaling ($P = 5 \times 10^{-6}$), (3) caveolar-mediated endocytosis signaling ($P = 2.6 \times 10^{-5}$) and (4) integrin signaling (4×10^{-5}). These pathways were associated to SRC, FYN and HCK kinases, unambiguously identified by mass spectrometry (Supplementary Table 1). Protein interactome networks predicted a number of regulatory proteins (hubs) including the PI3K-AKT signaling axis (Fig. 2). Importantly, SRC kinases controlled changes in the cytoskeleton and mitochondrial dysfunction through down-regulation of complex I NAPDH dehydrogenase subunits (Figs. 2, 3). These kinases were directly associated to various molecular nodes such as calmodulin, Hsp90, α-catenin and the proteasome (Fig. 4).

Confidence-based protein networks were reconstructed using STRING software [14], with up-regulated or down-regulated proteins. Both high and medium confidence links were considered (score >0.4), as the number of networks was limited to allow careful confirmation. About 10 distinct protein networks were organized around a central group of kinases that included SRC family members (Supplementary Fig. 1). These networks were associated to production of reactive oxygen species (ROS), protection against oxidative

damage, intracellular vesicle trafficking and aminoacid metabolism. Decreases in spliceseosomal proteins, carbohydrate metabolism, lysosomal function and MHC II antigen presentation were also evident.

KEGG pathway mapping was applied to up- and down-regulated proteins. KEGG analyses showed strong inhibition of cellular processes associated to inflammatory disorders and a decrease in metabolism of aminoacids (Fig. 5).

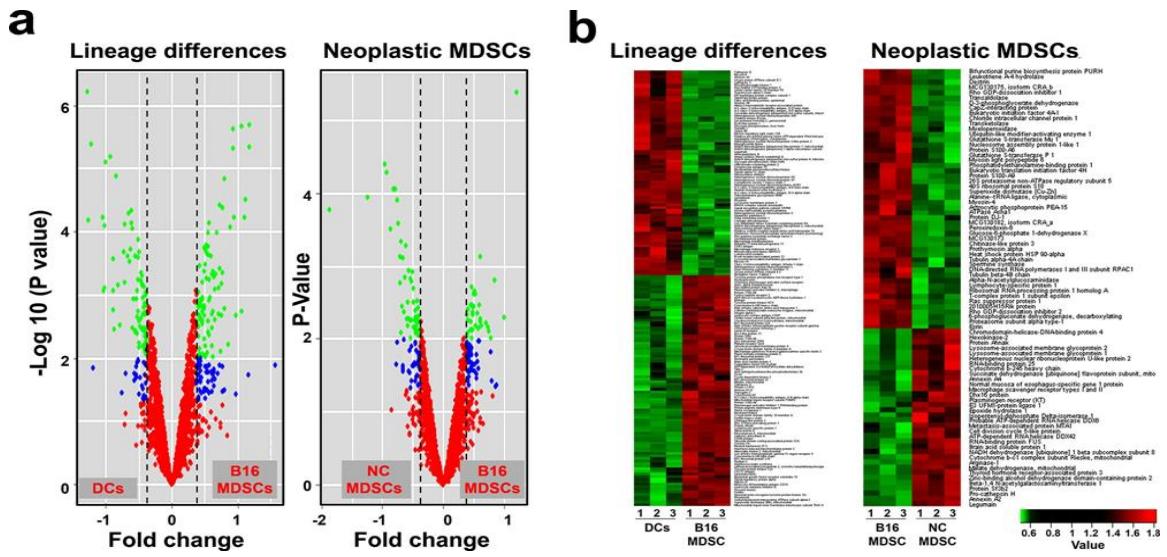


Figure 1: Differentially expressed proteins in MDSCs caused by lineage and cancer. a. *Volcano plots representing the fold-change of identified proteins with associated P values from the pair-wise quantitative comparisons of DCs vs B16-MDSCs (lineage differences, left plot) and NC-MDSCs vs B16-MDSCs (cancer-regulated differences, right plot). In green, very significantly changed proteins (P < 0.01), in blue, significantly changed proteins (P < 0.05) and in red, unchanged proteins between the pair-wise comparisons.* ***b.*** *Heat map representing the degree of change for the differentially expressed proteins (P < 0.01, Supplementary Table 1) between the indicated samples (independent biological triplicates are indicated as 1, 2 and 3; DCs, dendritic cells; B16-MDSCs, cancerous MDSCs; NC-MDSCs, non-cancerous MDSCs), as shown below. Legend (bottom right) indicates color-coded fold-change on Log_{10} scale. Red and green, up- and down-regulated proteins, respectively.*

Cancer-specific interactomes in MDSCs modeling tumor-infiltrating subsets

Our *ex vivo* system generates MDSCs that model tumor-infiltrating (B16-MDSCs) and non-cancerous NC- MDSCs (293T-MDSCs) [8, 9, 11]. It has to be pointed out that NC-MDSCs are not precursors of tumor-infiltrating MDSCs, but cells differentiated *ex vivo* in non-neoplastic conditions as described [8, 9]. Thus, a quantitative proteomic comparison between these two subsets was performed to highlight cancer-regulated pathways. These analyses uncovered 50 up- and 35 down-regulated proteins in B16-MDSCs compared to NC-MDSCs, and pathway reconstruction was performed using Ingenuity (Fig. 6, Supplementary Table 1). The top canonical pathways which differentiated neoplastic from non-cancerous MDSCs were: (1) the pentose phosphate pathway ($P = 6.4 \times 10^{-8}$), represented by G6PD, PGD and TALDO1 up-regulation; (2) epithelial adherence junction signaling with up-regulation of EZR, DSTN, tubulin and Rho-like proteins ($P = 2.4 \times 10^{-3}$). The two top associated molecular and cellular functions were (1) free radical scavenging and oxidative stress responses ($P = 1.1 \times 10^{-8}$) as indicated by up- regulation of SOD2, MPO, PRDX, GSTM5 and PARK7 amongst others, and (2) carbohydrate metabolism which was associated to the pentose phosphate pathway ($P = 2.5 \times 10^{-8}$). Interestingly, Ingenuity protein interaction networks included the kinases ERK1 and PKC isoforms as regulatory hubs (Fig. 6).

Similar results were obtained with STRING software (Supplementary Fig. 2). Most notably, up-regulation of the pentose phosphate pathway, changes in cytoskeletal proteins and down-modulation of oxidative phosphorylation. Results from KEGG pathway mapping highlighted increased glutathione metabolism, activation of the pentose phosphate pathway and a decrease in spliceosomal proteins (Fig. 7).

A kinase signature defines the neoplastic MDSC lineage

Systems biology analyses delineated a kinase signature of the MDSC lineage (AKT and the SRC family, which included SRC, HCK and FYN) and neoplastic MDSCs (ERK and PKC kinases). Overall, the expression of FYN, HCK and total and phosphorylated SRC agreed with proteomic data, as assessed by flow cytometry and immunoblotting. The predicted participation of AKT was also confirmed (Fig. 8a). AKT expression was particularly high in MDSCs modeling tumor-infiltrating subsets as detected by immunoblotting. ERK1 and PKC isoforms were predicted to be differentially expressed in tumor-infiltrating MDSCs. While total ERK expression was equivalent between B16-MDSCs and NC-MDSCs, phosphorylated (active) ERK1 was increased in B16-MDSCs (Fig. 8a). The expression of phosphorylated PKC isoforms (phosphorylated pan-PKCs) was tested by immunoblot. In agreement with proteomic data and Ingenuity analyses, phosphorylated PKCs were present at higher levels in MDSCs modeling neoplastic subsets (Fig. 8a).

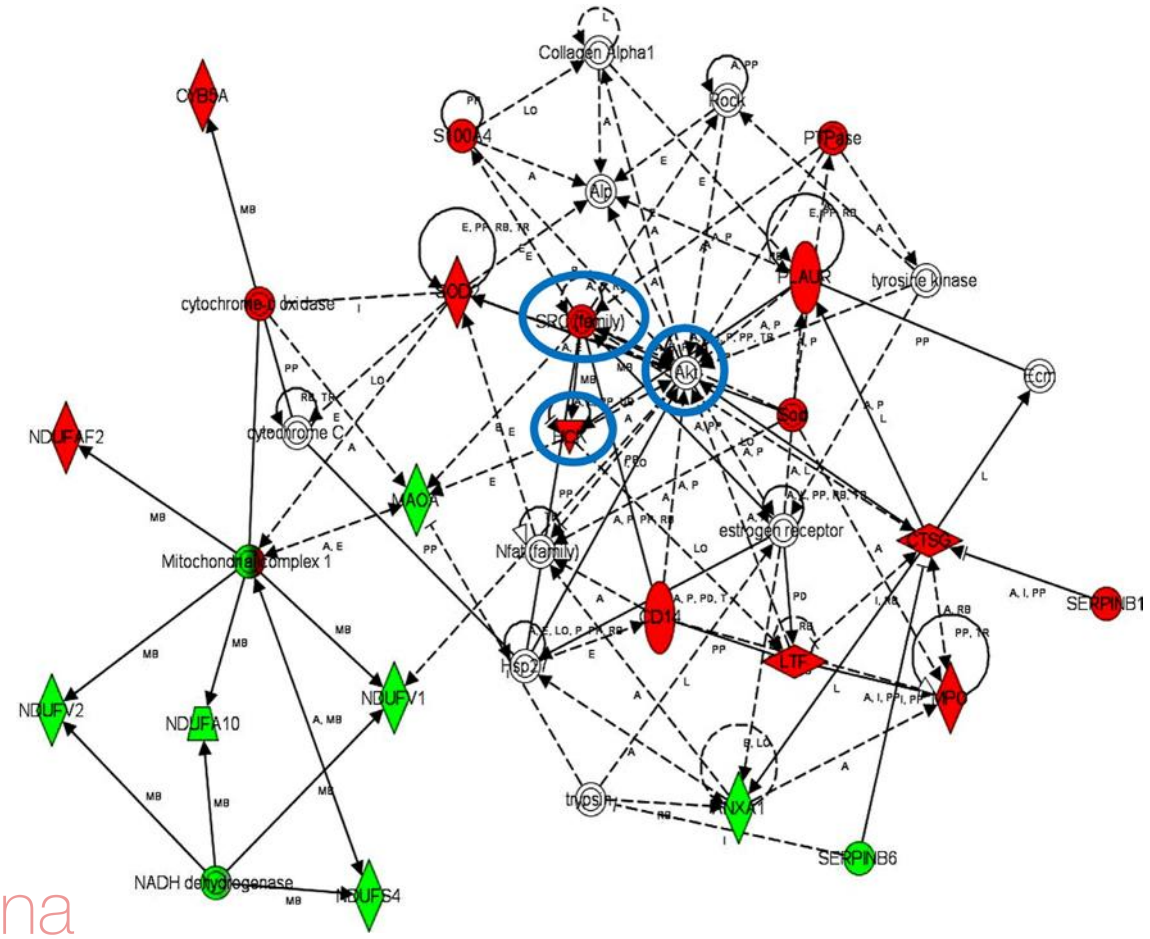


Figure 2: Functional MDSC lineage-specific interactome networks controlled by SRC, HCK and AKT kinases. *Graph represents functional interactomes constructed with Ingenuity Pathway Analysis tool using lineage specific (B16-MDSCs vs DCs) differentially expressed proteins, which shows detailed interaction relationships between the input nodes (differentially expressed proteins between MDSCs and DCs), and regulatory kinases encircled in blue. This interactome links AKT/SRC kinases with mitochondrial respiration and dysfunction, protection against oxidative stress and extracellular matrix remodeling. Nodes in red, up-regulated proteins. Nodes in green, down-modulated proteins. In white, predicted protein nodes. A, activation; B, binding; C, causes/leads to; CC, chemical- chemical interaction; CP, chemical-protein interaction; E, expression; EC, enzyme catalysis; I, inhibition; L, proteolysis; LO, localization; M, biochemical modification; MB, group/complex; P, phosphorylation/dephosphorylation; PD Protein-DNA binding; PP Protein-Protein binding; PR Protein-RNA binding; RB Regulation of Binding; RE Reaction; RR RNA-RNA Binding; T Transcription; TR Translocation. Dash arrows, indirect interactions.*

AKT and ERK1 specifically contribute to MDSC differentiation from myeloid precursors

To assess the contribution of MDSC-associated kinases to myeloid differentiation, a collection of kinase inhibitors were added to myeloid precursors committed towards DC or B16-MDSC differentiation. Inhibitors were added at concentrations reported to interfere with cancer cell growth. High resolution impedance-based real-time cell monitoring (RTCA) was used to continuously monitor myeloid differentiation, viability and to calculate IC50s (Fig. 8b and Table 1) [15]. Overall, all tested inhibitors affected equally to myeloid precursors differentiating towards DCs and MDSCs (Table 1). Treatments with the specific AKT inhibitor X or the MEK inhibitor PD0325901 were an exception. AKT inhibitor X was highly toxic to precursors differentiating towards MDSCs, while differentiating DCs remained unaltered (Fig. 8b and Table 1). Treatment with the MEK inhibitor PD0325901 selectively inhibited MDSC proliferation.

Overall, comparing the IC50s for differentiating DCs and MDSCs, AKT and MEK-ERK pathways specifically contributed to MDSC differentiation (Fig. 8c). Moreover, myeloid precursors committed to MDSC differentiation died within hours of adding the AKT inhibitor, strongly suggesting that AKT was involved in survival but not differentiation (Fig. 9a).

The results with kinase inhibitors were also confirmed with silencing shRNAs. Thus, myeloid precursors committed towards MDSC differentiation were expanded for three days from BM and transduced with a lentivector delivering immunoblot-validated shRNAs against AKT1 or ERK1 as described [8, 16] (Fig. 9b). Transduced myeloid precursors died 48 hours after delivery of the AKT1-specific shRNA. Likewise, ERK1 silencing significantly inhibited cell growth.

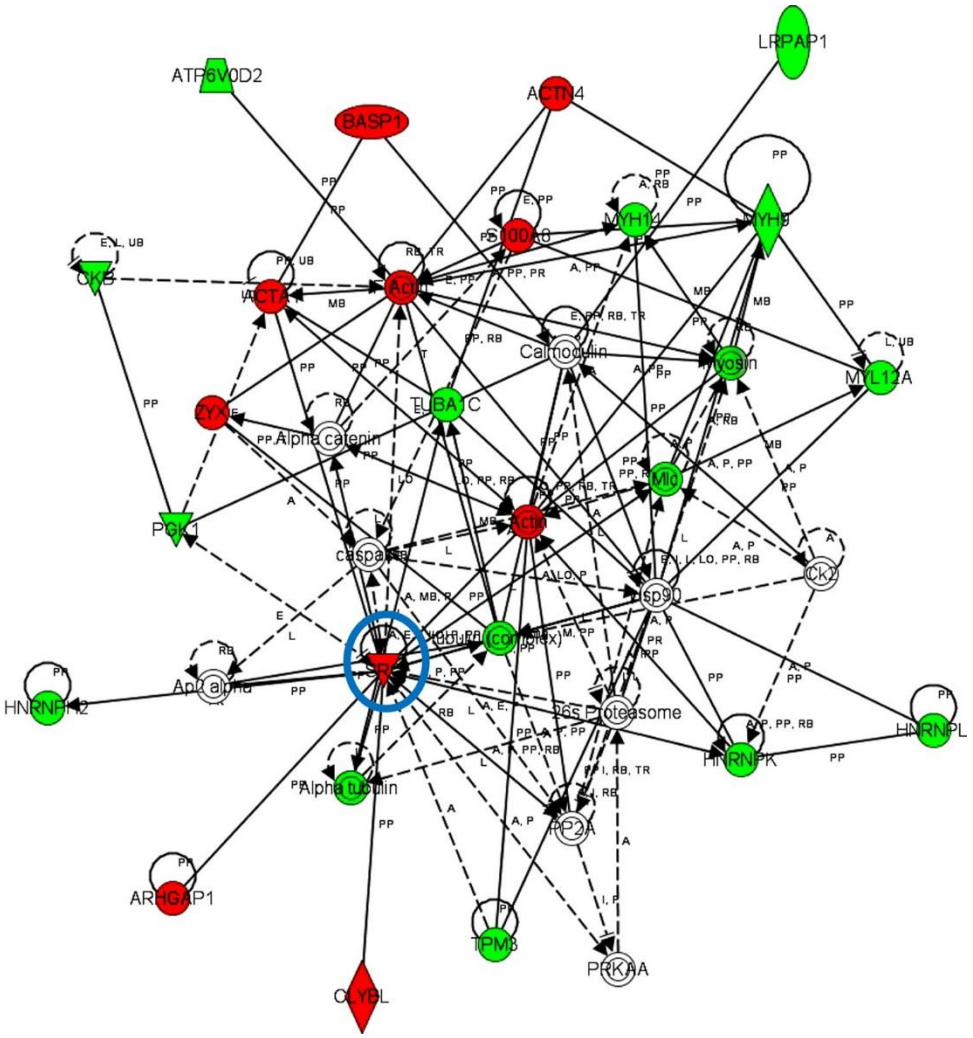


Figure 3: Functional MDSC lineage-specific interactome networks regulating cytoskeletal changes and controlled by SRC kinases. *Graph presents functional interactomes constructed with Ingenuity Pathway Analysis tool using lineage specific (B16-MDSCs vs DCs) differentially expressed proteins, which shows detailed interaction relationships between the input nodes (differentially expressed proteins between MDSCs and DCs), and regulatory kinases encircled in blue. This interactome links SRC kinases with protein transport, mRNA processing, cytoskeletal re-organization and decreased glycolysis. Nodes in red, up-regulated proteins. Nodes in green, down-modulated proteins. In white, predicted protein nodes. A, activation; B, binding; C, causes/leads to; CC, chemical- chemical interaction; CP, chemical-protein interaction; E, expression; EC, enzyme catalysis; I, inhibition; L, proteolysis; LO, localization; M, biochemical modification; MB, group/complex; P, phosphorylation/dephosphorylation; PD Protein-DNA binding; PP Protein-Protein binding; PR Protein-RNA binding; RB Regulation of Binding; RE Reaction; RR RNA-RNA Binding; T Transcription; TR Translocation. Dash arrows, indirect interactions.*

Inhibition of the ERK pathway interferes with MDSC differentiation and accelerates DC maturation

Inhibition of the ERK pathway interfered with MDSC growth. As the MEK inhibitor PD0325901 is currently used for the treatment of several human cancers in clinical trials, its effects on differentiation of myeloid cell lineages was further tested. Thus, the three main myeloid cell populations differentiated from bone marrow precursors was quantified by flow cytometry after a week of PD0325901 treatment; Namely, CD11b+ monocytic myeloid cells (Ly6Chigh Ly6Gneg), granulocytic myeloid cells (Ly6C+ Ly6Ghigh) and conventional DCs (Ly6C+ Ly6Gneg/low CD11c+) (Fig. 9c). Interestingly, PD0325901 treatment accelerated conventional CD11c+ DC differentiation. At the highest tested concentration, the MEK inhibitor was strongly cytotoxic to myeloid cells committed to MDSC differentiation, but not to those differentiating towards DCs which strongly up-regulated CD11c expression.

Overall, these results also confirmed that interference with the ERK pathway is inhibitory over MDSCs and promotes conventional DC differentiation.

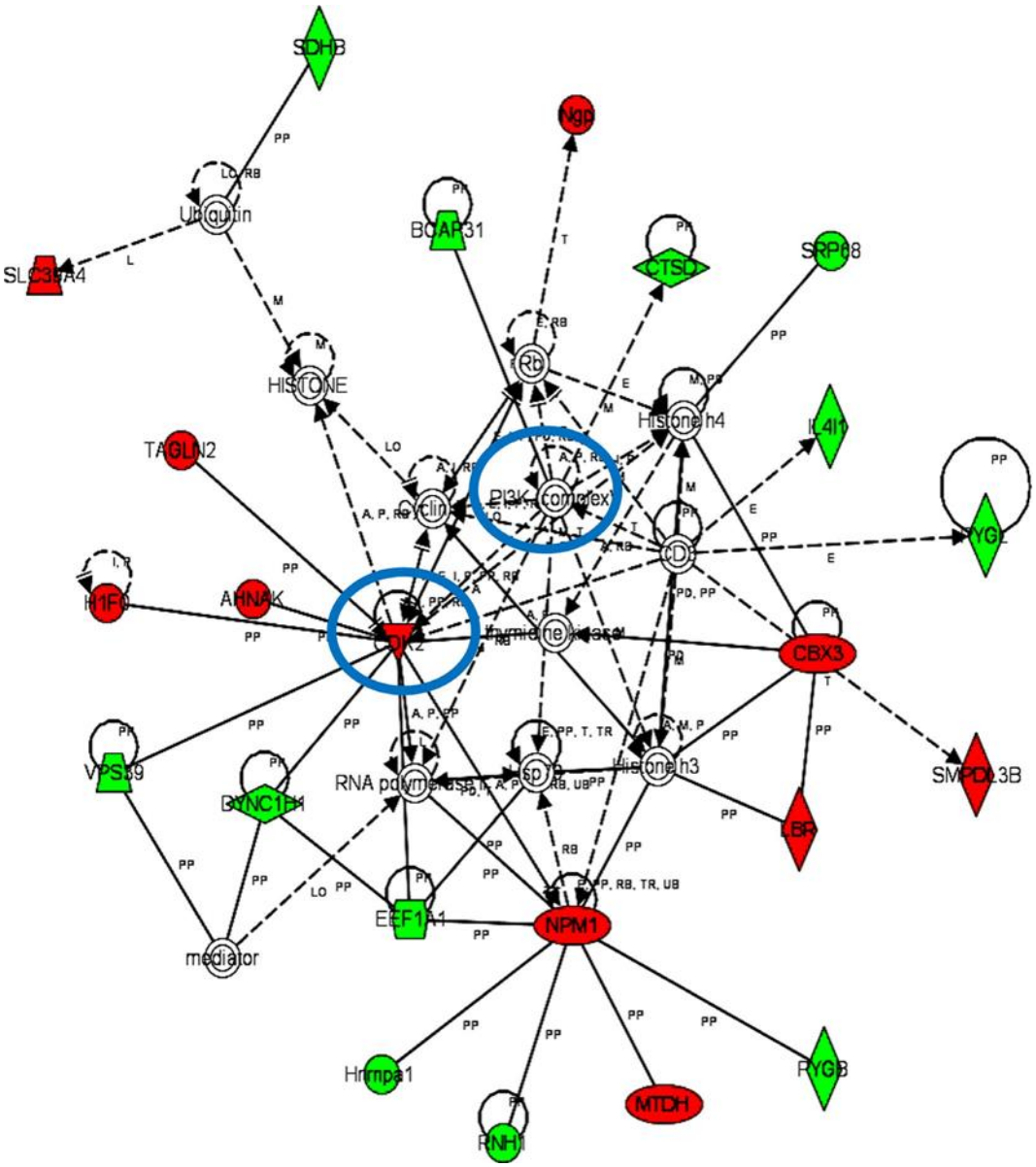


Figure 4: Functional MDSC lineage-specific interactome networks controlled by PI3K and CDK2 kinases. *Graph presents functional interactomes constructed with Ingenuity Pathway Analysis tool using lineage specific (B16-MDSCs vs DCs) differentially expressed proteins, which shows detailed interaction relationships between the input nodes (differentially expressed proteins between MDSCs and DCs), and regulatory kinases encircled in blue. This interactome links PI3K with*

cell cycle, protein synthesis and transport, survival and proliferation. Nodes in red, up-regulated proteins. Nodes in green, down-modulated proteins. In white, predicted protein nodes. A, activation; B, binding; C, causes/leads to; CC, chemical-chemical interaction; CP, chemical-protein interaction; E, expression; EC, enzyme catalysis; I, inhibition; L, proteolysis; LO, localization; M, biochemical modification; MB, group/complex; P, phosphorylation/ dephosphorylation; PD Protein-DNA binding; PP Protein-Protein binding; PR Protein-RNA binding; RB Regulation of Binding; RE Reaction; RR RNA-RNA Binding; T Transcription; TR Translocation. Dash arrows, indirect interactions.

DISCUSSION

High-throughput analyses of biological systems provide a unified view of biological processes, and can uncover novel molecular targets. However, sorting out meaningful information from large datasets is challenging and relies on choosing the right controls. In addition, some biological systems such as MDSCs are difficult to work with. Here we characterized the neoplastic B16 melanoma MDSC proteome by differentiating MDSCs modeling tumor-infiltrating subsets, and quantitatively comparing it with the proteomes of myeloid DCs and MDSCs modeling non-cancerous MDSCs. Myeloid DCs and NC-MDSCs provide the appropriate controls to discriminate pathways regulated by cell lineage or the tumor environment. We performed in-depth quantitative proteomics to construct highly detailed MDSC interactome maps. Regulatory networks were for the first time unambiguously ascribed to cell lineage or to a neoplastic environment.

Cell lineage differences were highlighted by comparing B16 melanoma-MDSCs with myeloid DCs. Mitochondrial dysfunction was a key characteristic of MDSCs, reflecting a shut-down of oxidative phosphorylation. MDSCs modeling non-cancerous subsets provided a convenient standard to discriminate cancer-specific pathways. Of these, the pentose phosphate pathway was one of the most prominent, probably used to produce NADPH for biosynthesis in the absence of oxidative phosphorylation. Decreased expression of mitochondrial NADPH

dehydrogenase, up-regulation of free radical scavenging proteins, and cellular stress were hallmarks of neoplastic MDSCs compared to NC-MDSCs.

As we found that NC-MDSCs differed from neoplastic MDSCs in cell stress pathways and inflammatory pathways, it is likely that NC-MDSCs are different to other subsets differentiated in non-neoplastic conditions such as cell stress and infection. Overall, published data agreed with our proteome maps [17–24].

Importantly, neoplastic MDSCs presented a specific kinase signature which controlled MDSC-related interactomes and clearly separated them from the myeloid DC lineage. While SRC, FYN, HCK, PI3K and AKT kinases differentiated MDSCs from DCs, ERK and PKC discriminated neoplastic MDSCs from non-cancerous subsets. The proteomic and systems biology data was confirmed by immunoblot and flow cytometry. Ingenuity analyses also predicted the PKC isoforms as a differential feature of neoplastic MDSCs. DCs and NC-MDSCs presented lower but detectable levels of phospho-PKC. As we used a pan-phospho-PCK antibody, we cannot rule out that some specific PKC isoforms discriminate neoplastic MDSCs from non-neoplastic counterparts. In fact, this is the case of 4T1 breast cancer MDSCs, for which there is evidence that isoforms beta and theta are specifically activated [22].

AKT was required for the survival of myeloid precursors differentiating into MDSCs, but was dispensable in precursors committed to DC differentiation. This is in agreement with the requirement of AKT for in vivo MDSC expansion [25], and with its anti-apoptotic role in hematopoietic cells [26]. Additionally, the MEK1 inhibitor PD032591 selectively affected differentiating MDSCs, while DC maturation was enhanced according to CD11c up-regulation. The ERK pathway is known to keep DCs immature and favor tumor progression [27–29]. Here we also demonstrated that ERK contributes to MDSC differentiation. Our results show that inhibition of ERK and AKT pathways could enhance anti-tumor immune responses by depleting MDSCs and activating DCs. Nevertheless, the other differentially expressed kinases may still participate in MDSC functions apart from differentiation and survival, which could be susceptible of therapeutic

intervention.

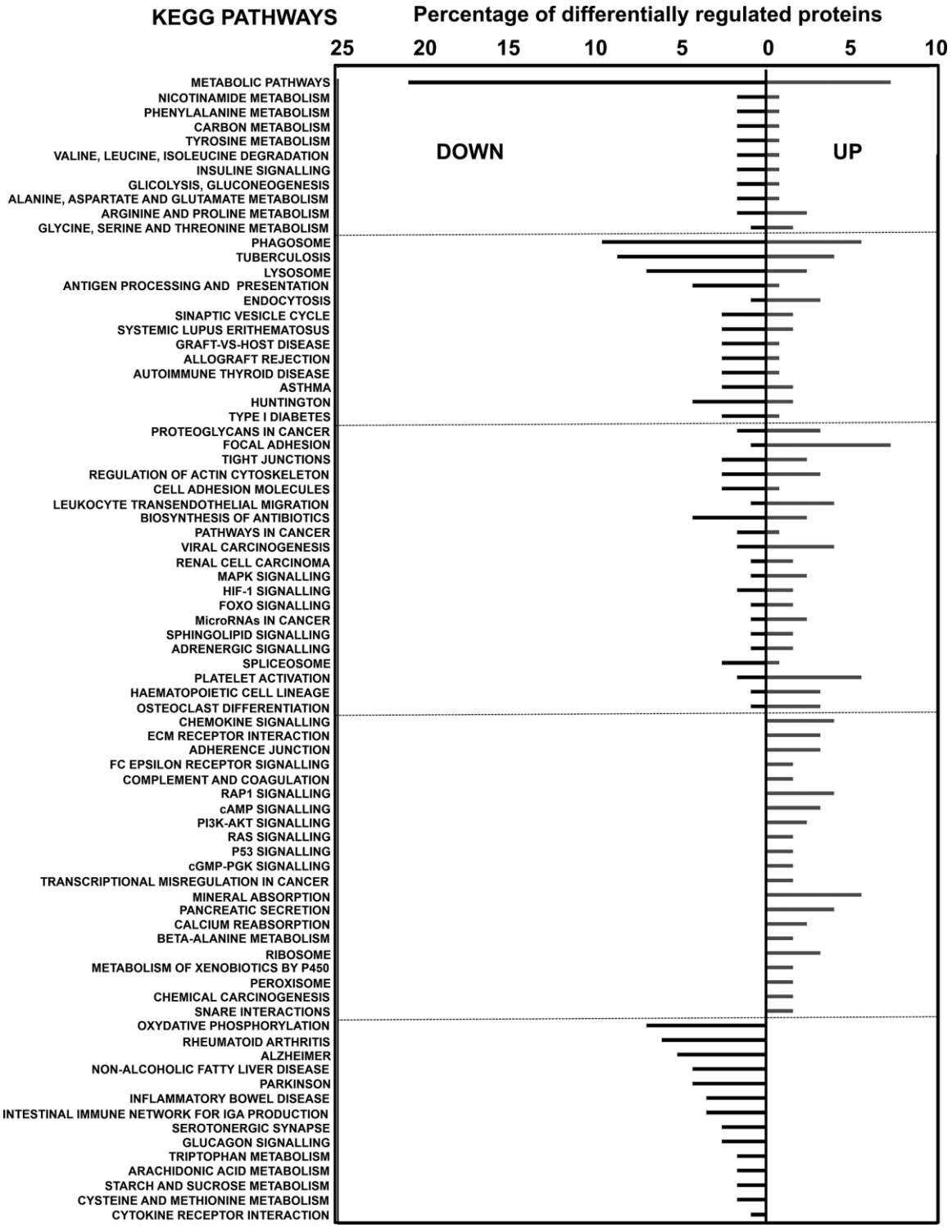


Figure 5: KEGG pathway analyses of differentially-expressed proteins in MDSCs compared to myeloid DCs. Graph represents the percentage of differentially up- or down-regulated (as indicated within the graph) proteins ascribed to the indicated KEGG pathways.

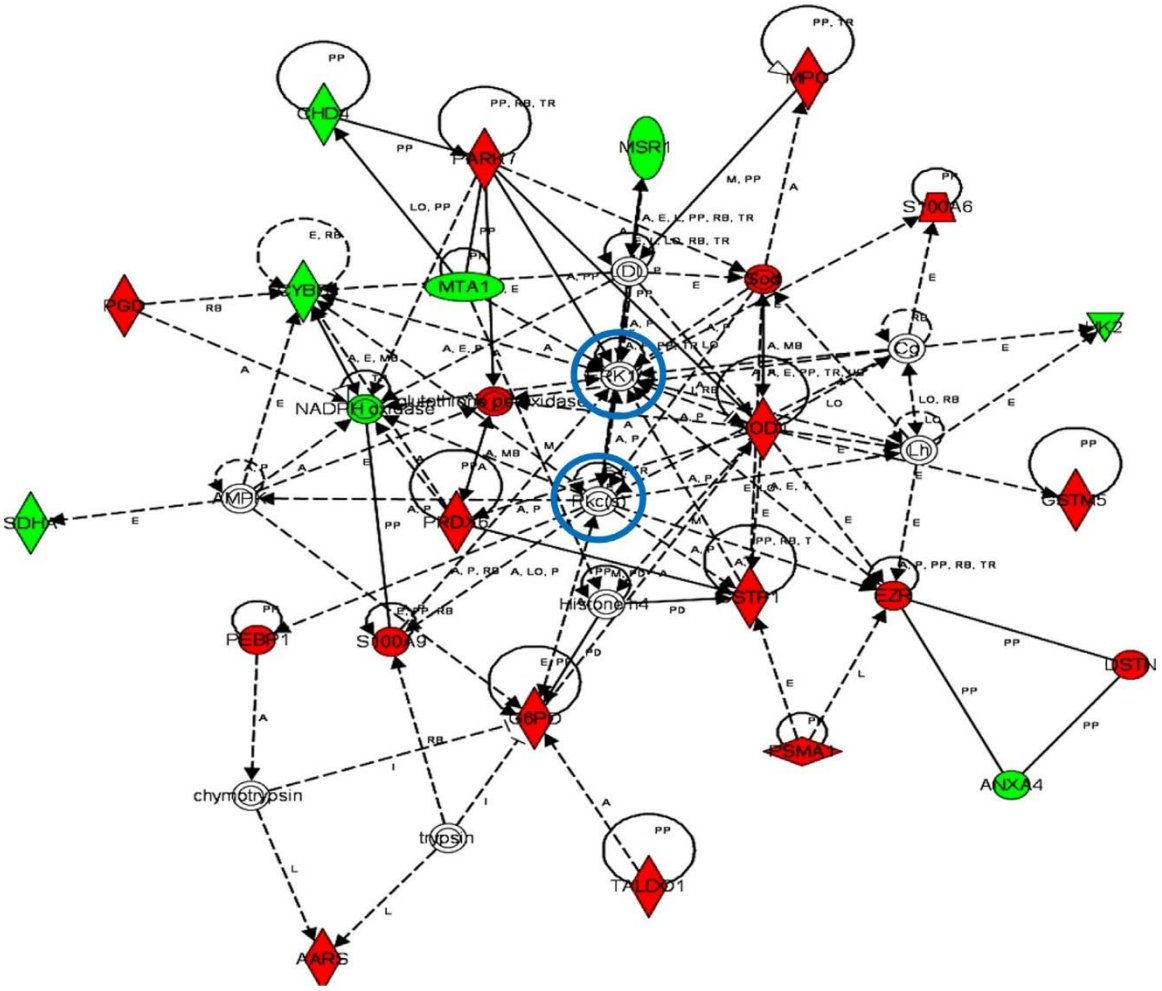


Figure 6: Functional interactomes with cancer-regulated (B16-MDSCs vs NC-MDSCs) differentially expressed proteins. *Ingenuity Analysis interactome linking ERK and PKCs with protection against oxidative stress, mitochondrial electron transport and NADPH oxidase activity, the pentose phosphate pathway and ROS generation. Regulatory kinases are encircled in blue. Nodes in red, up-regulated proteins. Nodes in green, down-modulated proteins. In white, predicted protein nodes; A, activation; B, binding; C, causes/leads to; CC, chemical-chemical interaction; CP, chemical-protein interaction; E, expression; EC, enzyme catalysis; I, inhibition; L, proteolysis; LO, localization; M, biochemical modification; MB, group/complex; P, phosphorylation/dephosphorylation; PD Protein-DNA binding; PP Protein-Protein binding; PR Protein-RNA binding; RB Regulation of Binding; RE Reaction; RR RNA-RNA Binding; T Transcription; TR Translocation.* Dash arrows, indirect interactions.

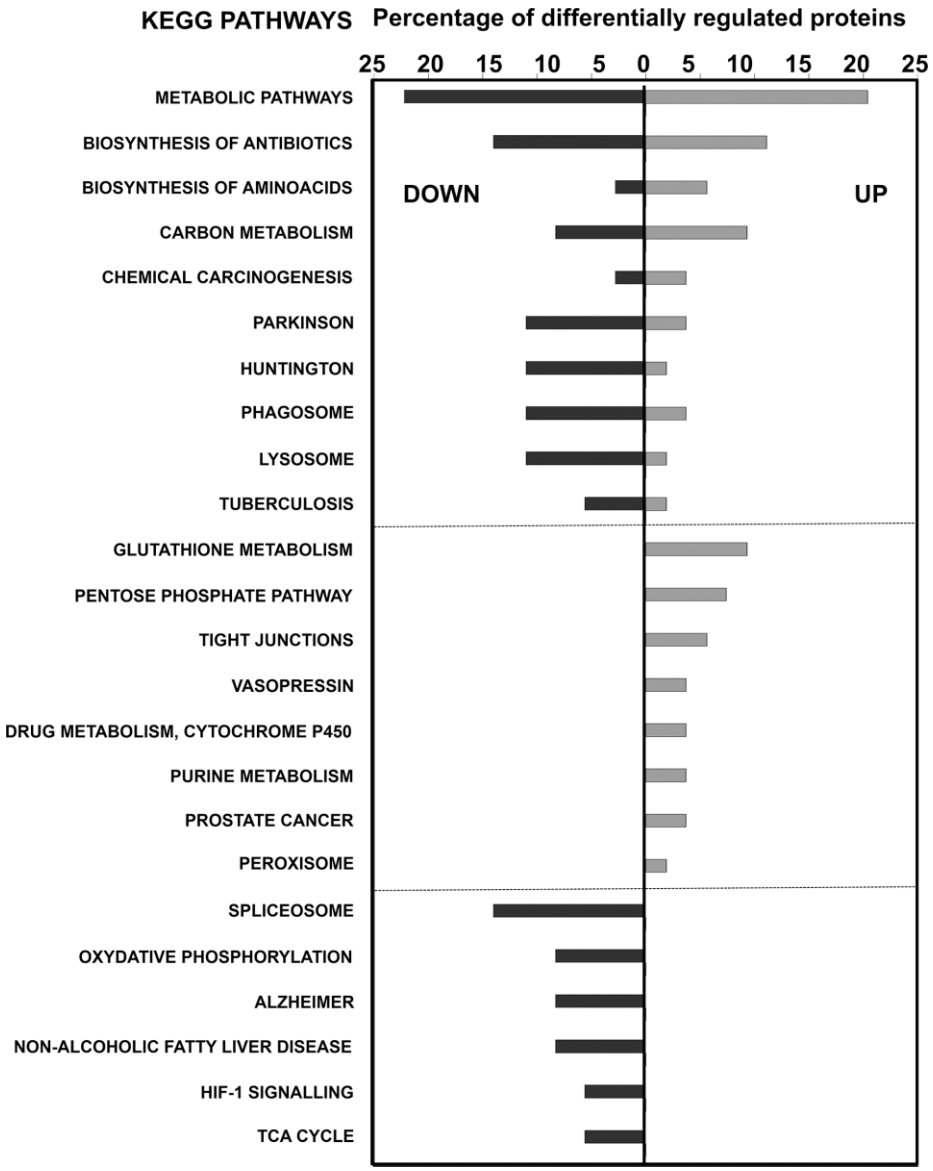


Figure 7: KEGG pathway analyses of differentially-expressed proteins in MDSCs modeling tumor-infiltrating subsets compared to NC-MDSCs. *The graph shows the percentage of differentially up- or down-regulated (as indicated within the graph) proteins ascribed to the indicated KEGG pathways.*

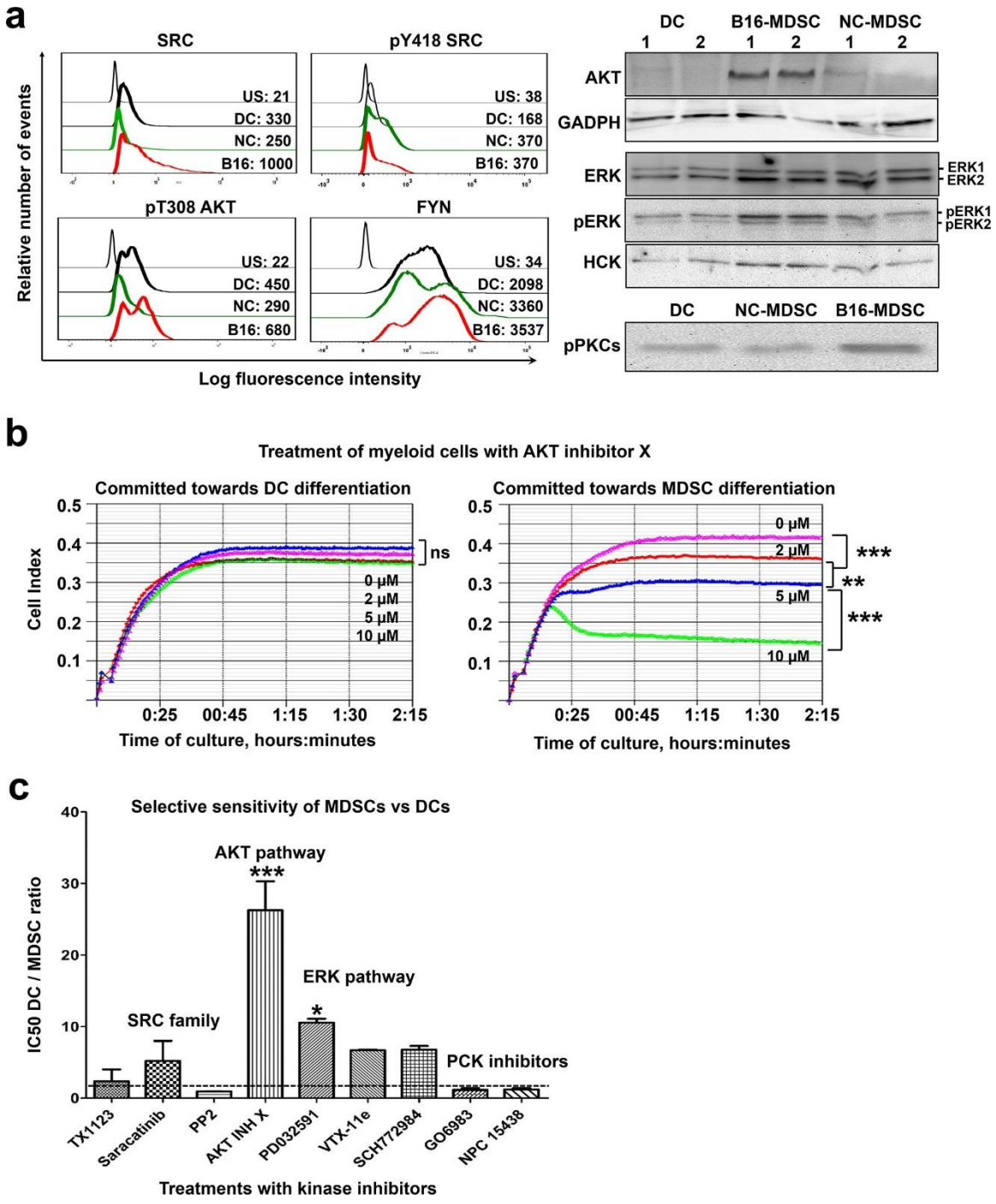


Figure 8: A kinase signature discriminates MDSCs from DCs. a. *Graphs on the left, flow cytometry histograms with expression profiles of the indicated kinases in DCs, NC-MDSCs and B16-MDSCs. Mean fluorescent intensities for each cell population are shown within the graphs. US, unstained control; DC, dendritic cells; NC, non-cancerous MDSCs; B16, MDSCs modeling tumor-infiltrating subsets. Blots on the right, detection of the indicated kinases by immunoblotting. Two preparations from the indicated cell populations (top of the immunoblots)*

were loaded and lanes were labelled as 1 and 2. An immunoblot for GADPH detection is shown as a reference control, on the same membrane used for AKT detection above. ***b.*** *Representative real-time cell monitoring (RTCA) results for myeloid precursors treated with the indicated concentrations of AKT inhibitor X, and grown either in DC-differentiation medium or in B16-MDSC conditioning medium as indicated on top. Data is plotted as means of cell index with error bars (standard deviations) from duplicate cultures, shown as a function of time. Relevant statistical comparisons are shown and indicated with *, **, and *** for significant (P < 0.05), very significant (P < 0.01) and highly significant (P < 0.001) differences, respectively.* ***c.****DC/MDSC IC50 ratios calculated for the indicated treatments. Ratios close to 1 (horizontal dotted line) indicate that treatments are equally inhibitory over MDSCs and DCs. Ratios higher than 1 indicate that MDSCs are more sensitive to the specific treatments than DCs.*

Table 1: IC50s of small molecule inhibitors over myeloid precursors committed towards DC or MDSC differentiation

Inhibitor	IC50, DCs	IC50, MDSC	Targeted kinases
AKT inhibitor X	>100 μM	3.9 ± 0.6 μM	AKT
TX1123	3.2 ± 1.4 μM	3.4 ± 3 μM	SRC eEF2-K PKA
Saracatinib	3.5 ± 3.4 μM	8.8 ± 8 μM	SRC FYN
PP2	46.5 ± 0.7 μM	45.4 ± 2 μM	FYN HCK
PD0325901	44.7 ± 4.5 nM	6.2 ± 2.8 nM	MEK
SCH772984	86.5 ± 19 nM	21 ± 15 nM	ERK1
VTX-11e	8 ± 0.15 μM	1.3 ± 0.14 μM	ERK1
Gö 6983	5.7 ± 1 μM	5.7 ± 2.3 μM	PKCα, β, γ, δ, ζ and μ
NPC-15437	8 ± 2.3 μM	8 ± 2 μM	PKC

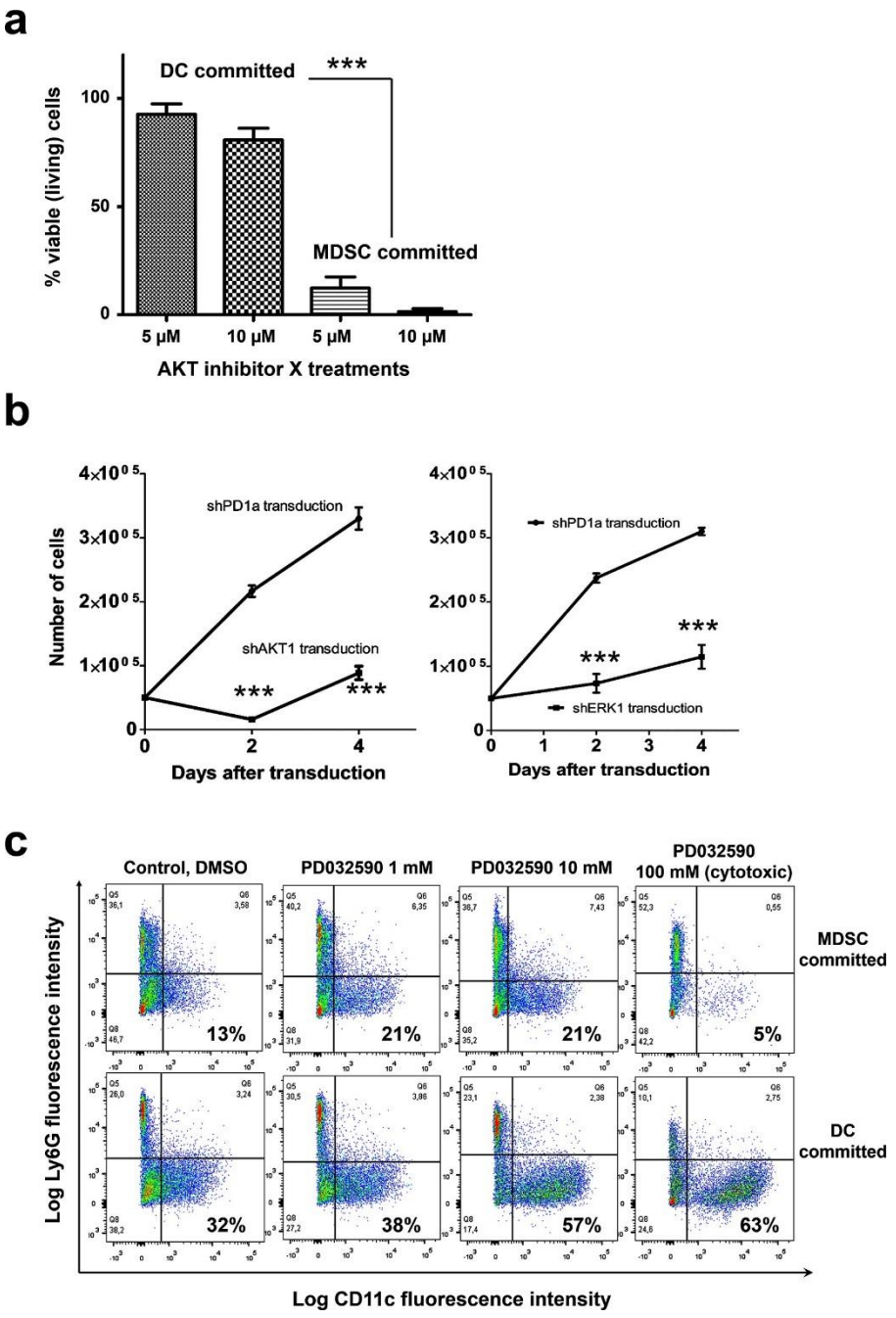


Figure 9: AKT is required for survival of myeloid cells committed to MDSC differentiation, while inhibition of the ERK pathway enhances conventional DC differentiation. a. The percentages of viable myeloid precursors treated with the indicated concentrations of AKT inhibitor X are indicated as a bar graph with standard deviations as error bars. Precursors were committed towards DC or MDSC differentiation as indicated on top of the bars. Viable cells were quantified following trypan blue staining. Relevant statistical comparisons are shown. b. Graph on the left, growth of myeloid cell precursors transduced with a lentivector

encoding a control shRNA (shPD1a), or an AKT1-specific shRNA (shAKT1). Data is plotted as means and standard deviations as error bars. The same is shown in the graph on the right, but delivering an ERK1-specific shRNA (shERK1). Relevant statistical comparisons are shown within the graph. **b.** Phenotype effects of sustained MEK inhibition with PD0325901 on myeloid precursors committed to MDSC differentiation or to DC differentiation. Ly6C+ cells were gated and the Ly6G-CD11c expression profiles are shown in flow cytometry density plots. Percentages of CD11c+ myeloid cells after 7 days of culture are highlighted within the graphs. ***, very highly significant differences.

MATERIALS AND METHODS

Cells and mice

293T, B16F0 cells and BM-DCs were grown as described [8, 27]. Approval for animal studies was obtained from the Animal Ethics Committee of the University of Navarra, and from the Government of Navarra. Non-cancerous MDSCs (NC-MDSCs, 293T-MDSCs) and B16-MDSCs were obtained from C57BL/6 murine BM cells as described [8].

Drug treatments of myeloid cell cultures and impedance-based real-time living cell monitoring (RTCA)

Myeloid hematopoietic precursors were expanded from BM cells using granulocyte-monocyte-colony stimulating factor (GM-CSF), stem cell factor (SCF) and leukaemia inhibitory factor (LIF) for 2 to 3 days, following published conditions [8, 30]. Then, myeloid precursors were seeded on two L8 cell culture chambers for the xCELLingence RTCA monitoring system (ACEA biosciences), at a density of 200000 cells per well. DC or B16-MDSC differentiation medium was added to myeloid precursors, and treatments were carried out simultaneously in duplicates. After 30 min, the indicated chemical inhibitors were added at concentrations reported to be cytotoxic to cancer cells. Control well were treated with carrier solution (either water or DMSO). The following inhibitors were used: AKT inhibitor X (Calbiochem), tyrosine kinase inhibitor TX-1123

(Calbiochem), MEK inhibitor PD0325901 (SIGMA), ERK inhibitors SCH772984 and VTX-11e [31], broad PKC inhibitor GÖ 6983 (Santa Cruz Biotechnology), PKC inhibitor NPC-15437 dihydrochloride (Santa Cruz Biotechnology), selective LCK and FYN inhibitor PP2 (Santa Cruz Biotechnology), and the SRC and FYN inhibitor Saracatinib (MedChem Express). IC50s for each inhibitor were calculated using the RTCA data and analysis software, using duplicates for each drug treatment.

Lentivector production and cell transduction

Lentivectors were produced and titrated by flow cytometry or Q-PCR as described [32]. The pHIV-SIREN system developed by our group [16] was used as a backbone to clone the following validated shRNAs against ERK1 (GCATGCTTAATTCCAAGGGCTATTCAAGAGATAG CCCTTGGAATTAAGCATGTTTTTTACGCGT) and AKT1 (GTCTGAGACTGACACCAGGTATTTCAAGAG AATACCTGGTGTCAGTCTCAGATTTTTTACGCGT). A control shRNA-encoding lentivector targeting the human PD1 transcript (SIREN-shPD1a) was used [33]. The same shRNAs were cloned into the pSIRACT-GFP shRNA-cloning lentivectors, which were derived from pHIV-SIREN constructs by replacing the PGK promoter by the Actinin 4 promoter. The lentivector backbone was changed because PGK was strongly down-modulated in MDSCs, while actinin4 was strongly expressed.

Immunoblot

Immunoblots were performed as described [27]. Anti-GADPH was purchased from Calbiochem. Rabbit anti-human HCK was purchased from Millipore. From BD bioscience, mouse anti-pan ERK, mouse anti-AKT and mouse anti-AKT pT308. From Cell Signaling, rabbit anti-mouse T202/Y204 p-P44/42 MAPK, phospho-pan-PKC rabbit mAb. Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from DAKO. Membranes were stripped and re-probed with antibodies for total and phosphorylated proteins, when required.

Cell staining and flow cytometry

Surface and intracellular staining were performed as described previously [27] using the indicated antibodies. From BioLegend: Alexa fluor 488-Ly6C, PE-Cy7-Ly6G, PE-Cy7-streptavidin, APC-streptatividin; From BD Pharmigen: APC-CD11b, PE-Cy7 anti-mouse CD11c, Rat anti-mouse CD16/CD32, PE-conjugated anti-Gr-1, Alexa 647-conjugated anti- PY418 SRC, PE-conjugated ant-AKT1, from Invitrogen: APC-CD11c, PE-streptavidin, FITC-streptavidin; from AbDSerotec: PE-CD62L; From Santa Cruz Biotechnology: PE-conjugated anti-Fyn. From Cell Signaling, Alexa 647-conjugated anti-SRC rabbit antibody (clone 36D10) and PE-conjugated anti-phospho AKT rabbit antibody (Ser473, clone D9E).

Mass spectrometry-based quantitative proteomics and bioinformatic analyses

A global experiment was carried out with three biological replicates in each experimental condition using B16-MDSC, NC-MDSC and DC cell pellets. The specific procedures for sample preparation, proteomic analyses, iTRAQ-based proteomic workflows and mass spectrometry using triple-TOF 5600 system (AB Sciex) have been published [8]. The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange. org) via the PRIDE partner repository with the data set identifiers PXD001103 and PXD001106.

Analyses of raw data (.wiff, AB Sciex) were performed with MaxQuant software [34]. For peak list generation, default AB Sciex Q-TOF instrument parameters were used except the main search peptide tolerance, which was set to 0.01 Da, and MS/MS match tolerance, which was increased up to 50 ppm. Minimum peptide length was set to 6. Two databases were used. A contaminant database (.fasta) was firstly used for filtering out contaminants. Peak lists were searched against UniProt murine database, and Andromeda was used as a search engine [35]. Methionine oxidation was set as variable modification, and the carbamidomethylation of cysteine residues was set as fixed modification. Maximum false discovery rates (FDR) were set to 0.01 at protein and peptide levels. Analyses were limited to peptides of six or more amino acids in length, and considering a maximum of two missed cleavages. Relative protein

abundance output data files were managed using R scripts for subsequent statistical analyses and representation. Proteins identified by site (identification based only on a modification), reverse proteins (identified by decoy database) and potential contaminants were filtered out. Only proteins with more than one identified peptide were used for quantification. For possible quantification data rescue, up to one missing value for each group was rescued replacing it by the mean of the rest in-group samples. Data was normalized and transformed for later comparison using quantiles normalization and log2 transform respectively. The Limma Bioconductor software package in R was used for ANOVA analyses. Significant and differential data were selected by a p-value lower than 0.01, fold changes of <0.77 (down-regulation) and >1.3 (up-regulation) in linear scale. These parameters were used for differential expression threshold with volcano and profile plots.

The proteomic information was analyzed using bioinformatic tools. Studies with the Kyoto Encyclopedia of Genes and Genomes (KEGGS) Pathway mapping tool were performed as described (http://www.genome .jp/kegg/tool/map_pathway1. html). The identification of specifically up- or dysregulated regulatory/metabolic networks in MDSCs was analyzed with the open access STRING (Search Tool for the Retrieval of Interacting Genes) analysis tool (v.9.1) [14] and with the Ingenuity Pathway Analysis Tool (Qiagen).

Statistical analyses

GraphPad Prism and SPSS software packages were used for plotting data and statistical analyses. No data was considered an outlier. Real time cell monitoring data (RTCA, ACEA biosystems) was analyzed by exporting the Cell Index data as a function of time. It was confirmed that Cell Index in a growing population of cells was highly homogeneous and normally distributed at any given time-point. Therefore, the data was analyzed by one-way ANOVA and Tukey's pair-wise comparisons. IC50s were estimated for each treatment (using three published active concentrations per compound) in duplicates by RTCA, and means with standard deviations were obtained. IC50s were also highly homogeneous and normally distributed. The relative IC50 ratios for DCs vs

MDSCs were also calculated, and compared by one-way ANOVA and Tukey's pair wise comparisons. Cell viability was quantified by trypan blue staining and data analyzed by one-way ANOVA and Tukey's pair wise comparisons. Triplicates per treatment were used for the analyses. Growth of myeloid cells transduced with lentivectors encoding shRNAs was compared by a two-way ANOVA with "time of growth" as a random factor with data from four independent transductions, as described previously [8].

ACKNOWLEDGMENTS

Maria Gato is funded by a contract from Gobierno de Navarra (BMED 033–2014). Xabier Martinez obtained a Technology Development Fellowship from Gobierno de Navarra. Idoia Blanco Luquin is funded with a post- doctoral contract from a Bioef (Gobierno Vasco) grant. Therese Liechtenstein was granted a UCL Overseas Research Scholarship. Isabel Zudaire was funded with a post-doctoral contract from the RefBio NTBM grant. Dr Chaikuad is supported by the European Union FP7 Grant No. 278568 "PRIMES".

Dr Knapp is grateful for financial support by the SGC, a registered charity (number 1097737) that receives funds from AbbVie, Bayer, Boehringer Ingelheim, the Canada Foundation for Innovation, the Canadian Institutes for Health Research, Genome Canada, GlaxoSmithKline, Janssen, Lilly Canada, the Novartis Research Foundation, the Ontario Ministry of Economic Development and Innovation, Pfizer, Takeda, and the Wellcome Trust [092809/Z/10/Z]. The authors thank all PRIDE Team for helping with the mass spectrometric data deposit in ProteomeXChange/PRIDE. Navarrabiomed Proteomics Unit belongs to ProteoRed, PRB2-ISCIII, supported by grant PT13/0001, and is member of the Spanish Human Proteome Project (SpHPP) (Chromosome 16 Consortium). The authors appreciate the help from the research groups of Drs Sarobe and Lasarte from the CIMA, University of Navarra.

FUNDING

This work was funded by the following fellowships and grants: Miguel Servet Fellowship (CP12/03114); FIS project grant (PI14/00579) from the Instituto de Salud Carlos III, Spain; Refbio transpyrenaic collaborative project grants (NTBM); Sandra Ibarra Foundation grant; Gobierno de Navarra Grant (BMED 033–2014); Gobierno Vasco BioEf project grant (BIO13/CI/014). Purchase of reagents was in part supported by the BD Bioscience Spring 2014 Immunology Award to David Escors. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICTS OF INTEREST

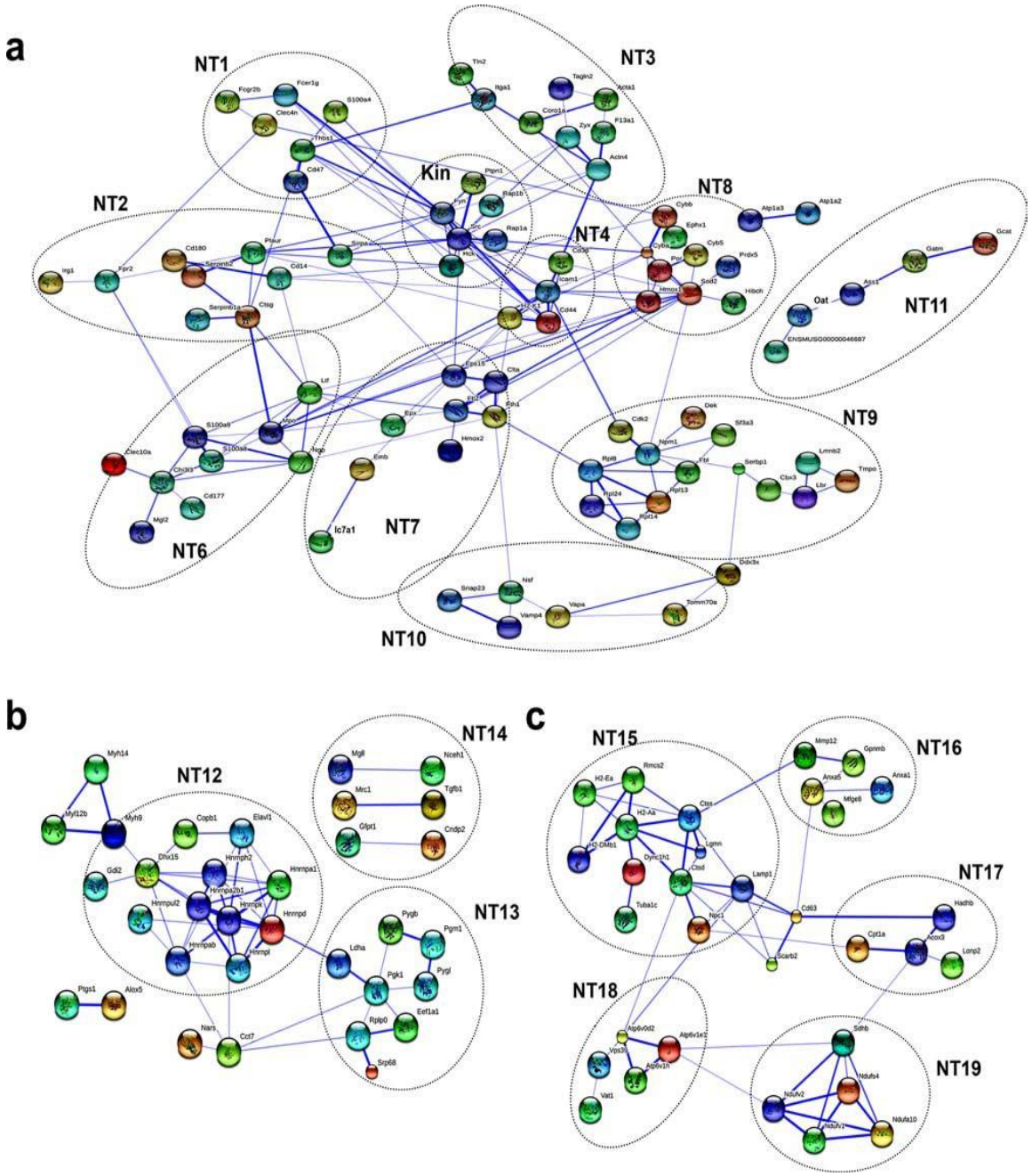
David Escors and Therese Liechtenstein are inventors of the MDSC production method (Patent file 14166221.3–1405). The other authors do not declare any conflict of interest.

AUTHORS' CONTRIBUTIONS

MGC designed, carried out experiments, collected, analyzed data and contributed to the writing of the manuscript. XMM analyzed mass spectrometry raw files, extracted proteomic information and generated the interactome maps. IBL designed, carried out experiments, collected, analyzed data and contributed to the writing of the manuscript. JFI carried out experiments, collected, analyzed mass spectrometry raw files, extracted proteomic information and generated the interactome maps. IZ carried out experiments, collected, analyzed data and contributed to the writing of the manuscript. TL designed, carried out experiments, collected, analyzed data and contributed to the writing of the manuscript. HA carried out experiments, collected and analyzed data. TL and NC generated data and contributed to the writing of the manuscript. SK and AC participated in the experiments with kinase inhibitors. DGS analyzed data. DE conceived the project, designed, carried out experiments, collected and analyzed

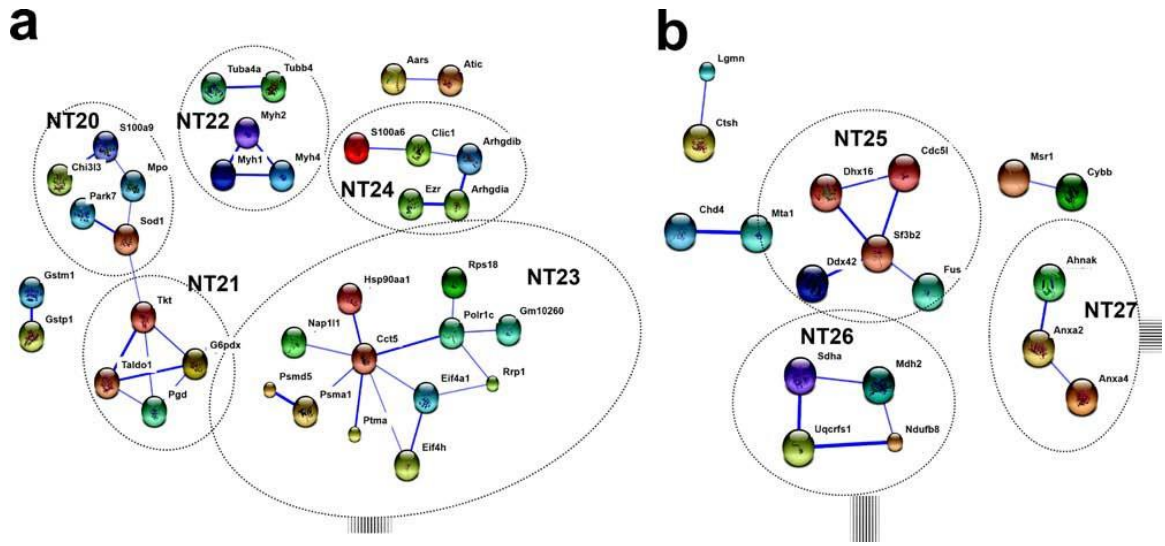
data and contributed to the writing of the manuscript. GK conceived the project, designed, carried out experiments, collected and analyzed data and contributed to the writing of the manuscript. ES conceived the project, designed experiments, analyzed mass spectrometry raw files, extracted proteomic information and generated the interactome maps.

SUPPLEMENTARY FIGURES AND TABLE



Supplementary Figure S1: STRING functional interactomes with lineage-specific differentially expressed proteins (B16-MDSC vs DCs). a. *Interactome using differentially up-regulated proteins as inputs. Medium (thin lines, score of 0.400) and high (thick lines, score of 0.700) confidence relationships between protein nodes are indicated in the graph. Internodal relationships were independently confirmed. Proteins were encircled and grouped as interconnected networks*

(NTs) to facilitate interpretation. A central network of kinases (Kin) is shown in the middle, which links the other networks. Networks NT1, NT2, NT3 and NT4 grouped membrane receptors together with associated signal transduction proteins. Networks NT6 and NT7 included S100A protein family members, c-type lectins, membrane receptors involved in phagocyte migration to sites of inflammation and phagocyte-associated enzymes. NT8 included redox proteins associated to reactive oxygen species (ROS) and protection against oxidative damage and xenobiotics. NT9 comprised ribosomal proteins and regulators of nuclear processes and cell division. NT10 included proteins involved in intracellular vesicle trafficking, while NT11 consisted in a network of aminoacid metabolic pathways. ***b.*** *Interactome map grouping differentially down-modulated proteins involved in splicesosome formation (NT12) and carbon metabolism (NT13).* ***c.*** *As in (b) with down-modulated proteins involved in MHC II antigen presentation (NT15), lysosomal functions (NT18) and mitochondrial complex I (NT19).*



Supplementary Figure S2: STRING Functional interactomes with tumor-regulated (B16-MDSCs vs NC-MDSCs) differentially expressed proteins. a. *STRING interactomes with up-regulated proteins. Medium (thin lines, score of 0.400) and high (thick lines, score of 0.700) confidence relationships between protein nodes are indicated in the graph. Internodal relationships were*

independently confirmed. Proteins were encircled and grouped as interconnected networks (NTs) to facilitate interpretation. NT20 grouped proteins involved in inflammatory receptor signaling and ROS scavenging proteins (SOD2, PARK7, MPO). NT21 grouped the pentose phosphate pathway. NT22 contained tubulin and myosin, pointing to changes in the cell cytoskeleton. NT23 included proteins involved in ribosomal RNA transcription, protein translation, folding and proteosomal degradation. NT24 contained proteins involved in membrane signaling, membrane structure and cytoskeleton rearrangements. ***b.*** *As in (a), NT25 contained proteins involved in gene expression and splicesosome organization. NT26 contained mitochondrial NADH dehydrogenase complex I and complex III proteins. NT27 contained cell growth-promoting and signal transduction proteins.*

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Annexin A5	Anxa5	24	0, 409148995	6, 08E-07
Rho guanine nucleotide exchange factor 2	Arhgef2	2	0, 414308614	6, 6E-05
Lactadherin	Mfge8	11	0, 429506653	4, 11E-06
Annexin A1	Anxa1	25	0, 481617476	6, 04E-05
Twinfilin-1	Twf1	2	0, 489112815	0, 000865
Tubulin alpha-1C chain	Tuba1c	18	0, 49021701	0, 0008
Class II histocompatibility antigen, M beta 1 chain	H2-DMb1;H2-DMb2	2	0, 491533209	8, 1E-05
H-2 class II histocompatibility antigen, E-U alpha chain	H2-Ea	5	0, 498582303	8, 08E-06
H-2 class II histocompatibility antigen, A-D alpha chain	H2-Aa	4	0, 522877994	6, 45E-06
Myosin regulatory light chain 12B	Myl12b;Myl9	9	0, 536878005	0, 000273
Glycogen phosphorylase, brain form	Pygb;Pygm	3	0, 538066216	0, 001733
Lon protease homolog 2, peroxisomal	Lonp2	2	0, 538215926	0, 000881
Solute carrier family 35 member F6	Slc35f6	2	0, 543109435	0, 003618
H-2 class II histocompatibility antigen, E-D beta chain		5	0, 557078509	1, 3E-05
Asparagine—tRNA ligase, cytoplasmic	Nars	4	0, 565052924	0, 000702
CD63 antigen	Cd63	2	0, 578425521	0, 003113
Fatty acid-binding protein, epidermal	Fabp5	3	0, 600473325	0, 000207
H-2 class II histocompatibility antigen, A-D beta chain	H2-Ab1	9	0, 609153728	7, 78E-05
Myosin-9	Myh9	111	0, 611970692	0, 000644
V-type proton ATPase subunit d 2	Atp6v0d2	7	0, 630823671	0, 000251
Heterogeneous nuclear ribonucleoproteins A2/B1	Hnrnpa2b1	14	0, 63694467	5, 2E-05
Myosin-14	Myh14	7	0, 642878205	0, 0003

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Uncharacterized protein	Gm8730;Rplp0	14	0, 648606361	0, 000193
Neutral cholesterol ester hydrolase 1	Nceh1	7	0, 651305733	4, 73E-05
Heterogeneous nuclear ribonucleoprotein A/B	Hnrnpab	7	0, 65477646	7, 23E-05
Monoglyceride lipase	Mgll	2	0, 657831546	0, 00342
Transmembrane glycoprotein NMB	Gpnmb	6	0, 66221021	9, 17E-05
von Willebrand factor A domain-containing protein 5A	Vwa5a	4	0, 663896759	0, 001158
Monoacylglycerol lipase ABHD12	Abhd12	6	0, 677723053	0, 003818
Ras-related GTP-binding protein C	Rragd;Rragc	2	0, 678167689	0, 004017
Lysosome membrane protein 2	Scarb2	4	0, 679787707	0, 000251
Heterogeneous nuclear ribonucleoprotein U-like protein 2	Hnrnpul2	7	0, 685215928	0, 001169
Cathepsin D	Ctsd	13	0, 691475625	0, 000874
Putative sodium-coupled neutral amino acid transporter 10	Slc38a10	3	0, 693741006	g 0, 004065
Signal recognition particle subunit SRP68	Srp68	2	0, 6969969	0, 00223
Alpha-2-macroglobulin receptor-associated protein	Lrpap1	20	0, 698359835	0, 000132
Elongation factor 1-alpha 1	Eef1a1;Eef1a2	18	0, 700559018	0, 000256
Cathepsin S	Ctss	6	0, 701751582	0, 001797
Estradiol 17-beta-dehydrogenase 11	Hsd17b11	4	0, 703096327	0, 000583
Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	Sdhb	9	0, 706493357	0, 000214
Transforming growth factor beta-1	Tgfb1	2	0, 707417988	0, 002654
Tropomyosin alpha-3 chain	Tpm3;Tpm3-rs7	21	0, 7087286	0, 006006

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
WASH complex subunit strumpellin	Kiaa0196	2	0, 710467852	0, 006814
Lysosome-associated membrane glycoprotein 1	Lamp1	4	0, 718359696	0, 003244
ELAV-like protein 1	Elavl1	5	0, 720005557	0, 000596
LEM domain-containing protein 2	Lemd2	4	0, 723410824	0, 002332
Aminopeptidase N	Anpep	26	0, 724568989	0, 000451
Heterogeneous nuclear ribonucleoprotein A1	Hnrnpa1	8	0, 728604525	0, 000505
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	Ndufa10	6	0, 730876406	0, 003613
Heterogeneous nuclear ribonucleoprotein H2	Hnrnph2	6	0, 731481958	0, 000661
Legumain	Lgmn	3	0, 733714602	0, 00072
ER membrane protein complex subunit 1	Emc1	6	0, 736539664	0, 006507
Serpin B6	Serpinb6a;Serpinb6	8	0, 739418148	0, 002021
Nicastrin	Ncstn	5	0, 739929041	0, 001134
Cytoplasmic dynein 1 heavy chain 1	Dync1 h1	36	0, 746509337	0, 000733
Macrophage metalloelastase	Mmp12	11	0, 747676988	0, 003364
L-amino-acid oxidase	Il4i1	4	0, 748410986	0, 003715
DnaJ homolog subfamily C member 11	Dnajc11	3	0, 74862864	0, 001608
Glutamine—fructose-6-phosphate aminotransferase [isomerizing] 1	Gfpt1	4	0, 749338067	0, 0075
Lymphocyte antigen 75	Ly75	4	0, 750276114	0, 007796
Macrophage mannose receptor 1	Mrc1	18	0, 750401561	0, 003811
Golgi membrane protein 1	Golm1	2	0, 751319227	0, 001423
Dipeptidyl peptidase 2	Dpp7	2	0, 751371795	0, 002028
Vam6/Vps39-like protein	Vps39	2	0, 753460592	0, 001331

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Amine oxidase [flavin-containing] A	Maoa	3	0, 753928115	0, 007226
Creatine kinase B-type	Ckb	8	0, 754517987	0, 001915
Nicotinamide phosphoribosyltransferase	Nampt	2	0, 755875358	0, 00465
Inosine triphosphate pyrophosphatase	Itpa	2	0, 760176881	0, 008895
Heterogeneous nuclear ribonucleoprotein L	Hnrnpl	7	0, 76047527	0, 001026
Ribonuclease inhibitor	Rnh1	7	0, 760502021	0, 000888
B-cell receptor-associated protein 31	Bcap31	11	0, 761263852	0, 00613
NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	Ndufs4	5	0, 762621458	0, 005491
V-type proton ATPase subunit E 1	Atp6v1e1	16	0, 763478047	0, 004022
Heterogeneous nuclear ribonucleoprotein K	Hnrnpk;Gm7964	12	0, 763856162	0, 005636
L-lactate dehydrogenase	Ldha	13	0, 764291243	0, 000965
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX15	Dhx15	7	0, 765273038	0, 001402
Phosphoglycerate kinase 1	Pgk1	18	0, 766086802	0, 007756
Glycogen phosphorylase, liver form	Pygl	16	0, 766633266	0, 0034
NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	Ndufv2	7	0, 76712425	0, 004833
NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	Ndufv1	10	0, 769871468	0, 004625
Mimitin, mitochondrial	Ndufaf2	2	1, 30381531	0, 002611
Mitochondrial import receptor subunit TOM70	Tomm70a	7	1, 304563258	0, 001894
Ras-related protein Rap-1b	Rap1b;Rap1a	6	1, 307217616	0, 003029
Tyrosine-protein phosphatase non-receptor type 1	Ptpn1	12	1, 310480124	0, 001443

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Lactotransferrin	Ltf	6	1, 311085	0, 009649
Vacuolar protein sorting-associated protein 33A	Vps33a	2	1, 317391275	0, 000905
Mitochondrial import inner membrane translocase subunit Tim8 A	Timm8a1;Timm8a2	2	1, 322616616	0, 003141
Argininosuccinate synthase	Ass1;Gm5424	9	1, 323069518	0, 004604
Ferritin	Ftl1;Ftl2	10	1, 323319619	0, 001567
Protein S100-A4	S100a4	3	1, 326852892	0, 002104
Nucleophosmin	Npm1;Gm5611	14	1, 328008535	0, 000918
Integrin alpha-1	Itga1	5	1, 33234292	0, 008921
Epidermal growth factor receptor substrate 15	Eps15	4	1, 335132857	0, 007084
Adenylate kinase 2, mitochondrial	Ak2	9	1, 339117363	0, 000355
Lamin-B receptor	Lbr	10	1, 340226149	0, 002185
Heme oxygenase 1	Hmox1	7	1, 341204992	0, 00124
Platelet receptor Gi24	4632428N05Rik	4	1, 350347861	0, 002464
Transgelin-2	Tagln2	12	1, 351776016	0, 001362
Low affinity immunoglobulin gamma Fc region receptor II	Fcgr2	6	1, 366625261	0, 002449
Leukocyte elastase inhibitor A	Serpinb1a	6	1, 37092156	0, 001528
Perilipin-3	Plin3	3	1, 373402061	0, 002905
Superoxide dismutase [Mn], mitochondrial	Sod2	8	1, 373760594	0, 001123
High affinity immunoglobulin epsilon receptor subunit gamma	Fcer1g	4	1, 374118744	0, 00872
Embigin	Emb	4	1, 377538576	0, 001189
Formyl peptide receptor 2	Fpr2	3	1, 379093299	0, 000644
Cytochrome b5	Cyb5a	6	1, 380498718	0, 001192
Chromobox protein homolog 3	Cbx3	6	1, 380952591	0, 001884
Acid sphingomyelinase-like phosphodiesterase 3b	Smpdl3b	4	1, 384287037	0, 002606

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Protein-arginine deiminase type-4	Padi4	9	1, 40155117	0, 000424
Protein LYRIC	Mtdh	7	1, 406115785	0, 002098
Bcl-2-like protein 13	Bcl2l13	3	1, 408599571	0, 001685
H-2 class I histocompatibility antigen, K-B alpha chain	H2-K1	8	1, 41010511	0, 001533
Napsin-A	Napsa	2	1, 411600269	0, 005092
Coronin-1A	Coro1a	12	1, 414013879	0, 00018
2-amino-3-ketobutyrate coenzyme A ligase, mitochondrial	Gcat	2	1, 421520698	0, 006462
Cyclin-dependent kinase 2	Cdk2	2	1, 426124115	0, 009262
Plexin domain-containing protein 2	Plxdc2	5	1, 42751035	0, 00033
Rho GTPase-activating protein 1	Arhgap1	5	1, 428460911	0, 000189
Histone H1.0	H1f0	3	1, 429352055	0, 00982
Peroxiredoxin-5, mitochondrial	Prdx5	10	1, 43101649	0, 000163
Cytochrome b-245 light chain	Cyba	5	1, 432458891	0, 000559
CD44 antigen	Cd44	3	1, 443457084	0, 000215
Zyxin	Zyx	4	1, 444345746	0, 008419
Cytochrome b-245 heavy chain	Cybb	10	1, 455588804	0, 000222
Chitinase-like protein 3	Chil3	18	1, 46074136	0, 000102
Tyrosine-protein kinase HCK	Hck	5	1, 464103351	0, 000238
Leukocyte surface antigen CD47	Cd47	2	1, 465022748	0, 003868
C-type lectin domain family 6 member A	Clec4n;Clec6a	2	1, 467063188	0, 00017
CD177 antigen	Cd177	5	1, 473425935	0, 002559
Lamina-associated polypeptide 2, isoforms beta/delta/epsilon/gamma	Tmpo	11	1, 476178931	0, 00123
Glycerol kinase	Gyk	2	1, 481175737	0, 000568
Alpha-actinin-4	Actn4	36	1, 485459058	7, 33E-05

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
ATP-dependent (S)-NAD(P)H-hydrate dehydratase	Carkd	2	1, 487792072	0, 004226
Actin, alpha skeletal muscle	Acta1	17	1, 490499494	0, 001138
Plasminogen activator inhibitor 1 RNA-binding protein	Serbp1	7	1, 515466638	0, 000249
Tyrosine-protein kinase Fyn	Fyn;Yes1	3	1, 524885079	0, 003341
Sodium/potassium-transporting ATPase subunit alpha-3	Atp1a3;Atp1a2	13	1, 525120684	0, 002015
60S ribosomal protein L8	Rpl8	5	1, 526316859	0, 00643
Urokinase plasminogen activator surface receptor	Plaur	2	1, 529089244	0, 000475
Vesicle-associated membrane protein 4	Vamp4	3	1, 538993145	0, 001874
ADP-ribosyl cyclase/ cyclic ADP-ribose hydrolase 1	Cd38	3	1, 539999685	0, 000433
60S ribosomal protein L24	Rpl24;Gm17430	4	1, 542903394	0, 009811
Plasminogen activator inhibitor 2, macrophage	Serpinb2	3	1, 562731137	0, 000522
60S ribosomal protein L13	Rpl13	3	1, 568815619	0, 000839
Coagulation factor XIII A chain	F13a1	5	1, 575022778	0, 002436
Protein S100-A9	S100a9	5	1, 585262138	0, 000482
C-type lectin domain family 10 member A	Clec10a	9	1, 608439704	0, 000107
3-hydroxyisobutyryl-CoA hydrolase, mitochondrial	Hibch	2	1, 633954389	0, 002052
Neuronal proto-oncogene tyrosine-protein kinase Src	Src	3	1, 641084449	0, 000776
Macrophage galactose N-acetyl-galactosamine specific lectin 2	Mgl2	13	1, 648910806	3, 73E-05
Monocyte differentiation antigen CD14	Cd14	3	1, 649302138	0, 000391

B16-MDSC vs DC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Signal-regulatory protein alpha	Sirpa	5	1, 667987603	0, 007842
High affinity cationic amino acid transporter 1	Slc7a1	2	1, 683193993	0, 002121
Cathepsin G	Ctsg	2	1, 684502903	0, 000662
Zinc transporter ZIP4	Slc39a4	2	1, 701167303	0, 000458
Ferritin heavy chain	Fth1	3	1, 70872916	4, 24E-05
Phostensin	Ppp1r18	4	1, 734747128	0, 001686
Myeloperoxidase	Mpo	14	1, 739785165	1, 48E-05
Citrate lyase subunit beta-like protein, mitochondrial	Clybl	2	1, 812987713	0, 00626
Brain acid soluble protein 1	Basp1	8	1, 855107248	8, 3E-05
Carbonic anhydrase 4	Ca4;Car4	10	1, 910244551	4, 79E-06
Lymphocyte-specific protein 1	Lsp1	12	1, 914761621	2, 34E-06
Protein DEK	Dek	3	1, 924824794	6, 07E-05
Talin-2	Tln2	7	1, 958388414	0, 002108
Protein Ahnak	Ahnak	95	2, 08080018	2, 1E-06
60S ribosomal protein L14	Rpl14	3	2, 097473177	0, 00187
Protein S100-A8	S100a8	6	2, 162403323	6, 12E-05
Eosinophil peroxidase	Epx	16	2, 166652695	3, 09E-05
Myeloid bactenecin (F1)	Ngp	3	2, 254673881	4, 64E-06
Interferon-induced transmembrane protein 3	Ifitm3	3	2, 270237035	2, 09E-06

NC-MDSC vs B16-MDSC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
MCG130182, isoform CRA_a	Gm5483	3	0, 589206137	0, 000126
26S proteasome non-ATPase regulatory subunit 5	Psmd5	2	0, 598593317	0, 000267
Eukaryotic translation initiation factor 4H	Eif4 h	4	0, 611736747	0, 001747
CapZ-interacting protein	Rcsd1	3	0, 612295734	0, 000745
Protein S100-A6	S100a6	2	0, 617006908	0, 008245
Rho GDP-dissociation inhibitor 2	Arhgdib	7	0, 618770272	0, 000466
Tubulin alpha-4A chain	Tuba4a	10	0, 626186143	0, 003667
Superoxide dismutase [Cu-Zn]	Sod1	3	0, 631095674	0, 000772
Nucleosome assembly protein 1-like 1	Nap1l1	5	0, 640182294	0, 004251
Protein S100-A9	S100a9	5	0, 648160088	0, 001437
Prothymosin alpha	Ptma	3	0, 651999552	0, 00052
Myosin light polypeptide 6	Myl6	8	0, 656424969	0, 005285
Astrocytic phosphoprotein PEA-15	Pea15	2	0, 666877728	0, 003191
DNA-directed RNA polymerases I and III subunit RPAC1	Polr1c	2	0, 67045724	0, 006728
Glucose-6-phosphate 1-dehydrogenase X	G6pdx	13	0, 670989902	0, 001161
Spermine synthase	Sms	2	0, 673833379	0, 005298
Leukotriene A-4 hydrolase	Lta4 h	13	0, 686384982	0, 001508
Myeloperoxidase	Mpo	7	0, 688935214	0, 005634
Alpha-N-acetylglucosaminidase	Naglu	3	0, 6900478	0, 009688
Eukaryotic initiation factor 4A-I	Eif4a1	13	0, 694111659	0, 001646
Tubulin beta-4B chain	Tubb4b;Tubb4a	14	0, 696428165	0, 005599
Transaldolase	Taldo1	10	0, 703041508	0, 001204
Rho GDP-dissociation inhibitor 1	Arhgdia	5	0, 708523919	0, 002027
6-phosphogluconate dehydrogenase, decarboxylating	Pgd	13	0, 709492257	0, 003216
Glutathione S-transferase P 1	Gstp1	3	0, 715568979	0, 006782
Alanine—tRNA ligase, cytoplasmic	Aars	9	0, 724055571	0, 00406
T-complex protein 1 subunit epsilon	Cct5	7	0, 725355973	0, 008302
Destrin	Dstn	4	0, 726632332	0, 005658
Ubiquitin-like modifier-activating enzyme 1	Uba1	16	0, 727024557	0, 005177

NC-MDSC vs B16-MDSC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
ATPase Asna1	Asna1	2	0, 72757609	0, 007588
Ezrin	Ezr	26	0, 730834893	0, 005634
Phosphatidylethanolamine-binding protein 1	Pebp1	3	0, 734400826	0, 005071
Ribosomal RNA processing protein 1 homolog A	Rrp1	2	0, 738249657	0, 006636
Transketolase	Tkt	11	0, 741843927	0, 00586
Peroxiredoxin-6	Prdx6	8	0, 743801046	0, 002992
Protein DJ-1	Park7	5	0, 749901306	0, 007964
Heat shock protein HSP 90-alpha	Hsp90aa1	23	0, 750975375	0, 003701
Chloride intracellular channel protein 1	Clic1	8	0, 751762163	0, 005866
40S ribosomal protein S18	Rps18;Gm10260	4	0, 753299597	0, 005027
Glutathione S-transferase Mu 1	Gstm1	7	0, 753382783	0, 008871
Ras suppressor protein 1	Rsu1	2	0, 753677287	0, 004846
Proteasome subunit alpha type-1	Psma1	6	0, 761777735	0, 007526
Bifunctional purine biosynthesis protein PURH	Atic	9	0, 765684291	0, 00599
Macrophage scavenger receptor types I and II	Msr1	6	1, 303825752	0, 009559
Protein Sf3b2	Sf3b2	8	1, 310630121	0, 004769
Malate dehydrogenase, mitochondrial	Mdh2	10	1, 316433466	0, 005662
ATP-dependent RNA helicase DDX42	Ddx42	2	1, 326602896	0, 0084
Beta-1, 4 N-acetylgalactosaminyltransferase 1	B4galnt1	5	1, 328280926	0, 003965
Legumain	Lgmn	5	1, 363515033	0, 003449
E3 UFM1-protein ligase 1	Ufl1	3	1, 368089173	0, 005859
Brain acid soluble protein 1	Basp1	8	1, 369738842	0, 007428
Cytochrome b-245 heavy chain	Cybb	8	1, 379745693	0, 007504
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	Ndufb8	2	1, 379768717	0, 009965
RNA-binding protein FUS	Fus	4	1, 383579198	0, 003469
Annexin A2	Anxa2	23	1, 387238034	0, 001975
Hexokinase-2	Hk2	12	1, 391029042	0, 004338
Protein Ahnak	Maoa	82	1, 397110044	0, 003518

NC-MDSC vs B16-MDSC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	Ckb	11	1, 400581193	0, 007454
Chromodomain-helicase-DNA-binding protein 4	Nampt	5	1, 401800102	0, 004666
Plasminogen receptor (KT)	Itpa	3	1, 417180455	0, 003521
Thyroid hormone receptor-associated protein 3	Hnrnpl	5	1, 418157791	0, 002434
Zinc-binding alcohol dehydrogenase domain-containing protein 2	Rnh1	5	1, 432808549	0, 002205
Normal mucosa of esophagus-specific gene 1 protein	Bcap31	2	1, 447669214	0, 006718
Pro-cathepsin H	Ndufs4	2	1, 448045875	0, 0024
Annexin A4	Atp6v1e1	20	1, 469858566	0, 004418
Isopentenyl-diphosphate Delta-isomerase 1	Hnrnpk;Gm7964	4	1, 484479927	0, 007223
Heterogeneous nuclear ribonucleoprotein U-like protein 2	Ldha	7	1, 512224046	0, 003377
Cytochrome b-c1 complex subunit Rieske, mitochondrial	Dhx15	6	1, 514801192	0, 000731
RNA-binding protein 25	Pgk1	3	1, 519285254	0, 005808
Lysosome-associated membrane glycoprotein 1	Pygl	4	1, 528207246	0, 005381
Dhx16 protein	Ndufv2	2	1, 535132714	0, 006593
Probable ATP-dependent RNA helicase DDX6	Ndufv1	2	1, 624024139	0, 006046
Cell division cycle 5-like protein	Ndufaf2	2	1, 657520224	0, 006832
Lysosome-associated membrane glycoprotein 2	Tomm70a	3	1, 684964459	0, 007944
ADP/ATP translocase 1	Rap1b;Rap1a	5	1, 714237166	0, 009989
Epoxide hydrolase 1	Ptpn1	2	1, 800951684	0, 001541
Metastasis-associated protein MTA1	Ltf	3	2, 002434335	0, 005013
Arginase-1	Vps33a	10	2, 305826701	4, 13E-06

NC-MDSC vs B16-MDSC differentially expressed proteins				
Protein names	**Gene names**	**Peptides**	**Fold change**	***P* value**
Myosin-4	Myh2;Myh4;Myh1	3	0, 272566219	0, 00017
Lymphocyte-specific protein 1	Lsp1	6	0, 421060939	0, 000113
MCG130173	Stfa2l1	2	0, 513643456	4, 06E-05
2010005H15Rik protein	2010005H15Rik	3	0, 525149229	5, 18E-05
Chitinase-like protein 3	Chil3	12	0, 550932031	0, 000314
D-3-phosphoglycerate dehydrogenase	Phgdh	5	0, 564754318	8, 73E-05
MCG130175, isoform CRA_b	BC100530	5	0, 579007568	8, 97E-05

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DISCUSSION

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Tumors escape from the immune attack by several means. In this Ph.D thesis I studied two of the mechanisms of tumor escape and disease progression; the establishment of immunosuppressive interactions between T cells and tumor cells, and the biology of MDSCs.

1. PD-L1 INTRACELLULAR SIGNALLING AS A MECHANISM OF TUMOR PROGRESSION

The first immunosuppressive barrier that I studied was the PD-L1/PD-1 interaction. The results from this Ph.D thesis have contributed to the understanding of this key interaction in two major points. First, PD-L1 expression in cancer cells contribute to the survival of cancer cells by conferring resistance to IFN toxicity. Second, two sequence motifs have been mapped within the intracytoplasmic domain of PD-L1 that participate in IFN-β resistance by transmitting putative intracellular signals to the cancer cell.

Surprisingly, very little is still known on the biological mechanisms of action of PD-L1/PD-1 interactions, compared to the extent to which PD-L1/PD-1 blockade is utilized in the clinic. Although this might be practical for the point of view of the patient, the lack of knowledge on how these interactions work can make several opportunities for therapeutic interventions to be missed.

There is a general consensus on the association of PD-L1 expression with tumor progression. The direct inhibition of T cells by PD-L1 has a strong enhancing effect on tumor progression by counteracting the immunological attack. Here, the direct participation of PD-L1 expression in the survival of cancer cells was studied.

1.1. PD-L1 intracellular motifs

Before this Ph.D thesis, there was a somewhat surprising lack of studies on PD-L1 intracellular signal transduction. Only a very few previous studies addressed this issue, some of them in a rather indirect manner. For example, there was evidence that PD-L1 intracellular signals to cancer cells favored their survival (Azuma et al. 2008), and regulated cancer cell aerobic glycolysis and autophagy through the AKT/mTOR signaling (Chen et al. 2014; Shi et al. 2013; Chang et al. 2016; Palmer et al. 2015; Thompson et al. 2004) (Figures 10 and 7B).

Indeed, no signaling motifs had been identified or mapped in the intracytoplasmic domain of PD-L1 before. While addressing this issue, we decided to employ a classical approach to identify specific motifs with potential signal transduction capacities. An alignment of the cytoplasmic region of 10 mammalian PD-L1 molecules readily highlighted three highly conserved sequence motifs. One of these motifs was the "RMLDVEKC" sequence. Its removal eliminated the capacities of PD-L1 to counteract IFN-β cytotoxicity, leading to cell death. The second motif, the "DTSSK" sequence was a strong inhibitor of PD-L1 anti-IFN-β functions. Its removal potentiated the growth and survival of cancer cells in the presence of IFN-β, suggesting that it was a negative regulator of PD-L1 functions. The third motif "QFEET" could be removed without having any functional consequence at least on cell growth and survival. Of note, the importance of the RMLDVEKC sequence was also indirectly highlighted by two facts: (1) The salmon PD-L1 molecule was a DTSSK/QFEET-deleted version of the mammalian counterparts; (2) no somatic mutations were found in databases of human cancer genomic sequences affecting any of the residues comprising this sequence. In contrast, most of the mutations affected the DTSSK motif. Indeed, considering mutations leading to disrupting aminoacid changes, 5 out of the 7 directly affected the human homolog of the inhibitory motif DTSSK (Figure 5A Cell Reports). These changes were potentially highly disruptive, and I tested two of them in the murine PD-L1 homologue positions (D276H and K280N). These mutations disrupted the inhibitory functions of

DTSSK, strongly enhancing resistance to type I and type II IFNs. Although there was no time to check the other mutations, it is likely that these may disrupt DTSSK inhibitory capacities.

Hence, selected somatic mutations in the PD-L1 gene in human carcinomas keep the RMLDVEKC motif unaltered, while disrupting the DTSSK inhibitory motif. These cancer cells will probably express hyperactivated PD-L1 mutants conferring cancer cells stronger resistance to IFN cytotoxicity. In this Ph.D thesis, I also tested if alternative sequence motifs could be found using bioinformatics databases. Interestingly, only Motif finder (http://www.genome.jp/tools-bin/search_motif_lib) identified a sequence in the intracellular domain of the murine PD-L1 gene with high similarity to a domain present in DNA-dependent RNA polymerase beta subunits. Even more, this polymerase-like sequence spanned the two of the motifs found in this Ph.D with signal-transduction activities (Figure 12). This result may indicate convergence towards a particular structural feature, although this is only speculation.

Other motifs that can affect PD-L1 functions are ubiquitination sequences, which may regulate PD-L1 stability. Indeed, cancer cells subjected to TNF-α upregulate COP9 signalosome 5 (CSN5), which stabilizes PD-L1 by favoring its de-ubiquitination (Lim et al. 2016). It has also been recently shown that the stability and functions of PD-L1 are regulated by stabilizing interactions with membrane proteins such as CMTM6 and CMTM4 (Mezzadra et al. 2017). These proteins inhibit PD-L1 ubiquitination by STUB1 at the plasma membrane, preventing its degradation in lysosomes (Burr et al. 2018; Mezzadra et al. 2017). Interestingly, the exact lysine residues that get ubiquitinated were not identified in any of the previous studies. In this Ph.D thesis I also addressed the possibility of ubiquitination to regulate PD-L1 intracellular signaling. There are three lysine residues susceptible of ubiquitination, two of them within the murine RMLDVEKC and DTSSK motifs, and one of them at the transmembrane-cytoplasmic interphase. By mutating the RMLDVEKC/DTSSK lysine residues to arginines (a similar aminoacid that cannot get ubiquitinated) I found them to be

negative regulators of the anti-IFN-β capacities of PD-L1. However, in this thesis it was not directly demonstrated whether these lysines do get ubiquitinated. Nevertheless, it could be hypothesized that PD-L1 ubiquitination is a major regulatory mechanism of its functions by either degrading it and inhibiting signal transduction (Sathianathen et al. 2017) or by modulating the recruitment of other signaling components (Powles et al. 2018). The existence of this regulation is strengthen by our data demonstrating that the inhibitory activity of DTSSK also depended on its lysine, and that one selected somatic mutation in the human DTSSK homologue disrupted the lysine residue in a cervix carcinoma. It could be interesting to test whether for example STUB1 can introduce ubiquitination in lysines 271 and 280 or CSN5 prevent their ubiquitination.

1.2. PD-L1 cross-talk with interferon signaling in cancer cells

The elucidation of the exact molecular mechanisms behind the signal transduction capacities of the motifs identified in this Ph.D thesis can be challenging. It could be possible that kinases or phosphatases physically bind these domains either directly or through adaptor proteins that may crosstalk with other signal transduction pathways. Proteins belonging to the mTOR signaling pathway may be good candidates according to the experimental evidence so far.

Therefore, I decided to approach this problem by first elucidating the stage at which PD-L1 interfered with IFN-β signal transduction, a common theme utilized by cancer cells for tumor progression. One of such mechanisms is IFNAR1 down-modulation, that overcomes oncogene-induced senescence in melanoma (Katlinskaya et al. 2016). However, PD-L1 overexpression did not alter IFNAR1 and IFNAR2 surface levels in B16 melanoma cells (Figure 1 Cell reports). Nevertheless, silencing of IFNAR1 or JAK1 completely protected melanoma cells from IFN-β-dependent apoptosis, similarly to the effects observed by PD-L1 overexpression (Figure 1 Cell reports).

The evidence provided in this thesis shows that lack of PD-L1 enhanced STAT3 expression and its tyrosine 705 phosphorylation without affecting STAT1 or STAT2 after IFN-β stimulation (Figure 3 Cell reports). Pfeffer et al showed that non-phosphorylated STAT3 also plays an important role in the IFN I response pathway (Pfeffer et al. 2017) and Ren et al proposed that apoptosis caused by IFN-β could be driven through STAT3 in breast cancer (Ren et al. 2017). Caspases 7 and 9 were strongly up-regulated and required for IFN-β-dependent apoptosis (Figure 13, Figure 3 Cell Reports). Recently, our results were corroborated by Luo et al. and Garcia-Diaz et al. using shRNA-based or CRISPR-based genetic screenings, respectively (Garcia-Diaz et al. 2017; Luo et al. 2018). JAK1 but not JAK2 was found to be the primary and essential mediator of STAT1, STAT3 and STAT5 phosphorylation following IFN-ϒ stimulation, resulting in PD-L1 upregulation. PD-L1 is transcriptionally up-regulated by STAT1/STAT2 and STAT3 (Garcia-Diaz et al. 2017) after IFN exposure. Our data adds that PD-L1 up-regulation would counteract the apoptotic branch of the IFN-signalling pathway, allowing cancer cells to survive. As many studies show that STAT3 and STAT5 phosphorylation in cancer cells are indeed pro-tumorigenic, especially when induced by JAK2 (Wellbrock et al. 2005; Yu et al. 2016), it could be tested whether this is in part caused by the anti-apoptotic effects of PD-L1 up-regulation. Indeed, these authors propose that PTPN2 (JAK2) inhibitors would potentiate JAK1 activities and the enhancement of IFN cytotoxicity (Luo et al. 2018; Manguso et al. 2017).

IFN-β induces the expression of TRAIL that triggers caspase 8 and 3 dependent apoptosis in melanoma and breast cancer cells (Bernardo et al. 2013; Chawla-Sarkar et al. 2001). In cervical carcinoma, IFN-β causes proliferative arrest and accumulation of the anti-apoptotic protein cFLIP and caspase 8 (Apelbaum et al. 2013). Our data shows that STAT3 clearly has an anti-oncogenic function by promoting apoptosis through caspases 7 and 9 in melanoma cells after IFN-β exposure.

As mentioned above, this seems to be a discrepancy with other published work. Phospho-STAT3 Y705 and sometimes S754 has been linked to oncogenic and anti-apoptotic capacities in several cancers (Hsia et al. 2017;

Ganguly et al. 2018; Ni 2018; Liu et al. 2018; Zhang et al. 2011; Bowman et al. 2000; Darnell et al. 2005; Bharti et al. 2013; Couronné et al. 2013; Pencik et al. 2016). Inhibition of the STAT3 pathway counteracts tumor progression in vitro, in vivo and in some clinical trials. Some examples are a phase I study of an oral STAT3 inhibitor OPB-31121, in patients with advanced solid tumours witch concluded preliminary antitumour activity and safety of it (Oh et al. 2015) and a phase I clinical trial of an oral STAT3 direct inhibitor (C188-9) for patients with advanced cancers that is currently on (https://clinicaltrials.gov/ct2/show/NCT03195699).

These discrepancies could have several explanations. First, STAT3 could have different functions within cancer cells, depending on the initiating stimulus. In our case, IFN-β is the initiating stimulus and its antitumor and pro-apoptotic functions through JAK/STAT pathway are well-known. Second, STAT3 can be phosphorylated in several sites, and here our data strongly suggests that tyrosine 705 phosphorylation is directly linked to apoptosis. Other authors link this phosphorylation to anti-apoptotic responses (Ganguly et al. 2018; Zhang et al. 2011). However, it could well be that specific combinations of phosphorylated/non-phosphorylated sites may confer differential functions to STAT3. Third, STAT3 could play a dual role by having a pro-apoptotic function first, but triggering a pro-tumorigenic effect by further causing PD-L1 transcriptional up-regulation. Indeed, there is evidence that lentiviruses-transduced-microRNA-3127-5p expression leads to PD-L1 elevation and suppresses autophagy through increase STAT3 phosphorylation, promoting human non-small lung cell carcinoma growth. However, the specific mechanism by which this mircroRNA achieves these effects were not described by the authors of the study (Tang et al. 2018). Other studies have demonstrated that STAT3 is involved in PD-L1 upregulation in several cancers (Fujita et al. 2015; Horlad et al. 2016). Our data corroborate those results but points to caspase 7 mainly and caspase 9. Four, other factors that were not studied in this thesis may also influence the outcome. For example, in head and neck cancer STAT3 SUMOylation increases STAT3 Y705 phosphorilation (Zhou et al. 2016). Other pathways such as MAPKs, PI3K, or NF-κB cooperate with JAK/STAT signaling

(Gough et al. 2008). IFNs also activate some protein kinase C isoforms (PKCs) and the multifunctional adaptor protein CrkL (Schreiber al. 2017; Suda et al. 2012; Barbosa et al. 2014). The contribution of some of these pathways was part of this PhD thesis, which is currently undergoing.

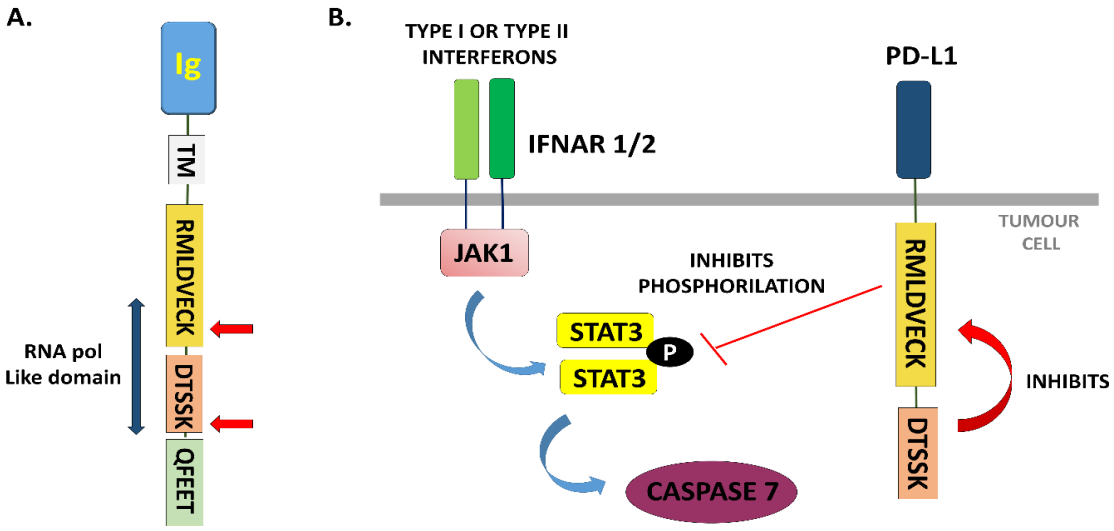

Figure 12. PD-L1 domains and cross-talk with interferon signaling in cancer cells. ***(A)*** *The domain structure of PD-L1 is represented in the figure. Ig, the extracellular immunoglobulin domain; TM, transmembrane domain. The RMLDVEKC, DTSSK and QFEET motifs are represented in the intracytoplasmic region of PD- L1. The RNApol-like motif identified by MotifFinder is indicated, which contains part of the RMLDVEKC motif and the whole DTSSK motif. Red arrows point the inhibitory lysines.* ***(B)*** *The mechanism by which PD- L1 counteracts interferon-mediated apoptosis is represented in the figure. A function associated to the RMLDVEKC motif is required to inhibit STAT3 phosphorylation, which in turns stops caspase7-mediated apoptosis. The DTSSK motif acts as a negative regulator of the RMLDVEKC motif.*

1.3. Consequences of PD-L1-dependent inhibition of the ifn signaling pathway in cancer cells

It is highly likely that PD-L1/PD-1 blockade with antibodies may sensitize cancer cells to IFN-β cytotoxicity similarly to silencing or abrogation of PD-L1. This has been shown *in vitro* in this Ph.D thesis by using anti-PD-L1 antibodies. Indeed, inactivating mutations in components of the IFNs signal transduction pathway enhance cancer progression, pointing to the necessity of disrupting this pathway in many cases for tumor progression (Nakanishi et al. 2007; Yao et al. 2009; Gao et al. 2016; Zaretsky et al. 2016). Some of these mutations contribute to resistance to anti-PD-1 therapy (Shin et al. 2017; Zaretsky et al. 2016). Other mechanisms of tumor escape include downregulation of IFN receptors after ubiquitination by SCF-bTrcp2/HOS E3 ubiquitin ligase (Kumar et al. 2003; Splawski et al. 2004; Kumar et al. 2007; Katlinskaya et al. 2016).

The group led by Dr Antoni Ribas proposed that loss of IFN signaling in cancer cells prevented adaptive up-regulation of PD-L1, becoming “PD-L1” negative tumors (Zaretsky et al. 2016). These tumors then would be intrinsically resistant to PD-L1/PD-1 blockade. However, this idea is difficult to reconcile with our current understanding of PD-L1/PD-1 interactions. It would be expected that PD-1+ T cells would be “free” to exert their cytotoxic activities over PD-L1-negative tumors. The data obtained in this Ph.D thesis offers a complementary interpretation. PD-L1/PD-1 blockade would sensitize cancer cells to IFN-induced apoptosis leading to cancer cell death. Only cancer cell variants with mutations in the IFN signal transduction pathway would be selected because no apoptosis will occur, in analogy to the B16 melanoma cells with silenced IFNAR1 or JAK1. These cells thrive even in non-physiologically high concentrations of IFNs (Gato-Cañas et al. 2017).

The data presented in this Ph.D thesis also offers mechanistic bases for combining PD-L1 blockade and IFN treatment. IFN-β has been used to treat melanoma as an adjuvant therapy prolonging survival (Uehara et al. 2017). Although most of the evidence suggests that IFNs are not effective at priming T cell responses, they are required when produced by T cells as they induce tumor cell apoptosis, neoantigen release, destruction of tumor vasculature and

increasing immune cell tumor infiltration (Fujimura et al. 2009; Escors et al. 2013). IFNs possess immunomodulatory activities by the induction of CCL5 and CXCR3 ligands (CXCL9-11) in melanoma, which could play a role in T-cell recruitment, enhancing anti-PD-L1 mAb treatment (Hong et al. 2011; Liu et al. 2015; Kakizaki et al. 2015). Increased tumor destruction by IFN-β provokes the priming of neoantigen-specific T-cells that can be mobilized with anti-PD-L1 mAb (Uehara et al. 2017). In agreement with this model, in this thesis cancer cells expressing a signal-null PD-L1 combined with intratumor delivery of lentivector encoding IFN-β was sufficient to counteract tumor growth even if PD-L1 could still engage PD-1 on T cells. Indeed, there is not a need to directly use IFNs. Type I IFN stimulators have potent anti-cancer activities that synergize with PD-L1/PD-1 blockade, including TLR3 agonists and STING (Stimulator of IFN genes) agonists (Woo et al. 2015; Leach et al. 2018; Curran et al. 2016; Allen et al 2017; Brockwell et al. 2017). This synergistic effect could be the result of a potentiated IFN signal transduction in cancer cells (Ager et al. 2017). Indeed, it has been recently demonstrated that murine lung cancer and melanoma tumors treated with local radiation activate the IFNβ-MHC I pathway, releasing neoantigens and synergizing with PD-1 blockade (Overwijk et al. 2013). There is evidence that radiation induces IFN-β production by tumor infiltrating DCs in a B16 melanoma model (Zhao et al. 2013).

1.4. Towards a complete mechanistic model

A key goal that could not be accomplished in this thesis was the immunoprecipitation of PD-L1 protein complexes from cancer cells after IFN-β treatment followed by mass spectrometry. Nevertheless, this has been carried out from human HEK 293T cells (Huttlin et al., 2015) and found that PD-L1 was associated to mTOR and kinases regulating DNA damage (such as ATR and ATM). A recent review by our group proposes a mechanism that integrates the results from Huttlin et al, and those obtained in this thesis. Briefly, mTOR and ATM bound to PD-L1 could associate with and regulate STAT1 and STAT3 and indirectly CASP3, CASP9 and CASP7 (Figure 13). Overall, PD-L1 could regulate

the anti-apoptotic response by the mTOR-AKT signaling core (Huttlin et al. 2015). A key question remains whether mTOR, ATM or any of the components co-immunocaptured with PD-L1 directly interacts with either RMLDVEKC or DTSSK sequence motifs. Or how these putative interactions activate mTOR-AKT. Nevertheless, the physical association of each component identified by Huttlin et al with PD-L1 will have to be independently validated in future experiments.

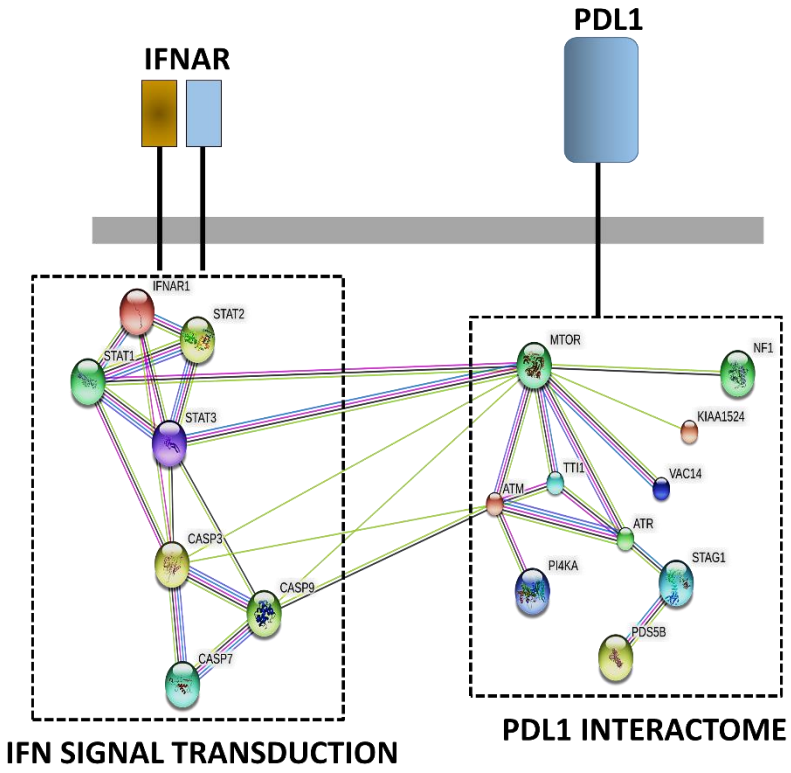

Figure 13: PD-L1 protection from IFNβ toxicity is mTOR dependent. *STRING functional interactome integrating type I IFN signal transduction components and PD-L1-interacting proteins. PD-L1-interacting protein mTOR can probably regulate STAT1 and STAT3 and indirectly CASP3, CASP9 and CASP7.*

2. MDSCs AS A MECHANISM OF RESISTANCE TO IMMUNOTHERAPY

The second theme of my Ph.D. thesis was the molecular study of MDSCs using an ex vivo differentiation system developed by our group, and quantitative proteomics. These cells are broad suppressors of immune responses, and negatively correlate with the efficacy of immune checkpoint blockade.

Using the same datasets obtained in a study published before the start of this Ph.D. thesis (Liechtenstein et al. 2014). The proteomic data was re-analyzed using MaxQuant, an integrated suite of algorithms specifically developed for high-resolution, quantitative MS data, which achieves mass accuracy in the part per billion range, a six-fold increase over standard techniques (Cox et al. 2008). The top molecular and cellular pathways in MDSC modelling melanoma subsets compared to DCs correlated with previous analyses by Liechtenstein *et al.* (Figure 9). The most important were cellular growth and proliferation (NPM1, CDK2), cell-to-cell signalling (Ctsg, Icam), migration to inflammatory sites (s100), angiogenesis, invasion/metastasis (c-type lectins), endocytosis (Clta, Actn4) and integrin signalling (TLR). These differentially-activated pathways correlated with most of the published literature on MDSC (Trikha et al. 2014; Rausch et al. 2012; Wesolowski et al. 2013; Dilek et al. 2012; Youn et al. 2010; Peranzoni et al. 2010) highlighting their importance for the differentiation, migration, and function of MDSCs within tumor-bearing hosts. These processes require high energy levels, but interestingly MDSCs down-regulated proteins participating in aerobic ATP production, aerobic cellular respiration, and glycogen/glucose metabolism, relative to BM-DCs. This could be observed by a decrease in expression levels of such proteins as coenzyme Q10 (ubiquinone), glycogen phosphorylase (Pygl) and phosphoglucomutase-1 (Pgm1).

Probably as a compensation mechanism, proteins that enhanced lipid metabolism (ApoB R, Perilipin-3) were increased in MDSCs, which provides energy and contributes to amino acid synthesis (D3-PGDH). This data was also confirmed by several other studies highlighting the lipid metabolism in MDSC as

an energy source (Boutte et al. 2011; Liechtenstein et al. 2014). However, these metabolic pathways produce a large number of toxic metabolites. Thus, MDSCs also exhibited high levels of detoxifying enzymes including P450R, ROS scavenger proteins such as Sod2 and free radical scavenging proteins (NADPH oxidase I). This data was recently corroborated by others (Hossain et al. 2015).

It is important to note that mitochondrial dysfunction by downmodulation of NADPH dehydrogenase complex I and III was a characteristic of MDSCs compared to conventional DCs, possibly as a reflection of the shut-down of oxidative phosphorylation. As expected, and compared to conventional DCs, MDSCs showed a decrease in proteins regulating antigen presentation by MHC II, including lysosomal proteins and enzymes (Ctsd, Lamp1, Lgmn…).MHC I molecule was up-regulated (H2-K1). This may be related to MDSC suppressive activities on CD8 T cells (by inducing anergy), which was shown to be mediated in an antigen-dependent way (Kusmartsev et al. 2005).

These functions indicate that MDSCs are highly active cells that obtain energy from lipid catabolism and as a result protect themselves from oxidative stress, in agreement with a previous study by our group and others (Gato-Canas et al. 2015; Liechtenstein et al. 2014; Boutte et al. 2011; Hossain et al. 2015; Trikha et al. 2014)(Figure 5 and suplem. 1 Oncotarget).

To study functions specifically associated to cancer-regulated pathways in MDSCs, we had first to generate MDSCs modelling non-neoplastic subsets, which is rather challenging. In a study previous to this Ph.D. thesis, we generated these no-neoplasic MDSCs using conditioning medium from the supernatant of 293T human cells modified with lentivectors to express GMCSF. Using this supernatant, MDSC-like cells can be generated from murine bone marrow. 293T cells are human embryonic kidney cells modified to express the T antigen of the SV40 virus, and immortalized with an integrated adenovirus. Although these are non-cancerous cells *per se*, they are immortal and of human origin. The choice of the producer cell line was not originally made for the

purposes described in this Ph.D. thesis, or the published studies. However, it was found some years ago that this supernatant generated conditioning media that differentiated MDSCs without some of the characteristics typical of cancer-specific MDSCs (Liechtenstein et al. 2014). Later, I developed a 3T3 murine fibroblast-based GM-CSF producer cell line with which I generated similar phenotypic and functional results. This 3T3-based model may provide a more adequate model for murine non-neoplastic MDSC differentiation.

Nevertheless, using this comparative control, the up-regulation of the pentose phosphate pathway (PPP) was one of the most prominent features (Figure 6 Oncotarget). This result agrees with the need of obtaining NADPH for biosynthesis in the absence of oxidative phosphorylation. Furthermore, the decreased expression of mitochondrial NADPH dehydrogenase complex I and III, the up-regulation of free radical scavenging proteins (SOD1), responses to cellular stress (Peroxiredoxin 6), epithelial adherence junction signaling (Tubulin, Myosin), DNA methylation (CHD4) and transcriptional repression pathways were all hallmarks of MDSCs modelling melanoma-infiltrating subsets compared to non-neoplastic MDSCs (Figure 6 Oncotarget). In addition, the glutathione metabolism was also a prominent feature, possibly in coordination with the PPP as an energy source, or for participation in the production of reactive oxygen and nitrogen species (Figure 7 Oncotarget).

Overall, published data agree with our results (Hitosugi et al. 2016; Aliper et al. 2014; Pilon-Thomas et al. 2011; Sawant et al. 2013; Wu et al. 2012; Cheng et al. 2014; Zhang et al. 2017; Zhai et al. 2017; Hammami et al. 2012; Sinha et al. 2008; Youn et al. 2012).

Importantly, the data provided in this Ph.D. thesis highlights a group of kinases that differentiate MDSCs from DCs which included SRC family members and the predicted implication of PI3K-AKT signalling axis (Figure 2-4 Oncotarget). ERK, PKC, and AMPK kinases were predicted to play major roles regulating neoplastic-specific MDSC functions, which also agrees with the role of these kinases in cells of the tumor microenvironment (Zhai et al. 2017; Pilon-Thomas et al. 2011; Mao et al. 2017; Slack et al. 2007; Thiel et al. 2007; Barbosa

et al. 2014)(Figure 6 Oncotarget). All these analyses provide a unified view of biological processes in MDSCs and uncover a large list of molecular targets susceptible of therapeutic intervention. Some multi kinase inhibitors were used to inhibit MDSC expansion like sunitinib (Ko et al. 2010).

By silencing or activating genes of interest or inhibiting enzymes, insight into each specific pathway over MDSC biology will be gained, followed by validation of attractive MDSC-specific targets.

Again, most of the published data agrees with the core MDSC-specific kinases as found in this thesis. SRC kinases were predicted to regulate MDSC differentiation when compared to conventional DCs, linked to mitochondrial dysfunction and changes in the cytoskeleton (Figure 8 Oncotarget). Using broad inhibitors of SRC, MDSC differentiation was also inhibited (Figure 8 Oncotarget), in agreement with other studies (Aliper et al. 2014; Mao et al. 2017).

PI3K signalling is known to regulate chemotaxis, phagocytosis, ROS production, and apoptosis in macrophages and neutrophils (Trikha et al. 2014). Murine and human monocytes can be converted into m-MDSC by activation of the PI3K-AKT-mTOR pathway following GM-CSF and IFN-Ɣ treatment (Ribechini et al. 2017). The PI3K pathway regulates transcription factors that control proliferation and survival of MDSCs; it, therefore, has been suggested that PI3K may play a central role in controlling MDSCs and that it may be an effective MDSC-depleting target (Trikha et al. 2014). Targeting PI3K with an inhibitor (NCT02637531) in myeloid cells overcoes resistance to checkpoint blocade therapy in various cancers (Henau et al. 2016).

AKT was predicted to regulate melanoma-specific MDSCs activity and proliferation according to our data, in agreement with most studies (Figure 4 Oncotarget) (Liu et al. 2012; Zhu et al. 2014; Zhai et al. 2017).

AKT and MEK/ERK inhibitors are being tested in human clinical trials for the treatment of several cancers (Arce et al. 2011; Arce et al. 2012; Escors et al. 2008) (NCT01781429 , NCT01392521, NCT01229150, NCT01668017) We confirmed that inhibition of the ERK pathway preferentially affected MDSCs over conventional DCs (Figure 8, 9 Oncotarget). In our data, ERK upregulation

discriminated neoplastic from non-neoplastic MDSCs, in agreement with other published studies (Fang et al. 2015). Protein kinase C (PKC) proteins were also upregulated in MDSCs modelling neoplastic subsets (Figure 6 Oncotarget) them are involved in MDSC suppressive cascade (Wang et al. 2016) and identified in other interactome analyses (Aliper et al. 2014).

Interestingly, AMPK appeared as another core regulatory kinase of MDSC functions, in agreement with Hammami *et al.* (Figure 6 Oncotarget) (Hammami et al. 2012). AMPK is induced by hypoxia, low glucose levels, or H_2O_2 oxidation and counteracts metabolic stress as it plays a crucial role in NADPH homeostasis (Kang et al. 2015).

Changes in mitochondria were remarkable. The MDSC mitochondrial machinery was turned to high ROS production, while MDSCs up-regulated protective proteins towards oxidative damage. Indeed, ROS can also drive the carbohydrate flux to the PPP. Hydrogen peroxide can activate by oxidation G6pdx, which is up-regulated in our melanoma MDSCs (Figure 6 Oncotarget) (Hitosugi et al. 2016). G6pdx is a key regulatory enzyme of the PPP and its activation increases NADPH levels and can thereby counteracts ROS damage (Hitosugi et al. 2016). It is worth noting that similar processes take place in cancer cells, which exhibit mitochondrial metabolic reprogramming, production of ROS and glucose metabolism through the PPP (Martin- Bernabe et al. 2014). Overall, the detrimental effects of ROS and RNS on NK and T cells are well established, which potentiate immune suppression in the tumor microenvironment by MDSCs and other tumor-infiltrating myeloid cells.

The data presented in this Ph.D. thesis includes several different proteins related to MDSC activities that could be targeted. These include HSP70, HSP90 (Figure 1-3 Oncotarget), shown to expand and activate MDSCs following pro-inflammatory responses (Ociennikowska et al. 2015; Diao et al. 2015). HSP90 inhibitors are used to deplete MDSC (Rao et al. 2012). Retinoblastoma (Rb) regulates m-MDSC to g-MDSC differentiation (Youn et al. 2013), and the S100 family of UP-regulated MDSC proteins. These last family of proteins participate in MDSC migration to sites of inflammation (KO et al. 2010; Sinha et al. 2008)

and enhanced ROS production (Gabrilovich et al. 2012).

Several studies have analyzed MDSCs by mass spectrometry or gene expression arrays in different experimental models (Boutté et al. 2011; Burke et al. 2014; Chornoguz et al. 2011), particularly of circulating and peripheral MDSCs which differ significantly from tumor-infiltrating subsets (Aliper et al. 2014). A study compared splenic and tumor-infiltrating MDSCs by gene arrays, further emphasizing the difference between these MDSC populations. This study predicted key transcription factors, kinases, and proteases within the MDSC populations following the inferred interactomes from the gene expression arrays (Aliper et al. 2014). While it is important to assess MDSCs *in vivo*, the use of our *ex vivo* MDSCs substantially facilitates their study and use.

3. COMBINATION OF THERAPIES TARGETING BOTH BARRIERS

Therapeutic targeting of the MDSC pathways could be used in combination with PD-L1/PD-1 blockade. Preclinical studies indicate that this is a promising approach (Clavijo et al. 2017; Meyer et al. 2014; Martens et al. 2018; Ajona et al. 2017). Indeed, these strategies may synergize as some studies show that PD-L1 blockade attenuates the suppressive activity of MDSCs (Toor and Elkord 2018). Some examples of these strategies are enumerated as follows: PI3K targeting with PD-1 inhibitors in murine models of colorectal and breast cancers (Kim et al. 2014); Anti-PD-L1 efficacy enhanced by Inhibition of MDSCs with a selective inhibitor of PI3Kδ/γ (Yang et al. 2015); PI3Kδ/γ inhibition and PD-L1 blockade in head and neck cancer (Medsker et al. 2016); depletion of g-MDSC with CXCR2-specific antibodies and anti-PD-1 in a murine rhabdomyosarcoma (Highfill et al. 2014); MDSC depletion with anti-GR-1 antibody in combination with anti-PD-1 antibody in glioma (Kamran et al. 2017); and histone deacetilases with anti-PD-1 or anti-PD-L1 in lung and renal carcinoma models or small cell lung cancer (Orillion et al. 2017; Briere et al. 2018). Other strategies combine several anti-MDSC agents, some of them are multi-kinase inhibitors, in combination with PD-L1/PD-1 blockers with positive

outcomes in several experimental models (Guan et al. 2017; Yang et al. 2018; Larkin et al. 2015; Zhou et al. 2018). These last studies corroborate the importance of kinase activities in MDSCs function and survival as I have concluded in this thesis.

In contrast, few studies have targeted MDSC in combination with immune checkpoint inhibitors in cancer patients yet (Martens et al. 2018; Meyer et al. 2014; Noelle et al. 2014; J. Zhou et al. 2018; Eissler et al. 2016). A summary of some MDSC-targeting approaches with immune checkpoint inhibition in clinical trials is shown in Table 2.

AUTHOR, YEAR	CANCER MODEL	ICH	MDSC
Highfill,2014	Rhabdomyosarcoma mice	PD1	CXCR2 Ab
Kim,2014	Colon,breast mice	PD1	PI3K Inh
LeMercier,2014	Melanoma mice,human	PD1/CTLA4	VISTA Ig
Eissler,2016	Neuroblastoma human	PD1	CSF1R Inh
Kamran,2016	Glioma mice, human	PD1	Gr1 Ab
Clavijo,2017	Head and neck mice	CTLA4	Ly6G Ab
Davis, 2017	Head and neck mice	PDL1	PI3K Inh
Guan,2017	Osteosarcoma mice	PD1	Anti-IL18
Lu,2017	Prostate mice	PD1	Multikinase Inh
Orillion,2017	Lung adenocarcinoma mice	PD1	HDAC I
Poon,2017	Colorectal mice	CTLA4	MEK Inh
Briere,2018	NSCLC mice	PDL1	HDAC I/IV
Yang,2018	Gastric mice	PD1	Anti-CCR5
Zhou,2018	Hepatoma mice,human	PDL1	CCRK/CDK20 Inh

Table 2. Combination therapies of MDSC inhibition or depletion and Immune Checkpoint Inhibitors. *Specific examples of studies using combination of anti-MDSC with immune checkpoint inhibitors are enumerated. NSCLC,no-small-cell-lung-cancer; CXCR2, IL8receptor; PI3K, phosphatidylinositol-3-kinase; VISTA, V-domain Ig suppressor of T cell activation; CSF1R, colony stimulating factor 1 receptor; LY6G, Lymphocyte antigen 6 complex locus G6D; Ab,antibody; Inh,inhibitor; HDAC, histone deacetylase; MEK, mapkinase; CCR5, chemokine receptor type 5; CCRK,cell cycle-related kinase; CDK,cyclin-dependent kinase; IL18,interleukin.*

CONCLUSIONS

CONCLUSIONS

CONCLUSIONS PART I

1. PD-L1 delivers intrinsic intracellular signals to cancer cells that promote their growth and survival. It represents a barrier against IFN cytotoxicity that can reinforce its inhibitory properties to T cells when engaged to PD-1 on T cells.

2. PD-L1 contains two phylogenetically conserved sequence motifs within its carboxy terminus which regulate signal transduction capacities and cross-talk with the IFN signal transduction pathway in cancer cells. Somatic mutations affecting the inhibitory DTSSK motif are selected in some human carcinomas that disrupt its regulatory functions and hyperactivate PD-L1 anti-IFN activities.

3. PD-L1 inhibits IFN signal transduction in cancer cells at the STAT3 phosphorylation step and inhibits IFN-dependent apoptosis mainly affecting caspase 7 expression.

4. Intrinsic signal activities of PD-L1 contribute to tumor progression *in vivo* and protect cancer cells against IFNs in the tumor environment.

5. PD-L1 silencing or blockade with antibodies is sufficient to sensitize cancer cells to IFNs. Therefore, any adaptation of cancer cells to either inhibit the IFN signaling pathway or potentiate PD-L1 activities will favor their escape from the immune attack.

CONCLUSIONS PART II

1. Differential proteomes from conventional *ex vivo*-differentiated murine DCs and MDSCs resembling melanoma tumor-infiltrating and no-tumoral subsets have been obtained and quantitatively compared.

2. MDSC-specific targets affecting MDSC differentiation and functions were identified.

3. MDSCs exhibit an altered metabolism adapted to energy consumption in the absence of oxygen, high production of ROS and NOS species, and activation of protective pathways against oxidative stress.

4. MDSCs express a core of specific kinases that regulate their differentiation from conventional DCs (AKT, PI3K, and SRC) or their immunosuppressive functions within the tumor environment (ERK, PKC, AMPK).

5. MDSC-specific kinases can be targeted with inhibitors that selectively affect MDSCs and not conventional DCs *in vitro*.

OVERALL CONCLUSION

I propose that targeting/depleting MDSCs in cancer patients could be a prerequisite for initiation of immune checkpoint therapies.
Additionally, enhancenment of IFN responses in combination with PD-L1 blockade should improve therapeutic responses in human patients compared to monotherapies.

CONCLUSIONES

CONCLUSIONES

CONCLUSIONES PARTE I

1. PD-L1 transmite intrínsecamente señales intracelulares a las células cancerosas que promueven su crecimiento y supervivencia. Representa una barrera frente la citotoxicidad del IFN y puede reforzar sus propiedades inhibitorias frente las células T cuando se une a PD-1 en las células T.

2. PD-L1 contiene dos motivos en su secuencia filogenéticamente conservados dentro de su extremo carboxi que regulan las capacidades de transducción de señal e interaccionan con la vía de transducción de señal de IFN en células cancerosas. Las mutaciones somáticas que afectan el motivo inhibidor de DTSSK se seleccionan en algunos carcinomas humanos, estos alteran sus funciones reguladoras e hiperactivan las actividades anti-IFN de PD-L1.

3. PD-L1 inhibe la transducción de señal de IFN en células cancerosas en la el paso de fosforilación de STAT3 e inhibe la apoptosis dependiente de IFN que afecta principalmente a la expresión de caspasa 7.

4. Las señalizcion intrínseca de PD-L1 contribuye a la progresión tumoral in vivo y protege a las células cancerosas contra los IFN en el entorno tumoral.

5. El silenciamiento o bloqueo de PD-L1 con anticuerpos es suficiente para sensibilizar las células cancerosas a los IFN. Por lo tanto, cualquier adaptación de las células cancerosas para inhibir la vía de señalización de IFN o potenciar las actividades de PD-L1 favorecerá su escape del ataque inmune.

CONCLUSIONES PARTE II

1. Se han obtenido los proteomas de DCs murinas convencionales diferenciadas ex vivo y MDSCs de melanoma infiltrantes de tumor y otras MDSCs no tumorales y se han comparado cuantitativamente.
2. Se identificaron dianas específicas de MDSC que afectan la diferenciación y funciones de MDSC.
3. Las MDSC exhiben un metabolismo alterado adaptado al consumo de energía en ausencia de oxígeno, alta producción de especies ROS y NOS, y activación de vías protectoras contra el estrés oxidativo.
4. Los MDSC expresan un núcleo de quinasas específicas que regulan su diferenciación de las DC convencionales (AKT, PI3K, SRC) o sus funciones inmunosupresoras dentro del entorno tumoral (ERK, PKC, AMPK).
5. Las quinasas específicas de MDSC se pueden tarjetear con inhibidores que afectan selectivamente a los MDSC y a las DC convencionales *in vitro.*

CONCLUSIÓN GENERAL

Propongo que tarjetear / eliminar las MDSC en pacientes con cáncer podría ser un requisito previo para el inicio de las terapias de “immune checkpoint inhibitors”.

Además, la potenciación de las respuestas de IFN en combinación con el bloqueo de PD-L1 debería mejorar las respuestas terapéuticas en pacientes humanos en comparación con las monoterapias.

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