IMMUNOTHERAPY IN MELANOMA:

THE ROLE OF NK CELLS

INÊS ESTEVES DOMINGUES PIRES DA SILVA

Tese para obtenção do grau de Doutor em Medicina

na Especialidade de Biomedicina

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"Imagination is more important than knowledge. Knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution."

Albert Einstein

To my family and friends

For their daily support and love

To my patients

For their strength and generosity

This thesis includes methods and techniques presented in the following publications and presentations:

Publications

- Salhi A, Farhadian JA, Giles KM, Vega-Saenz de Miera E, Silva IP, *et al*. RSK1 activation promotes invasion in nodular melanoma. The American Journal of Pathology 2015;185(3):704-716.
- Silva IP, Gallois A, Jimenez-Baranda S, Khan S, Anderson AC, *et al*. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. Cancer Immunology Research 2014;2(5):410-422.
- Moogk D, Silva IP, Ma MW, Friedman EB, Vega-Saenz de Miera E, *et al*. Melanoma expression of matrix metalloproteinase-23 is associated with blunted tumor immunity and poor responses to immunotherapy. Journal of Translational Medicine 2014;12(342):1-10.
- Gumaste P, Fleming N, Silva IP, Shapiro RL, Berman RS, *et al*. Analysis of recurrence patterns in acral versus nonacral melanoma: should histologic subtype influence treatment guidelines? J Natl Compr Canc Netw 2014;12:1706-1712.
- Fleming NH, Zhong J, Silva IP, Vega-Saenz de Miera E, Brady B, *et al*. Serum-based miRNAs in the prediction and detection of recurrence in melanoma patients. Cancer 2015;121(1):51-59.
- Scanlon P, Tian J, Zhong J, Silva IP, Shapiro R, *et al*. Enhanced immunohistochemical detection of neural infiltration in primary melanoma: is there a clinical value? Hum Pathol 2014;45(8):1656-1663.
- Gallois A, Silva IP, Osman I, Bhardwaj N. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. OncoImmunology 2014;3(7):1-3.
- Jiménez-Baranda S, Silva IP, Bhardwaj N. Plasmacytoid dendritic cells lead the charge against tumors. J Clin Invest. 2012;122(2):481-484.

Oral presentations

- Silva IP, Gallois A, Jimenez-Baranda S, Anderson AC, Kuchroo V, *et al*. Melanoma progression is associated with NK cell exhaustion. SITC 29th annual meeting 2014; National Harbor, USA.
- Silva IP, Bapodra A, Lui K, Pavlick A, Zhong J, *et al*. Avaliação da resposta clínica e imunológica da associação do anti-CTLA-4 com radioterapia ou corticoterapia em doentes com melanoma avançado. Encontros da Primavera 2015; Évora, Portugal.

Poster presentations

- Silva IP, Gallois A, Lui K, Shapiro R, Pavlick A, *et al*. The effect of ipilimumab on natural killer cells identifies the subset of advanced melanoma patients with clinical response. 2015 ASCO Annual Meeting, Abstract #9065; Chicago, USA.
- Silva IP, Gallois A, Lui K, Shapiro R, Berman R, *et al*. Association of natural killer (NK) cell exhaustion with melanoma progression. 2014 ASCO Annual Meeting, Abstract #9099; Chicago, USA.
- Silva IP, Salhi A, Lui K, Ismaili N, Wu C, *et al*. Integration of melanoma genotyping in clinical care. 2014 ASCO Annual Meeting, Abstract #9095; Chicago, USA.
- Bapodra A, Silva IP, Lui K, Pavlick A, Zhong J, *et al*. Clinical outcome and $CD4^+$ differentiation in anti-CTLA-4/radiation and anti-CTLA-4/steroid therapy. 2014 ASCO Annual Meeting, Abstract #3019; Chicago, USA.
- Fleming N, Silva IP, Vega-Saenz de Miera E, Brady B, Han S, *et al*. Clinical utlity of serum miRNAs for the prediction and early detection of recurrence in melanoma patients. 2014 ASCO Annual Meeting, Abstract #9046; Chicago, USA.
- Fleming N, Lui K, Shapiro R, Berman R, Pavlick A, Zhong J, Krogsgaard, Silva IP, Osman I. Dissecting the effect of age on immune response in melanoma patients. 2014 ASCO Annual Meeting, Abstract #9058; Chicago, USA.
- Moogk D, Ma M, Silva IP, Friedman E, Vega-Saenz de Miera E, *et al*. Matrix metalloproteinase-23 as a new immunotherapeutic checkpoint target in melanoma. 2014 ASCO Annual Meeting, Abstract #3030; Chicago, USA.

- Gumaste P, Fleming N, Silva IP, Shapiro R, Berman R, *et al*. Analysis of recurrence patterns in acral versus non-acral melanoma: Should histologic subtype influence treatment guidelines? 2014 ASCO Annual Meeting, Abstract #9052; Chicago, USA.
- Gallois A, Silva IP, Jimenez-Baranda S, Khan S, Anderson AC, *et al*. Reversal of NK cell exhaustion in advanced melanoma patients by Tim-3 blockade. 2014 AACR Annual Meeting, Abstract #4862; Chicago, USA.
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PREFACE

I decided to become an oncologist on the fourth year of medical school, under the wings of Dr. Vera Tomé, a great oncologist who ended up being my mentor. At that time, I had no idea I would like to focus on the melanoma field. My inspiration? Two young mothers, both with advanced melanoma, brain metastases and both treated with temozolamide, but unfortunately died soon after, in my second year of residency. I will never forget them. At that time there were very few therapeutic strategies for melanoma and a lot of disappointing trials.

I have always liked research, but I forgot that feeling for a few years when I fell in love with the clinical work and I mistakenly thought we could follow only one career, doctor or researcher. When I heard about the Programme for Advanced Medical Education, I saw it as a great opportunity to combine two passions, clinical work and research, and this way develop a real translational project. I had the invaluable support of my family and friends and I decided to apply to the Programme for Advanced Medical Education in 2009, a PhD program for medical doctors, sponsored by *Fundação Calouste Gulbenkian*, *Fundação Champalimaud*, *Ministério da Saúde* and *Fundação para a Ciência e Tecnologia*. The initial six months of the programme included classes and seminars on various topics, from basic science to translational research and epidemiology. The goal of this educational period was to provide the students with solid scientific basis in order to develop research projects oriented for specific clinical questions.

The decision to interrupt my residency for 3 years to develop my research project was not an easy one to make, but seemed the best one for me at that time. The switch from a purely clinical thinking to an integrated clinical and scientific thinking was hard but in my opinion, completely essential in order to develop a good project. This decision allowed me to go abroad and join a renowned laboratory in the melanoma field. At that time I had little knowledge and experience regarding good laboratories and centers in melanoma, so I decided to search them. After a first selection of some laboratories and contacting them, I got three interviews. The first one at New York University (NYU), with Iman Osman, that went extremely well and made me feel at home right away. The second was at Memorial Sloan Kettering and it went also well. I was completely divided between these two excellent centers when I went to the third one at the Ludwig Institute in New York City. This interview was

with Dr. Lloyd Old, an impressive man and researcher, pionner in TNF blockade who asked me whether I wanted to study immunology by using melanoma as a model, or if I wanted to focus on melanoma through immunology pathways/immunotherapy. My answer was the latter and he immediately advised me to go to NYU and to work with Dr. Nina Bhardwaj and Dr. Iman Osman. I will be always grateful to Dr. Lloyd Old. I have never regretted my decision.

During the first three years I worked in the Bhardwaj labortory and I have to highlight the extraordinary people I met there. Working with them allowed me to learn a wide variety of techniques and to become familiar with the latest technologies in the field. I was also able to establish different collaborations, within NYU and with other institutions, and to participate in the development of phase I trials using vaccines. After these three years, I had completed most of the work for my research project, and I was ready to go back home. At that time, unexpectedly, I was invited to stay one extra year for a clinical and research fellowship position in melanoma. I accepted it, did the United States Medical Licensing Examination (USMLE) exams and stayed. During that year, I managed to accompany melanoma patients, while doing clinical research, and I was able to finalize and develop some other projects.

My overall experience in New York City exceeded my best expectations. Besides an amazing research experience, those four years were a milestone in my personal life. New York is just an extraordinary city with an overwhelming cultural life and everyone I met during this period made this experience even richer. Undoubtedly, this was one of the most important periods of my life that I tried to share with my family and friends, by writing a weekly newsletter that became known as “Semanário Nova-Iorquino” that reached its 168th edition.

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I would like to express my deepest gratitude to Dr. Nina Bhardwaj, my PhD supervisor, and Dr. Iman Osman, the director of the Interdisciplinary Melanoma Cooperative Group (IMCG) and Co-supervisor of my PhD, who welcomed me to their laboratories and guided me in my training as an oncologist-scientist. I am forever grateful for the opportunities they gave me throughout my stay in New York City. It was a privilege to be part of the Bhardwaj and Osman laboratories and a member of the IMCG.

I wish to express my profound gratitude to Professor José Luís Passos Coelho, supervisor of my PhD, for his mentorship during these 4 years of research work. He is the director of the Oncology Department at *Hospital da Luz* and *Hospital Beatriz Ângelo*, and has been an example of a dedicated oncologist. I am thankful for his encouragement and support of my research.

I am forever thankful to Dr. Vera Tomé and Dr. Isabel Sargento, my mentors in Oncology, for their support of my research and for their generosity taking care of all our oncologic patients while I was in New York City. Their exceptional dedication to their patients and clinical thoroughness, have been an inspiration and an example to follow.

I wish to thank Dr. Anna Pavlick, for her mentorship in the melanoma clinical fellowship at NYU Cancer Center during my last year in New York City. She is an incredibly compassionate physician and an excellent teacher.

I am thankful to the Programme for Advanced Medical Education and particularly to Professor Leonor Parreira for her generous support and advice. The outstanding scientific training provided by the Programme was crucial for my research work in New York City. I wish to acknowledge the sponsors of the Programme, *Fundação Calouste Gulbenkian*, *Fundação Champalimaud*, *Ministério da Saúde* and *Fundação para a Ciência e Tecnologia*, for their institutional and financial support.

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I wish to express my sincere and deepest gratitude to my portuguese friends Sílvia, Célia, Neide, Vítor, Joana, Gonçalo, Miguel, Mônica and to my "almost" portuguese friends Pola, Sonia, Anne, Amel, Shan and Olivier for making me feel at home in New York City.

I would like to thank all my friends from the Programme for Advanced Medical Education, from my Medical School class, from *Instituto Português de Oncologia* and friends from different pieces of my life, in particular, to Ana and Carla, for their loyalty and friendship.

I wish to express my profound gratitude to my parents, brother, sister-in-law and nephews, for everything, in particular their patience and love.

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ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µL	microliter
2B4 (CD244)	Non-major histocompatibility complex binding natural killer cell receptor
7AAD	7-aminoactinomycin D
ADCC	Antibody-dependent cell-mediated cytotoxicity
aGVHD	Acute graft-versus-host disease
AJCC	American Joint Committee on Cancer
ALM	Acral-lentiginous melanoma
AML	Acute myeloid leukemia
AP-1	Adaptor protein-1
APC	Antigen presenting cell
AS15	Vaccine adjuvant containing CpG 7909, monophosphoryl lipid and QS-21
ASCO	American Society of Clinical Oncology
Bat3	HLA-B-associated transcript 3
BATF	Basic leucine zipper transcription factor
BCG	Bacillus Calmette-Guérin
BLIMP-1	B lymphocyte-induced maturation protein-1
BRAF	Proto-oncogene B-Raf
BTLA	B- and T-lymphocyte attenuator
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
CD62L	L-selectin, cell adhesion molecule
CD94-NKG2A	Lectin-like inhibitory receptor
CDK4	Cyclin-dependent kinase 4
CDKN2A,2D	Cyclin-dependent kinase inhibitor 2A, 2D
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CFSE	Carboxyfluorescein succinimidyl ester

CIN	Cervical intraepithelial neoplasia
CR	Complete responder
CS1 (CD319)	Member of signalling lymphocyte activation molecule
CSD	Chronic sun-induced damage
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCR1 (IL-8RA)	CXC chemokine receptor 1, interleukine 8 receptor alpha
DAMP	Damage associated molecular pattern
DAP10, 12	DNAX-activating protein 10, 12
DC	Dendritic cell
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
DNAM-1	DNAX accessory molecule-1
DSS	Disease-specific survival
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-linked immunosorb assay
EOMES	Eomesodermin
FACS	Fluorescence-activated cell sorting
FasR	Fas receptor
FasL	Fas ligand
FBS	Fetal bovine serum
FcR	Fc receptor
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
Flt3	Fms-like tyrosine kinase 3
Gal-9	Galectin-9
GM-CSF	Granulocyte-macrophage colony-stimulating fator
GranB	Granzyme B
GVAX	GM-CSF gene vaccine
HR	Hazard Ratio
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HD	Healthy donor

HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HLA-C	Human leucocyte antigen-C
HMGB1	High mobility group box 1
HSV	Herpes simplex virus
IDO	Indoleamine 2,3-dioxygenase
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL	Interleukin
IL-2R	Interleukin-2 receptor
IL-2Rα (CD25)	Interleukin-2 receptor alpha chain
IL-2Rβ (CD122)	Interleukin-2 receptor beta chain
IL-2Rγ (CD132)	Interleukin-2 receptor gamma chain
IL-7Rα (CD127)	Interleukin-7 receptor alpha chain
IL-8Rα	Interleukin-8 receptor alpha chain
IL-15R	Interleukin-15 receptor
iNK	Immature natural killer cell
ITIM	Immunoreceptor tyrosine-based inhibitory motif
ITSM	Immunoreceptor tyrosine-based switch motif
IRB	Institutional Research Board
IRRC	Immune-related response criteria
KIR	Killer cell immunoglobulin-like receptor
KIR2DL1,2	Killer cell immunoglobulin-like receptor 2DL1,2
KIR2DL3	Killer cell immunoglobulin-like receptor 2DL3
KIR3DL1	Killer cell immunoglobulin-like receptor 3DL1
KIT	Proto-oncogene c-Kit and stem cell growth factor receptor
LAG-3	Lymphocyte activation gene 3
Lamp-1 (CD107a)	Lysosomal-associated membrane protein 1
LCMV	Lymphocytic choriomeningitis virus
LDH	Lactate dehydrogenase
LMM	Lentigo maligna melanoma
Ly49H	Lymphocyte antigen 49H
mAb	Monoclonal antibody

MAGE-A3	Melanoma associated antigen 3
MAPK	Mitogen-activated protein kinase
MD	Melanoma donor
MDSC	Myeloid-derived suppressor cell
MEK	MAPK/Erk kinase
MFI	Mean fluorescence intensity
mg	milligram
MHC	Major histocompatibility complex
MICA	MHC class I chain-related genes A
MICB	MHC class I chain-related genes B
min	Minute
MIP-1α	Macrophage inflammatory protein-1α
MITF	Microphthalmia-associated transcription factor
mL	Milliliter
mM	Micromolar
mm	Millimeter
mm^2	Square millimeter
NCI	National Cancer Institute
NCR	Natural cytotoxicity receptors (NKp30, NKp44, NKp46)
NEJM	New England Journal of Medicine
NK	Natural killer
NKG2D	Natural-killer group 2D
NKG2DL	Natural-killer group 2D ligand
NKp30	Natural-killer p30
NKp44	Natural-killer p44
NKp46	Natural-killer p46
NKT cells	Natural-killer T cells
nM	Nanomolar
NM	Nodular melanoma
Non-CSD	Non-chronic sun-induced damage
NRAS	Neuroblastoma RAS oncogene
NSCLC	Non–small-cell lung cancer
OKT3	Anti-CD3 monoclonal antibody

OS	Overall survival
OX40 (CD134)	Tumor necrosis factor receptor superfamily member 4, costimulatory molecule
OX40L (CD252)	OX40 ligand
p	*p*-value
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-1	Programmed (cell) death 1
PD-L1	Programmed (cell) death-ligand 1
PD-L2	Programmed (cell) death-ligand 2
PE	Phycoerythrin
PEG-IFNα-2b	Pegylated interferon-α2b
PFA	Paraformaldehyde
pNK	Precursor NK cell
PR	Partial responder
PRR	Pattern recognition receptor
PTEN	Phosphatase and tensin homolog
PtdSer	Phosphatidylserine
RAGE	Receptor for advanced glycation endproducts
RECIST	Response Evaluation Criteria in Solid Tumors
Rh	Recombinant human
ROS	Reactive oxygen species
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
RT	Radiotherapy
Src	Non-receptor tyrosine kinases
SHP-1	Src homology region 2 domain-containing phosphatase-1
SHP-2	Src homology region 2 domain-containing phosphatase-2
SLAM (CD150)	Signalling lymphocyte activation molecule
SLN	Sentinel lymph node
sMICA	Soluble MHC class I chain-related genes A
SSM	Superficial spreading melanoma

TAM	Tumor-associated macrophage
T-bet	T-box transcription factor
TBI	Total Body Irradiation
TCR	T cell receptor
TGF-β	Transforming growth factor-beta
TGN	Trans Golgi network
Th	T helper
TILs	Tumor-infiltrating lymphocytes
Tim-3	T cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptor
TME	Tumor microenvironment
TNF-α	Tumor necrosis factor alpha
TNFR1	TNF receptor 1
TNFRSF	TNF receptor superfamily
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	T regulatory cells
TRIM	Transmembrane adaptor T-cell receptor interacting molecule
T-VEC	Talimogenelaherparepvec
ULBP	UL16 binding protein
ULBP2	UL16 binding protein 2
ULBP4	UL16 binding protein 4
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WBC	White blood cells

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ABSTRACT

Melanoma is the fifth most common cancer in men (seventh in women) and its incidence is increasing more rapidly than that of any other malignancy. It is the most aggressive type of skin cancer; although it accounts for 2% of all skin cancers it leads to more than 70% of their deaths. In fact, melanoma responds poorly to systemic therapy and the overall prognosis of patients with distant metastasized melanoma remains poor; the 2-year overall survival rate ranges from 18 to 40%.

In the context of cancer two opposite immune responses exist: an acute T helper (Th) 1 anti-tumoral response that requires a substantial autoimmune attack; and a pro-tumorigenic inflammatory response, characterized by an immune tolerance via active immunosuppressive mechanisms. During tumor progression there is an imbalance with a stronger pro-tumorigenic inflammatory response, while the anti-tumoral Th1 cells display a dysfunctional phenotype. The new concept of "T cell exhaustion", that has been developped in the past few years, can explain T cell dysfunction. This "T cell exhaustion", either caused by environmental influence or other factors, was first described in chronic infectious diseases and more recently in different types of cancer, particularly in melanoma. This phenotype is characterized by early loss of proliferative capacity, cytotoxic potential, and the ability to produce interleukin (IL)-2. The concept of T cell exhaustion in the context of metastatic cancer has been reinforced by the recent success of immunotherapies targeting the exhaustion markers cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed (cell) death 1 (PD-1) in advanced melanoma. However, the phenotype and function of natural killer (NK) cells in different phases of tumor progression and their relationship with prognostic factors is still unknown.

NK cells are innate immune cells, prone to target tumor cells, whose function depends on the balance between activating and inhibitory receptors, and on the expression of the respective ligands in their microenvironment. T cell immunoglobulin domain and mucin domain 3 (Tim-3), another T cell exhaustion marker along with CTLA-4 and PD-1, behaves as an inhibitory receptor in T cells and is key in the maintenance of immune tolerance. Tim-3 is also expressed in NK cells; however its role in modulating the function of these innate effector cells remains unclear, particularly in human disease.

The aims of this study were: to characterize the phenotype and function of NK cells from melanoma patients; to identify potential players in the mechanism of NK cell exhaustion as prognostic markers; to define the role of Tim-3 expression in NK cells from advanced melanoma patients; and to study the effect of checkpoint blockade in NK cell phenotype and function in the context of advanced melanoma patients.

Our data showed that NK cells from patients with advanced melanoma display an exhausted phenotype that is characterized by upregulation of inhibitory receptors (Killer cell Immunoglobulin-like receptor 3DL1 and 2DL3 - KIR3DL1 and KIR2DL3, respectively), downregulation of activating receptors (natural-killer group 2D (NKG2D), natural killer p46 (NKp46) and DNAX accessory molecule-1 (DNAM-1)), unresponsiveness to IL-2 stimulation, downregulation of the transcription factors T-box (T-bet) and eomesodermin (Eomes), and defective function. The latter includes the main NK cell functions, cytotoxicity, interferon gamma (IFN-γ) production and proliferation.

NK cells acquire this exhausted phenotype gradually as disease advances from early (stages I and II) to later stages (stages III and IV). Moreover, the exhaustion of NK cells is associated with clinicopathological parameters that have known prognostic value, such as thickness and presence/absence of metastases (regional and distant). In addition, high serum levels of soluble MHC class I chain-related genes A (sMICA), an NKG2D ligand (NKG2DL) that may be involved in the mechanism of NK cell exhaustion, identify patients with shorter disease-free and overall survival.

We also identified Tim-3 as an inhibitory receptor over-expressed in NK cells from advanced melanoma patients that plays a key role in the exhausted phenotype of these cells. Significantly, we demonstrated that Tim-3 blockade partially reversed this exhausted phenotype. Therefore, in average, it was possible to enhance cytotoxicity by 20%, IFNγ production by 15% and proliferation by 60%.

Finally, we characterized the effect of ipilimumab (anti-CTLA-4) on exhausted NK cells from advanced melanoma patients. Remarkably, NK cells from responders to ipilimumab treatment have higher levels of IL-2 receptor (IL-2R), and consequently, are more responsive to IL-2 stimulation and more cytotoxic.

These data open exciting avenues for new NK cell-based therapies, including targeting Tim-3 in the context of melanoma. Moreover, a better understanding of the mechanism behind NK cell exhaustion will help defining new prognostic markers and therapeutic strategies.

Keywords: melanoma, immunotherapy, natural killer cells, Tim-3, immune cell exhaustion, soluble MICA, ipilimumab.

RESUMO

O melanoma é o quinto tumor maligno mais comum nos homens (sétimo nas mulheres) e a sua incidência está a aumentar mais rapidamente do que a de qualquer outro cancro. É o tipo de cancro cutâneo mais agressivo; corresponde a apenas 2% de todos os cancros de pele, mas é responsável por mais de 70% das mortes causadas pelo cancro cutâneo. O melanoma responde mal à terapia sistémica e tem mau prognóstico nos doentes com metástases à distância, com uma taxa de sobrevivência global aos 2 anos que varia entre os 18 e os 40%.

No contexto do cancro identificamos duas respostas imunológicas opostas: uma resposta aguda anti-tumoral com predomínio de células Th1 podendo, consequentemente, levar a uma reacção auto-imune; e uma resposta inflamatória pró-tumoral, caracterizada por uma tolerância imunológica através de mecanismos imunossupressores. Durante a progressão do tumor verifica-se um desequilíbrio com um predomínio da resposta inflamatória pró-tumoral e disfunção das células anti-tumorais Th1. O conceito de "exaustão dos linfócitos T" tem sido desenvolvido nos últimos anos e pode explicar a disfunção das células Th1. Este conceito de "exaustão dos linfócitos T", consequência do microambiente tumoral ou de outros factores, foi descrita pela primeira vez em doenças infecciosas crónicas e mais recentemente em diferentes tipos de cancro, em particular no melanoma. Este fenótipo é caracterizado pela perda progressiva da capacidade de proliferação, do potencial citotóxico e da capacidade de produzir interleucina 2 (IL-2). A "exaustão dos linfócitos T" no contexto do cancro metastático tem sido reforçado pelo recente sucesso de imunoterapias dirigidas aos marcadores de exaustão expressados pelos linfócitos T, incluindo o CTLA-4 e o PD-1, em doentes com melanoma avançado. No entanto, o fenótipo e a função das NK em diferentes fases da progressão do tumor e a sua relação com fatores de prognóstico ainda é desconhecida.

As células NK pertencem ao sistema imune inato e têm um papel importante na resposta anti-tumoral. A função destas células depende do equilíbrio entre os receptores de activação e de inibição, e da expressão dos respectivos ligandos no microambiente tumoral. O Tim-3, outro marcador de exaustão dos linfócitos T juntamente com o CTLA-4 e o PD-1, comporta-se como um receptor inibitório nos linfócitos T e tem um papel fundamental para a manutenção da tolerância imunológica. O Tim-3 também é expresso nas células NK, no entanto o seu

papel na modulação da função destas células permanece pouco claro, em particular no contexto do cancro.

Os objetivos deste estudo foram: caracterizar o fenótipo e a função das células NK em doentes com melanoma; identificar moléculas, que possam estar envolvidas no mecanismo da exaustão das células NK, como potenciais marcadores de prognóstico no melanoma; definir o papel do Tim-3 expresso nas células NK de doentes com melanoma avançado; e estudar o efeito do bloqueio de *checkpoints* imunológicos no fenótipo e função das células NK no contexto dos doentes com melanoma avançado.

Os nossos dados mostraram que as células NK de doentes com melanoma avançado exibem um fenótipo de exaustão imunológica que é caracterizado por uma expressão aumentada dos receptores inibitórios KIR3DL1 e KIR2DL3, por uma expressão diminuida dos receptores de activação NKG2D, NKp46 e DNAM-1, por uma resposta deficiente à estimulação com IL-2, por uma regulação negativa dos fatores de transcrição T-bet e Eomes, e por um défice de função (citotoxicidade, produção de IFN-γ e proliferação).

As células NK adquirem gradualmente este fenótipo de exaustão imunológica à medida que a doença avança dos estadios iniciais (estadios I e II) para estadios mais avançados (estadios III e IV). Curiosamente, este fenótipo de exaustão das células NK está associado as parâmetros clinico-patológicos com valor prognóstico conhecido, como a espessura e a presença/ausência de metástases (regionais e à distância). Além disso, níveis elevados de MICA solúvel (ligando do receptor NKG2D) no plasma podem estar envolvidos no mecanismo de exaustão das células NK e identificam doentes com sobrevivência global e tempo de livre de doença mais curtos.

Neste trabalho também identificamos o Tim-3 como um receptor inibitório e com uma expressão aumentada nas células NK de doentes com melanoma avançado, e que desempenha um papel chave no fenótipo de exaustão destas células. Mais importante, mostramos ainda que o bloqueio do receptor Tim-3 consegue reveter, em parte, este fenótipo de exaustão, com um aumento da citotoxicidade em 20%, da produção de IFN-γ em 15% e da proliferação em 60%.

Finalmente, caracterizamos o efeito do ipilimumab (anti-CTLA-4) no fenótipo de exaustão das células NK de doentes com melanoma avançado. Notavelmente, as células NK de doentes

respondedores ao tratamento com ipilimumab apresentaram níveis mais elevados de IL-2R e, por conseguinte, maior citotoxicidade em resposta à estimulação com IL-2.

Estes dados abrem caminho para novas estratégias terapêuticas com base nas células NK, incluindo o bloqueio do receptor Tim-3 no contexto de melanoma. Além disso, uma melhor compreensão do mecanismo subjacente à exaustão das células NK irá definir novos marcadores de prognóstico e estratégias terapêuticas.

Palavras-Chave: melanoma, imunoterapia, células NK, Tim-3, exaustão de células imunológicas, MICA solúvel, ipilimumab.

CHAPTER 1:
INTRODUCTION & STUDY AIMS

1.1. Introduction

Melanoma is the deadliest skin cancer and its incidence is increasing dramatically. The median age at diagnosis is 59 years, and on average, an individual loses 20.4 years of potential life as a result of melanoma mortality, that is four years more than the average for all other malignancies[1].

Immune responses to cancer have been shown to play a role in control of tumor development (immunosurveillance). However, tumors continue to grow despite this immune pressure (immunoevasion). Two key processes are responsible for this immunoevasion phenomenon: (1) immunoediting, which refers to the role of the immune response in selecting tumor variants that have become poorly immunogenic (such as, loss or downregulation of human leucocyte antigen (HLA) class I molecules); and (2) immunosubversion, in which tumor cells or the tumor microenvironment (TME) suppress the immune response[2]. In fact, the cells responsible for anti-tumor immune responses, mainly T cells, NK cells and natural-killer T cells (NKT cells), seem to be dysfunctional in different advanced stages of tumors, partly as a result of the influence of the tumor[2].

The resistance of melanoma to standard treatments and the relevance of the immune response against melanoma, due to its high immunogenicity[3,4], have led to the evaluation of different immunotherapies such as cytokine therapies, targeted antibody therapies, cellular therapeutics, as well as vaccines. A recent advance is the approval of anti-CTLA-4 and anti-PD-1 antibodies, which have shown efficacy in the treatment of a substantial percentage of patients with advanced melanoma[5], both individually and in combination[6]. However, the successful treatment of patients with advanced melanoma will require additional novel interventions. Therefore, identification of key molecules that modulate antitumor immunity and that could be used as a therapeutic target, as well as predictive markers of response to immunotherapy are essential.

1.1.1. Melanoma

Melanoma is the sixth most common cancer in men and women, and is the second most common cancer in women aged 20 to 29 in the United States. The incidence of melanoma has increased more than 690% since 1950, and it is estimated that 1 in 34 men and 1 in 53 women will be diagnosed with melanoma during their lifetime (from a recent epidemiological analysis between 2009-2011)[7]. Melanoma accounts for less than 2% of all skin cancer cases, but is responsible for the vast majority of skin cancer deaths[8]. A positive family history of melanoma, prior melanoma, multiple clinically atypical moles or dysplastic nevi and rarely inherited genetic mutations [cyclin-dependent kinase inhibitor 2D (CDKN2D) and cyclin-dependent kinase 4 (CDK4)][9] are some of the risk factors for melanoma. Sun exposure also contributes to the development of melanoma, suggesting that the interaction between genetic susceptibility and environmental exposure contributes to the development of melanoma[10]. However, melanoma can occur in any ethnic group and in areas of the body without substantial sun exposure.

1.1.1.1 Mechanisms of melanoma progression

According to the Clark model of melanoma progression, the first change is the development of benign nevi, which results from benign controlled melanocyte proliferation. In this phase, proto-oncogene B-Raf (BRAF) mutations and the activation of the mitogen-activated protein kinases (MAPK) pathway may occur. The second step is the formation of a dysplastic nevus, which is considered a premalignant lesion and has some random atypia. These lesions have an aberrant growth and may reflect the loss of cyclin-dependent kinase inhibitor 2A (CDKN2A) and phosphatase and tensin homolog (PTEN). The third step consists of the radial-growth phase, where melanoma cells acquire the ability to proliferate intraepidermally. This phase is associated with decreased differentiation and acquisition of melanoma markers regulated by microphthalmia-associated transcription factor (MITF). The fourth step represents the vertical-growth phase, where melanoma cells acquire the ability to invade the dermis and form an expansive nodule. Finally there is the metastatic phase, where melanoma cells successfully proliferate and establish metastases. These two last phases are notable for striking changes in the control of cell adhesion[11].

1.1.1.2 Clinical-histopathological subtypes

Melanoma has been classically categorized into four major clinical-histopathological subtypes: superficial spreading melanoma (SSM), nodular melanoma (NM), acral-lentiginous melanoma (ALM) and lentigo maligna melanoma (LMM)[12]. SSM is the most frequent histological type of melanoma (about 65% of all cases) and is characterized by the presence of a radial growth phase mostly composed of large epithelioid melanocytes distributed at all levels of the epidermis in a pagetoid fashion. It is associated with pre-existing nevi (congenital and dysplastic) in about 25% of the cases. It commonly occurs on the trunk in males and in lower extremities in females. NM is the second most frequent histological subtype of melanoma (it corresponds to 10 to 15% of all melanomas in caucasians). Unlike the other three main histological types, NM only has a vertical growth phase. The absence of a radial growth phase is the pathological landmark of this type of melanoma. ALM is a relatively rare type of melanoma (about 5% of all cases). It arises in the palms of the hands, in the soles of the feet and in the nailbed (subungual melanoma). ALM occurs rarely in caucasians, but encompasses 35% of the melanomas that develop in black people, hispanics or asians. The dismal prognosis of this variant of melanoma has been explained by its location at acral glabrous sites; a delayed diagnosis may contribute to worsen the prognosis. The radial growth phase of ALM is histologically characterized by a lentiginous proliferation of atypical melanocytes along the basal layer of the epidermis, and is associated with marked acantosis of the epidermis and elongation of the rete ridges. The vertical growth phase often presents a spindle cell component. Finally, LMM typically occurs in older people, on sun-exposed areas, mostly head and neck. LMM was considered to have a better prognosis than the other forms of melanoma, but recent studies have suggested that the different biologic behavior may be related to thinner tumors at the time of diagnosis. This variant of melanoma is histologically characterized by a confluent growth of atypical melanocytes along the dermal-epidermal junction. The invasive component may be composed of spindle cells or may be associated to a desmoplastic reaction[13].

More recently, melanomas have been divided according to a different classification based on the etiology. There are four main subtypes: chronic sun-induced damage (CSD), melanomas with chronic sun-induced damage characterized by the presence of marked solar elastosis; non-chronic sun-induced damage (non-CSD), melanomas without chronic sun-induced

damage; acral melanomas on the soles, palms, or sub-ungual sites; and mucosal melanomas on mucosal membranes. Interestingly, these subtypes display a different mutational profile. Therefore, while the non-CSD subtype melanomas has the highest proportion of BRAF mutations (56%) compared to CSD, acral, and mucosal subtypes (6%, 21%, and 3%, respectively), the incidence of KIT (proto-oncogene c-Kit or stem cell growth factor receptor) aberrations was 28%, 36%, and 39% in CSD, acral, and mucosal subtypes, respectively, but 0% in non-CSD subtypes. NRAS (Neuroblastoma RAS oncogene) mutations were found in 5% to 20% of the subtypes[14]. In the context of melanoma, the histologic subtypes have limited effect on the prognosis and treatment. However, recent studies have shown how histology driven molecular characterization can impact clinical decision-making in other solid tumor types[15]. For example, histopathological subtypes of lung cancer have been shown to be correlated with specific mutational profiles that determine the response to targeted therapies[16].

1.1.1.3 Staging

Melanoma staging is determined by the American Joint Committee on Cancer (AJCC) system, that incorporates tumor thickness, ulceration, mitotic index, the lymph node status and distant metastases[17]. This staging system categorizes melanoma patients into three main groups: localized disease with no evidence of metastases (stage I–II), regional disease (stage III) and distant metastatic disease (stage IV) (**Figure 1.1**).

In localized melanoma (stage I or II), tumor thickness, ulceration and mitotic rate are the three most important characteristics of the primary tumor-predicting outcome. Ulceration in melanoma corresponds to an interruption of the surface epithelium by tumor cells. Mitotic rate (MR) is a marker of tumor proliferation and is measured as the number of mitoses per mm^2. MR greater than or equal to 1.0 per mm^2 is independently associated with worse disease-specific survival (DSS), especially in patients with melanoma less than or equal to 1.0 mm thick, and this is the criterion for upstaging patients with melanomas less than or equal to 1.0 mm in thickness from IA to IB[18,19].

Classification	Thickness (mm)	Ulceration Status/Mitoses
T		
Tis	NA	NA
T1	≤ 1.00	a: Without ulceration and mitosis < 1/mm²
		b: With ulceration or mitoses ≥ 1/mm²
T2	1.01-2.00	a: Without ulceration
		b: With ulceration
T3	2.01-4.00	a: Without ulceration
		b: With ulceration
T4	> 4.00	a: Without ulceration
		b: With ulceration
N	No. of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: Micrometastasis*
		b: Macrometastasis†
N2	2-3	a: Micrometastasis*
		b: Macrometastasis†
		c: In transit metastases/satellites without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/satellites with metastatic nodes	
M	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	Normal
M1b	Lung metastases	Normal
M1c	All other visceral metastases	Normal
	Any distant metastasis	Elevated

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase.
*Micrometastases are diagnosed after sentinel lymph node biopsy.
†Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically.

	Clinical Staging*				Pathologic Staging†		
	T	N	M		T	N	M
0	Tis	N0	M0	0	Tis	N0	M0
IA	T1a	N0	M0	IA	T1a	N0	M0
IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
IIC	T4b	N0	M0	IIC	T4b	N0	M0
III	Any T	N > N0	M0	IIIA	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Any T	N3	M0
IV	Any T	Any N	M1	IV	Any T	Any N	M1

*Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases.
†Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial (ie, sentinel node biopsy) or complete lymphadenectomy. Pathologic stage 0 or stage IA patients are the exception; they do not require pathologic evaluation of their lymph nodes.

Figure 1.1. **AJCC staging system for cutaneous melanoma.** (**Left**) Tumor (T) Nodes (N) Metastases (M) staging. (**Right**) Clinical and pathological staging[17].

In stage III melanoma, the most important predictors of survival are the number of metastatic nodes and clinical nodal status (nonpalpable vs. palpable). In the case of a positive sentinel lymph node, the number of positive nodes, tumor burden in the sentinel node, primary tumor thickness, mitotic rate, ulceration, and patient age are the most important prognostic factors. For patients with clinically positive nodes, prognostic factors include number of positive nodes, primary tumor ulceration, and patient age.

The site of metastases is the most significant predictor of outcome among patients with distant metastases (stage IV). The three risk categories recognized by the AJCC are skin / soft tissue / remote nodes, visceral-pulmonary, and visceral-nonpulmonary. Elevated lactate dehydrogenase (LDH) is also an independent predictor of poor outcome in patients with stage IV disease and has been incorporated into the AJCC staging system[17].

There are other pathological factors that seem to influence the prognosis. Some of these factors include, tumor-infiltrating lymphocytes (TILs), angiovascular infiltration, microscopic satellites and regression. With respect to infiltration of TILs, melanomas are normally

classified as: brisk or diffuse response, defined as lymphocytes throughout the tumor; non-brisk or focal, defined as only foci of infiltration rather than through the whole of the tumor; and absent TILs, defined by "no lymphocytes present"[20]. On a multivariate analysis of data from a prospective clinical trial, TILs response was a significant predictor of sentinel lymph node (SLN) metastasis, not a significant independent factor predicting disease-free survival (DFS) or overall survival (OS)[21]. However, the prognostic significance of TILs response in cutaneous melanoma is still controversial[22]. Microscopic satellites are defined as dermal or subcutaneous nests of metastatic cells more than 0.05 mm in diameter that are clearly separated from the main lesion by at least 0.3 mm, and the survival outcome is comparable to that of patients with clinically detectable satellite metastases. Regression corresponds to a partial host response to a malignant neoplasm, resulting in focal decrease of the process. Histologically, the changes are similar to those seen in a scar, with an infiltrate of lymphocytes admixed with histiocytes and pigment-laden macrophages underlying an atrophic epidermis with flattened rete ridges[23]. The prognostic value of regression is still controversial. While some results suggested that those melanomas with regression have a lower frequency of sentinel node involvement, others indicate that regression is associated with poor prognosis because the disappearance of a portion of the tumor may lead, at least in some cases, to an underestimation of the original Breslow thickness[24].

Patients with stage I melanoma have an excellent prognosis, with 5-year survival achieved in more than 90% of the patients. For patients with localized melanomas thicker than 1.0 mm (stage II), survival rates range from 50% to 90%. Within stage III, 5-year survival rates range from 20% to 70%, depending primarily on the nodal tumor burden (**Figure 1.2**). Long-term survival in patients with distant metastatic melanoma, taken as a whole, is less than 10%, however, this scenario is changing with new therapeutic strategies[25,17].

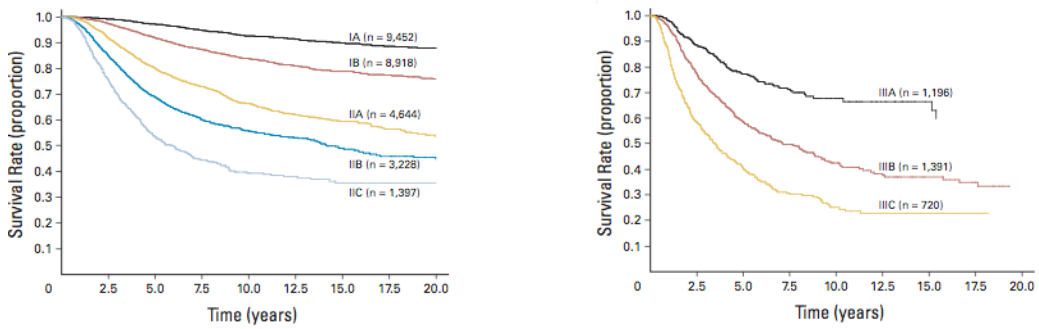


Figure 1.2. Survival curves using the AJCC melanoma staging. (**Left**) Different stage groupings for stages I and II melanoma. (**Right**) Different stage groupings for stage III[17].

1.1.1.4 Treatment

The primary treatment for local melanoma (stage I, II and III) consists of surgical excision with safety margins: margins of 0.5 cm for *in situ* melanomas, of 1 cm for tumors with a Breslow thickness of up to 2 mm and 2 cm for thicker tumors[26]. SLN biopsy aims to identify patients with subclinical nodal metastases at higher risk of recurrence, who could be candidates for complete lymph node dissection or adjuvant systemic therapy. SLN biopsy is necessary for precise staging in melanomas with a tumor thickness of >1 mm and/or ulceration[27]. In the cases that the sentinel node was found positive for metastases, a complete lymphadenectomy of regional lymph nodes was the standard treatment. However, a recent study showed that, even though a complete lymphadenectomy leads to a better disease control in the regional lymph node basin, it is not associated with improved distant metastases-free survival, recurrence-free survival or melanoma-specific survival[28].

For patients with stage III melanoma, adjuvant treatment may be considered. These therapeutic options include, observation, clinical trials or interferon alfa (IFN-α). Even though melanoma is poorly radiosensitive, adjuvant radiotherapy (RT) may be considered for selected patients with clinically positive nodes and features predicting a high risk of nodal basin relapse[29]. Adjuvant high-dose and pegylated interferon are both appropriate options for patients with completely resected stage III disease, either positive sentinel nodes or clinically positive nodes[30,31]. Many different treatment options, mostly locoregional, are available to patients presenting with stage III in-transit metastases. Treatment is based on the size,

location, and number of tumor deposits, but evidence is limited and there is no consensus on the optimal approach. Excision with clear margins is the mainstay for resectable regional recurrence. Although in-transit disease has a high probability of clinically occult regional nodal involvement, and a positive sentinel node in the presence of in-transit metastasis portends a more ominous prognosis, the impact of SLN biopsy on outcome remains unknown. A number of non-surgical local approaches are being used. These include intralesional local injections with bacillus Calmette-Guérin (BCG), IL-2 or IFN-α, laser ablation and topical imiquimod[32]. Isolated limb perfusion or infusion are techniques to regionally administer high doses of chemotherapy (melphalan is the drug most widely used for this technique) to an affected extremity while avoiding systemic drug exposure[33]. Moreover, ipilimumab (10mg/Kg) was also studied in the adjuvant setting in a double-blind phase 3 trial (NCT00636168), and showed an improved recurrence-free survival. Nevertheless, the distant metastasis-free survival and overall survival endpoints need to be clearly assessed in order to define the risk-benefit ratio of ipilimumab in this context[34].

Disseminated disease can be managed by systemic therapy, clinical trial, or best supportive care. Preferred regimens include ipilimumab (anti-CTLA-4)[35], nivolumab or pembrolizumab (anti-PD-1), vemurafenib or dabrafenib (BRAF inhibitor), cobimetinib or trametinib (MAPK/Erk kinase (MEK) inhibitor), or even BRAF and MEK inhibitors combination therapy. Vemurafenib and dabrafenib are recommended only for patients with documented V600 BRAF mutations[36,37]. BRAF inhibitors can be used alone, or in combination with MEK inhibitor for patients who have progressed from previous treatment with BRAF inhibitors[38]. Phase III data pointed out that combination therapy is superior over targeted monotherapy in terms of toxicity and efficacy[39,40]. Trametinib monotherapy can be used in patients who show intolerance to toxicities related to vemurafenib or dabrafenib. The recommendation for first-line systemic therapy of melanoma is based on several factors, including the BRAF mutation status, the kinetics of disease, and the presence/absence of cancer-related symptoms. Therefore, patients with low-volume, asymptomatic metastatic melanoma may be good candidates for immunotherapy (anti-CTLA-4 or anti-PD-1), independently of their BRAF status, as there may be time for a durable antitumor immune response to emerge. Patients with BRAF-mutant melanoma who have symptomatic disease or who have progressed despite immunotherapy should be considered for targeted therapies. Clinical trials are underway to address unanswered questions regarding the optimal sequencing and/or combination of these

agents[41].

1.1.2. Immunotherapy in melanoma

Cancer immunotherapy includes different strategies that aim to stimulate the immune system to obtain a specific and lasting antitumor response, with consequent clinical benefit. This concept originated in 1891 with William Coley, a surgeon from New York, who observed the regression of sarcomas in patients who developed erysipelas, and tried to reproduce this effect by injecting live or inactivated *Streptococcus pyogenes* and *Serratia marcescens* in cancer patients. Since then various attempts to boost the immune system, mainly by vaccines and cytokines, were made in the context of clinical trials, but with disappointing results. In recent years, we witnessed an important inflection point in the history of cancer immunotherapy, wherein new strategies mainly immune checkpoints blockade, have shown long-term clinical responses in different tumors[5,35].

1.1.2.1 Regulation of antitumor immune response

Understanding the development and regulation of the anti-tumoral immune response is essential for devising strategies that can improve anti-tumor immunity. An immune response occurs in multiple stages and simply can be divided into three phases: (1) maturation of dendritic cells (DC) for effective antigen presentation; (2) priming and activation of anti-tumor T cells; (3) an adaptive immune response to overcome the immunosuppressive mechanisms in the TME. **Figure 1.3** illustrates this "immunity cycle" in more detail.

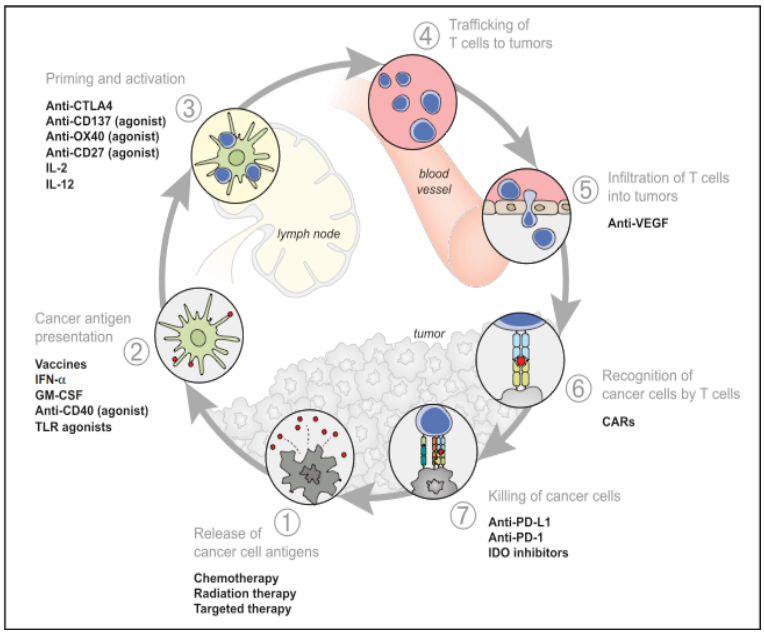


Figure 1.3. Anti-tumor immune response and different immunotherapy strategies[2].

In the first step there is capture of tumor antigens by DCs. These antigens include proteins derived from mutated or non-mutated genes, preferentially expressed by tumor cells. In order to process the antigens properly and effectively, DCs must receive a "maturation" sign, for example, by stimulation of toll-like receptors (TLR) and CD40 (costimulatory molecule). These cells migrate to the draining lymph nodes and present the processed antigens as peptides to T lymphocytes via major histocompatibility complexes (MHC) class I and II. For the generation of effective T cells against the tumor to occur, an interaction between costimulatory molecules present on T cells (CD28, OX40 (CD134)) and on DCs (CD80/CD86 and OX40L (CD252)) is needed. Finally, activated T cells are mobilized and infiltrate the tumor, in order to destroy it. However, many tumor cells develop mechanisms of escaping this immune response, such as increased programmed (cell) death-ligand 1 (PD-L1) expression and secretion of vascular endothelial growth factor (VEGF) by tumor cells, and secretion of IDO (indoleamine 2,3-dioxygenase) by T regulatory cells (Tregs), creating an immunossupressive TME[2].

In the context of melanoma, different strategies of immunotherapy have been developed to operate in each of these different phases in order to control tumor progression. They are divided into four main groups: (1) vaccines[42]; (2) cytokines (IL-2 and IFN-α)[43]; (3) adoptive

cell transfer[44]; (4) immune checkpoint inhibitors (ipilimumab, nivolumab and pembrolizumab)[45,46].

1.1.2.2 Vaccines

The goal of vaccination is to generate an anti-tumor response, based on $CD4^+$ and $CD8^+$ T lymphocytes against tumor-specific antigens, that is sufficiently robust to be able to produce long-lasting clinical responses[42]. Here we review these approaches, and highlight some of the failures and promising new vaccines.

The vaccine containing the melanoma associated antigen 3 (MAGE-A3) peptide was studied in a phase I/II clinical trial with 32 $MAGE\text{-}A3^+$ melanoma patients. Only 26 patients received at least four vaccinations, and one partial response and four mixed responses were observed[47]. In a phase II study, the combination of AS15 adjuvant (containing CpG 7909, monophosphoryl lipid and QS-21) plus MAGE-A3 peptide enhanced the immune response and was associated with tumor regression[48]. This provided the rationale for the development of a phase III trial with this vaccine in the context of melanoma and lung cancer. Unfortunately this trial failed to meet its endpoints and was halted.

Another vaccine based on irradiated melanoma cells, called Canvaxin™, was studied in a non-randomized trial and showed promising results compared to the historical control (overall survival at 5 years of 39% patients with the vaccine compared to 19% without the vaccine)[49]. However, this advantage was not confirmed in a phase III trial where this vaccine was used in the adjuvant setting in 1656 patients with resectable stage III/IV melanoma. In order to improve the quality of adjuvants, vaccines have been developed consisting of melanoma cells that secrete granulocyte-macrophage colony-stimulating fator (GM-CSF), often referred to as G-VAX. While the GM-CSF gene vaccine (GVAX) alone has not secured a high level of resposnes, it is still being considered in combination with other interventions e.g. an ongoing phase I trial with allogeneic melanoma cell GVAX with or without cyclophosphamide in resected high-risk melanoma[50].

The T-VEC vaccine (talimogenelaherparepvec), consisting of attenuated herpes simplex virus (HSV) associated with GM-CSF, was studied in a phase III trial (2:1) with 436 unresectable stage IIIA/B/C melanoma patients. The vaccine or the GM-CSF alone were injected into the

tumor, with an overall objective response of 26.4% and 5.7% for the arms of the T-VEC vaccine and GM-CSF, respectively[51]. This vaccine is now being studied in combination with immune checkpoints (ipilimumab) in advanced melanoma patients[52], and also in the neoadjuvant setting[53].

1.1.2.3 Cytokines

Cytokines are a diverse group of proteins important for immune system regulation. Two cytokines, IL-2 and IFN-α, have been approved by the Food and Drug Administration (FDA) for patients with stage IV and unresectable stage III melanoma, respectively.

IL-2 stimulates the proliferation and activity of T and NK cells and is associated with regression of some immunogenic tumors, such as kidney cancer and melanoma. High dose recombinant IL-2 was approved by the FDA in 1998 for patients with stage IV melanoma with good performance status[54]. Up to 5% of patients can exhibit a response to IL-2 but its use is less ferequent due to its toxicity and the advent of other treatments.

Pegylated interferon-α2b (PEG-IFNα-2b) is IFNα-2b conjugated with a polyethylene glycol molecule which confers a longer half-life. This compound was approved by the FDA in March 2011 for the adjuvant treatment of patients with stage III melanoma based on a randomized, multicenter, phase III clinical trial. This study was positive, with significant improvement in the primary endpoint, disease-free interval [hazard ratio (HR)=0.86; p=0.036)]; however, the difference in survival rate between the groups of patients treated with PEG-IFNα-2b and patients who remained under observation was not statistically different (HR = 0.98; p = 0.78)[30]. Thus, the approval of PEG-IFNα-2b for patients with stage III melanoma was based on a longer and clinically significant disease-free interval. Subsequently, a meta-analysis that included 14 trials with interferon showed a slight, but statistically significant, advantage in disease-free interval in patients with high risk cutaneous melanoma[31].

Currently, with new therapeutic options, cytokines are not as used in the treatment of melanoma as a single agent, but they remain a valid option in combination with other drugs.

1.1.2.4 Adoptive transfer of cells

This therapy consists of the extraction of TILs, their expansion and activation *ex vivo*, followed by infusion of these cells into the patient. In a National Cancer Institute (NCI) clinical trial, patients were previously submitted to Total Body Irradiation (TBI - 2 Gy or 12Gy) and to a non-myeloablative regimen with fludarabine and cyclophosphamide, while the cells were expanded with the T-cell stimulating anti-CD3 monoclonal antibody (OKT3) and IL-2. In this study, objective responses according to Response Evaluation Criteria in Solid Tumors (RECIST), were observed in 49% to 72% depending on the conditioning regimen prior to the cells' infusion. This study showed that the persistence of clones of T cell infused, as well as the length of the telomeres were associated with better clinical responses[44,55,56].

1.1.2.5 Immune checkpoints

Immune checkpoints are a family of inhibitory receptors that play a fundamental role in the maintenance of immunological tolerance, by modulating the duration and amplitude of the physiological immune response, in order to prevent autoimmunity. Consequently, they can prevent a robust and effective antitumor response, contributing for tumor progression. This family of immune checkpoints includes different molecules, such as CTLA-4, PD-1, Tim-3, lymphocyte activation gene 3 (LAG-3), B- and T-lymphocyte attenuator (BTLA), amongst others[45,46].

CTLA-4 is exclusively expressed by T cells, varying according to the activation state and subtype of these cells. This receptor is mostly intracellular, but following activation it is rapidly expressed in the membrane. Both CTLA-4 and CD28 (an activating receptor), bind to CD80 and CD86 receptors on DCs, however the affinity of CTLA-4 to CD80/CD86 is approximatly 40 times higher compared with the affinity of CD28 to the same receptors. For this reason even a low level expression of CTLA-4 in the membrane of T cells can block the CD28-CD80/CD86 interaction, preventing the activation of these cells[45]. The effect of CTLA-4 can be observed in two different contexts: inhibition of $CD8^+$ and $CD4^+$ Th and activation of Treg (regulatory T cells). CTLA-4 regulates immune responses in an early and critical phase of T cell activation, as evidenced by the phenotype developed by CTLA-$4^{-/-}$ mice,

which are characterized by a fatal lymphoproliferative disorder in 3-4 days[57]. In preclinical studies it was shown that blockade of this receptor by monoclonal antibodies led to an improvement of the activity of $CD4^+$ T cells, increased $CD8^+$ T cells:Tregs ratio and inhibition of Tregs in tumor models with some endogenous immune response, or even in poorly immunogenic tumors when combined with a vaccine.

These pre-clinical findings led to the development of two anti-CTLA-4 monoclonal antibodies: tremelimumab and ipilimumab. In a phase III trial the effect of tremelimumab (n=328; 15mg/kg 3/3 months) was compared with that of chemotherapy (n=327; dacarbazine 1000 mg/m^2, or temozolomide 200 mg/m^2) in patients with advanced melanoma. Although several patients had long-lasting responses, there was no improvement in survival (p=0.127)[58], probably due to the dosing schedule and patient selection[59].

The first phase III trial with ipilimumab for previously treated patients with unresectable stage III/IV melanoma was published in 2010 in the New England Journal of Medicine (NEJM). This study compared three arms: ipilimumab (n = 137; 3mg/kg 3/3 weeks, 4 cycles), gp100 vaccine (n = 136) and the combination ipilimumab + gp100 vaccine (n = 403). There was a distinct advantage of the arms that contained ipilimumab compared with the vaccine, with a survival rate at 2 years of 14%, 22% and 24% in the vaccine, combination and ipilimumab groups, respectively. The median survival on the ipilimumab arm was 10.1 months compared with 6.4 months in the arm of the gp100 vaccine (HR=0.68; p<0.001)[35] (**Figure 1.4**). Based on these results the FDA approved this drug for the treatment of metastatic melanoma in March 2011. Another Phase III trial included 502 patients with untreated advanced melanoma that were randomized to treatment with dacarbazine (850mg/m^2) plus placebo or dacarbazine (850mg/m^2) plus ipilimumab (10mg/kg). The primary objective of this study was OS and this was higher in the dacarbazine + ipilimumab arm (HR=0.72; p<0.001). There was also a higher percentage of adverse effects in this group, mainly gastrointestinal disorders (p <0.001)[60].

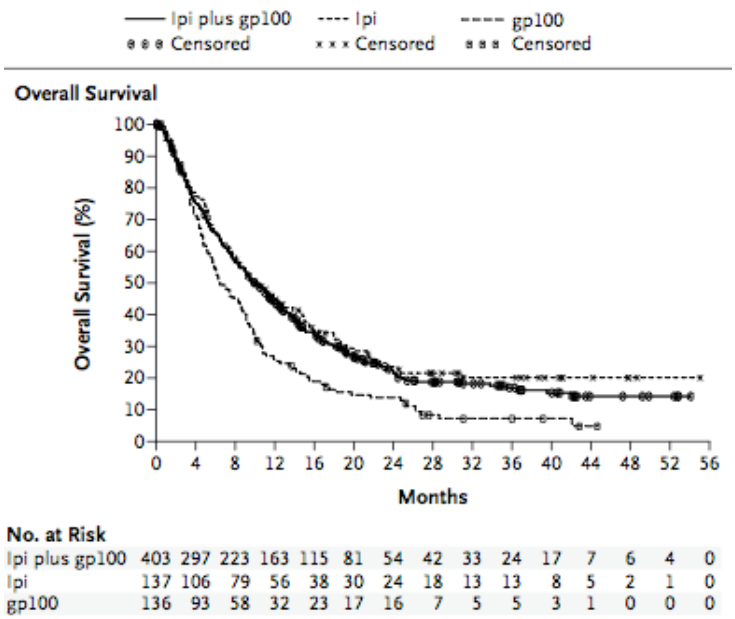


Figure 1.4. Overall survival for advanced melanoma patients treated with: vaccine gp100; ipilimumab; vaccine gp100 + ipilimumab[35].

More recently, **PD-1** emerged as a new therapeutic target with improved response rates and a better toxicity profile. PD-1 limits the activity of T cells at different stages of the immune response through interaction with their natural ligands, PD-L1 and programmed (cell) death-ligand 2 (PD-L2). This inhibition occurs in the peripheral tissues and in the TME, by inhibiting signaling pathway dependent kinases that activate T cells through the activity of a non-receptor tyrosine kinases (Src) homology region 2 domain-containing phosphatase-1 and -2 (SHP-1 and SHP-2). Contrary to CTLA-4, PD-1 is expressed in other immune cells other than T cells, including B and NK cells. Therefore PD-1 blockade will have an effect on the function of these different cells[45].

Several anti-PD-1 monoclonal antibodies have been developed, of which pembrolizumab and nivolumab have been approved by the FDA in September and December 2014, respectively. A phase II study published in NEJM 2012, assessed the safety and antitumor activity of nivolumab with a dose ranging from 0.1-10.0mg/kg 2/2 weeks. This study enrolled 236 patients (76 lung cancer, 94 melanoma, 33 kidney cancer) and had a 18% response rate for lung cancer, 28% for melanoma and 27% for kidney cancer[61]. Survival analysis in this study showed a median survival of 16.8 months, with survival rates at 1 and 2 years of 62% and

43%, respectively[62]. Two other studies compared nivolumab with chemotherapy in different contexts. In one of the trials, treatment with nivolumab (n=268; 3mg/kg) was compared with chemotherapy (n=102; dacarbazine 1000 mg/m^2; or carboplatin AUC 6 + Paclitaxel - 175 mg/m^2). There was an advantage in the nivolumab arm with a response rate of 32% compared with 11% in the chemotherapy arm in melanoma patients who progressed under ipilimumab[63]. In the other study, nivolumab (3 mg/kg) was compared with dacarbazine (1000 mg/m^2), in 418 patients with melanoma BRAF^{WT} without previous treatment. This study showed an improvement in survival at one year in the nivolumab group (72.9%) compared with 42.1% in the dacarbazine group (HR=42, p<0.001)[64]. In the trial with the pembrolizumab (formerly lambrolizumab), 135 patients with advanced melanoma (with or without pretreatment with ipilimumab) were included and different doses were tested: 10 mg/kg every 2/3 weeks or 2 mg/kg every 3 weeks. There was a 38% response rate according to RECIST with the highest response rate in the cohort who received the highest dose (10 mg/kg). There was no significant difference between patients previously treated or untreated with ipilimumab[65].

PD-L1 is one of the natural ligands of PD-1 and CD80, which is expressed by the tumor cells, stromal cells and immune cells in the TME. It limits PD-1 activity on tumor-infiltrating CD4$^+$ or CD8$^+$ cells. For this reason PD-L1 has become an interesting therapeutic target; the anti-PD-L1 monoclonal antibodies inhibit not only the binding of the receptor to PD-1, but also to CD80. In a phase I trial 207 patients with solid tumors (55 advanced melanoma patients) were recruited and treated with anti-PD-L1 (doses ranging from 0.3 to 10mg/kg). This antibody showed response rates between 6-17% and stabilization of the disease in 12-41% of cases of melanoma, lung and kidney, and a safe profile, with grade 3/4 adverse events experienced by 9% of the patients[66].

1.1.2.6 Immunotherapy Dynamics

The pattern of the clinical response to immunotherapy is completely different from that seen with chemotherapy. Through immunotherapy the immune system is modulated in order to effectively fight the tumor, which can lead to different responses that may affect therapeutic decisions. Firstly, sometimes, before a clinical and radiological response takes place, there may be an increase in tumor size or even the appearance of new lesions. Therefore, new

response evaluation criteria for evaluating immunotherapy - the immune-related response criteria (IRRC) - were proposed. Secondly, the responses to immunotherapy are often late responses and may happen after several weeks or months of treatment. Finally, these responses tend to be long-lasting[67]. Recently a pooled analysis that included 1861 patients treated with ipilimumab, from different studies (10 prospective and retrospective 2, including two clinical phase III trials), showed a survival rate at 3 years of 22%, which remained in a plateau up to a maximum follow-up of 10 years[68] (**Figure 1.5**).

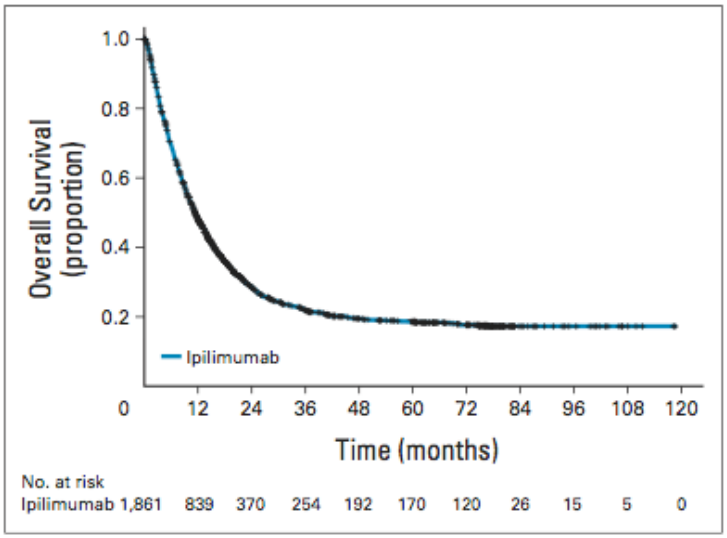


Figure 1.5. Overall survival of patients treated with ipilimumab from 12 different studies (pooled analysis)[68].

1.1.2.7 Immunological adverse effects

There is a clear long-term advantage in using the above-mentioned recent immunotherapy strategies, however they also present a downside. These treatments are associated with different side effects as a result of a nonspecific and exaggerated immune response, resulting in an autoimmune reaction. The percentage of grade 3-4 adverse events related to immunotherapy is similar to that observed with chemotherapy (approximately 10% to 20%), however they show different patterns. The most common toxicities that result from immunotherapy include itching with or without skin rash, diarrhea, colitis, hepatitis and endocrinopathies[35,61,69]. Dermatological toxicity is the most common and has an early onset (few weeks). It is characterized by an erythematous, pruritic, maculopapular rash on the trunk

and limbs and is generally treated with topical corticosteroids and antihistamines. Patients with severe rashes, such as Stevens-Johnson syndrome and toxic epidermal necrolysis, have to be admitted and treated with intravenous corticosteroids and hydration. The second most frequent side effect is diarrhea that is often associated with colitis, which usually appears after the 6th week. Mild cases of diarrhea are controlled with oral steroids, while more severe cases may need hospitalization and treatment with intravenous corticosteroids and infliximab. The elevation of transaminases (AST and ALT) is more frequent during treatment with anti-CTLA-4 treatment than with anti-PD-1, and is often asymptomatic. When these cases of hepatitis are symptomatic they should be treated with oral or intravenous steroids according to their severity, and mycophenolate mofetil should be added when necessary. The endocrinopathies may reach different glands including thyroid, adrenal and pituitary. Hormone monitoring and replacement must be done according to the clinical picture[69].

1.1.2.8 Response markers to immunotherapy

We are approving powerful therapeutic weapons that are extremely expensive from an economic point of view and that are also associated with important adverse effects. For this reason, these new strategies should be offered only to patients who will respond to the treatment; therefore biomarkers predictive of response need to defined. Regarding treatment using ipilimumab, absolute lymphocyte count has been described as a pharmacodynamic biomarker and an increase of this cell count is associated with improved survival[70]. In the case of anti-PD-1, PD-L1 seems to play this role. In a phase I clinical trial Topalian *et al* showed, in a small cohort (n=42), that the lack of PD-L1 expression by the tumor cells is associated with no clinical response to nivolumab (anti-PD-1)[61,62]. Later Weber *et al*. studied a larger cohort (n = 90) and set a new cut-off, showing that 67% of patients with 5% PD-L1^{+} tumor cells responded to the nivolumab while only 19% of PD-L1^{-} patients responded to the same treatment[71]. Moreover, in a recent phase I trial, pembrolizumab (anti-PD-1) was shown to be more effective in Non–Small-Cell Lung Cancer (NSCLC) with more than 50% PD-L1^{+} tumor cells[72]. The controversy remains and the best method of quantification of PD-L1 expression in the tumor, as well as the best cut-off and the true relationship with response to therapy, still need to be determined. Recent studies have shown that tumor mutational load and, more

importantly, a signature of somatic neoantigens are associated with clinical response to treatment with immune checkpoint inhibitors[73,74].

1.1.2.9 Strategies based on the combination

Immune checkpoint inhibitors have been effective against more immunogenic tumors, such as melanoma, kidney and lung cancer. Thus, several strategies have been designed to render tumors more immunogenic, such as low-dose chemotherapy, radiotherapy or even vaccines. The combination of these monoclonal antibodies with conventional chemotherapy was shown to be associated with liver toxicity without significant clinical response. The same was observed with the combination of ipilimumab (immune checkpoint inhibitor) with vemurafenib (BRAF inhibitor); moreover, the liver toxicity was so severe that the treatment had to be stopped[75]. On the other hand, a few retrospective studies have showed an advantage in clinical response and survival in patients who received radiotherapy in combination with ipilimumab[76]. These studies constitute the rationale for conducting clinical trials with this combination which are currently in enrollment phase[77]. The results of a phase II study that enrolled 245 patients with stage III/IV unresectable melanoma (naïve of treatment) treated with ipilimumab (10mg/kg) +/- GM-CSF were presented at American Society of Clinical Oncology (ASCO) meeting in 2013. Although no significant difference was observed in the response rate between the two groups, the group treated with the combination therapy showed greater overall survival rate (17.5 months) compared with the isolated ipilimumab group (12.7 months)[78].

Although CTLA-4 and PD-1 belong to the same family of immune checkpoints, they have different roles. While the interaction between CTLA-4 and its ligand B7 occurs in the lymph node, the PD-1 binding to PD-L1 occurs specifically in the TME. We are faced with two different but complementary mechanisms of T lymphocyte inhibition by the CTLA-4 and PD-1; therefore, there is a biological rationale behind the combination of anti-CTLA-4 with anti-PD-1. From a clinical point of view, this combination was associated with great results, with 53% objective response (all with 80% tumor reduction) in the cohort with the highest dose combination (nivolumab 1mg/kg; ipilimumab 3mg/Kg)[79]. The first results of OS of this study were presented at the ASCO meeting in 2014, with 2 years survival greater than 80% in some schemes[80], however these clinical responses were associated with significant toxicity. Grade

3-4 side effects were observed in more than 50% of patients and were qualitatively similar to previous studies with monotherapy and generally reversible. More recently, the results of the phase III study of the combination ipilimumab plus nivolumab were presented at the ASCO meeting in 2015 and published in the NEJM. Among previously untreated patients with metastatic melanoma, nivolumab alone (3mg/kg q2w) or combined with ipilimumab (Nivo 1mg/Kg q2w + IPI 3mg/Kg q3w) resulted in significantly longer progression-free survival than with ipilimumab alone (IPI 3mg/Kg q3w). Remarkably, in patients with PD-L1^{-} tumors, the combination of PD-1 and CTLA-4 blockade was more effective than either agent alone, while in patients with PD-L1^{+} tumors nivolumab alone seemed to be as effective as the combination (**Figure 1.6**)[81].

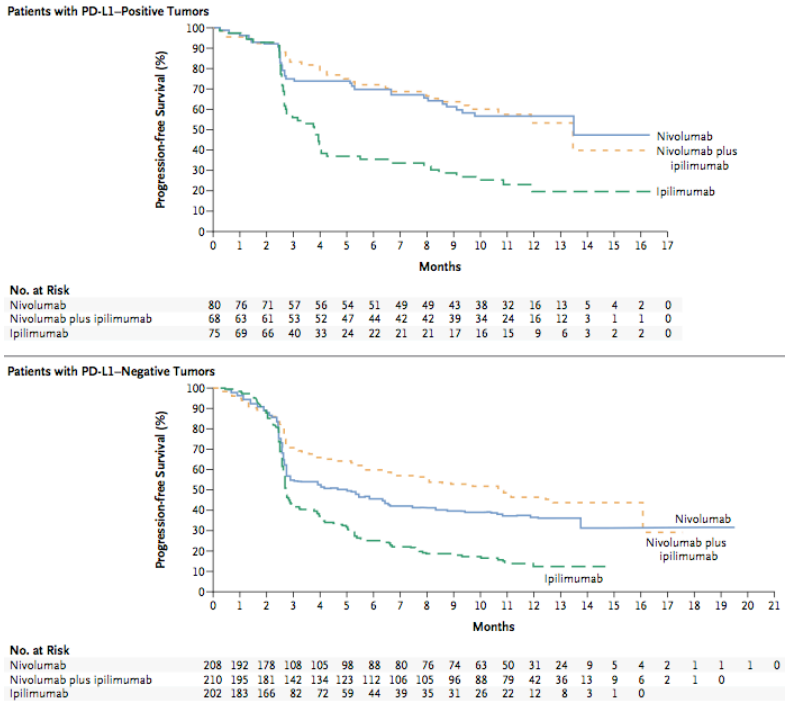


Figure 1.6. Progression-free survival of patients treated with ipilimumab, nivolumab or the combination ipilimumab+nivolumab in PD-L1-positive tumors (Upper plot) and in PD-L1-negative tumors (Lower plot)[81].

Immunotherapy is gaining increasing attention as a treatment of different types of cancer, as it is associated with long-lasting clinical responses. However, much still needs to be done, not only to be able to identify the long term responders, but also to increase the proportion of this group of patients and to identify markers of resistance. The answer may lie in the combination

of different immunotherapy strategies as well as in the association of immunotherapy with other types of treatment.

1.1.3. Immune exhaustion

Various states of T cell dysfunction have been described as a consequence of altered activation and differentiation processes including exhaustion, tolerance, anergy, senescence, and even ignorance. Tolerance in self-antigen specific T cells is a dysfunctional state required to prevent autoimmunity (self-tolerance), as a consequence of both central and peripheral immune tolerance mechanisms. In central immune tolerance, the thymocytes expressing T cell receptors (TCR) of too high affinity for self-antigen/MHC complexes are eliminated. Peripheral immune tolerance consists of deletion or suppression of self-reactive T cells that escape negative selection. Self-ignorance, in contrast to self-tolerance, is a state of no cell dysfunction, and is a consequence of antigen-inexperience. Therefore, "self-ignorant" T-cells persist as naïve, although they are potentially functional in the periphery. Anergy is commonly described as the dysfunctional state of T cells stimulated in the absence of co-stimulatory signals. Senescence is another cellular process that induces T cell impairment as a consequence of low-grade antigenic stimulation. Numerous mechanisms have been proposed to cause cellular senescence, such as repeated cell division, telomere shortening, and damage by reactive oxygen species (ROS)[82]. Senescent T cells lose the co-stimulatory molecule CD28 and their proliferative capacity, maintaining their cytotoxic activity and secretion of tumor necrosis factor alpha (TNF-α) and IFN-γ[83]. Finally, T cell exhaustion results from a chronic high-grade antigenic stimulation and refers to a state wherein T cells fail to respond to cytokines, proliferate or exert their effector cell functions (further details will be developed in the next section).

1.1.3.1 Immune cell exhaustion

Exhausted T-cell responses have been documented following numerous infections, including infections with human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), and have also been observed in patients with malignancies[84,85], as a consequence of chronic antigenic stimulation. Therefore, the levels and duration of antigenic

stimulation are critical determinants of this process and decreasing antigen availability, as occurs during treatment, generally helps the exhausted T-cell population regain their functional phenotype[86,87]. $CD4^+$ T cells have multiple effects on the overall immune response following infection and are often required for optimal $CD8^+$ T-cell responses. As a consequence, any abnormalities in, or loss of, $CD4^+$ T cells during the course of an infection, which can occur as these cells succumb to exhaustion, render $CD8^+$ T cells less effective. Moreover, severe chronic infections and tumors are associated with a higher expression of immunosuppressive cytokines, such as IL-10 and transforming growth factor-beta (TGF-β). IL-10 has multiple effects and it has been shown to reduce pro-inflammatory cytokine production, impede the functions of antigen-presenting cells (APCs), dampen T-cell responses and also affect B cells[88]. This cytokine is produced by $CD4^+$ T cells, including regulatory T cells, as well as by many other cell types such as DCs, macrophages, B cells and $CD8^+$ T cells. The relationship between IL-10 and T-cell exhaustion has been well studied using the lymphocytic choriomeningitis virus (LCMV) system, since chronic LCMV infection is associated with T-cell exhaustion and elevated levels of IL-10. Moreover, the administration of antibodies that block the IL-10 receptor immediately following infection, as well as a therapeutic regimen given after the infection had taken hold, resulted in lower viral loads, decreased expression of PD-1, and improved the functionality of the virus-specific T cells[89]. The immunosuppressive cytokine TGF-β has been implicated in regulating the size of the pathogen-specific T-cell responses and the propensity of these cells to undergo apoptosis. The significance of the TGF-β pathway on the development of exhaustion has been further dissected following LCMV clone 13 infection. Interestingly, elevated levels of TGF-β were observed under these conditions and the TGF-β pathway appears to regulate the extent of the response but not directly influence the functional capacity of the cells.

T-cell exhaustion is a progressive and stepwise process, where IL-2 production and proliferative ability are the first effector activities to be extinguished, followed by TNF-α production and cytotoxic activity (decreased production of perforin and granzymes), whereas the ability to produce IFN-γ is more resistant to inactivation[90]. In addition, as T cells acquire this exhausted phenotype, they start expressing inhibitory receptors, including Tim-3, PD-1 and LAG-3, while losing some activating receptors, such as CD43 (sialoglicoproteina), CD44 (cell adhesion molecule) and CD69. Furthermore, changes in the profile of transcription factors is also observed, with lower levels of T-bet and Eomes, and higher levels of B

lymphocyte-induced maturation protein-1 (BLIMP-1)[91]. The grade of functional impairment is a consequence of antigen load, duration of the infection and of $CD4^+$ T cell help and translates into distinct patterns of co-expression of inhibitory receptors (**Figure 1.7**). T cells also display an "exhausted phenotype" in the context of advanced cancers due to high tumor-antigen load and immunosuppressive factors in the TME. Therefore, T cells isolated from human tumors as well as experimental tumor models share many phenotypic and functional characteristics of exhausted T cells in chronic infections[92,93].

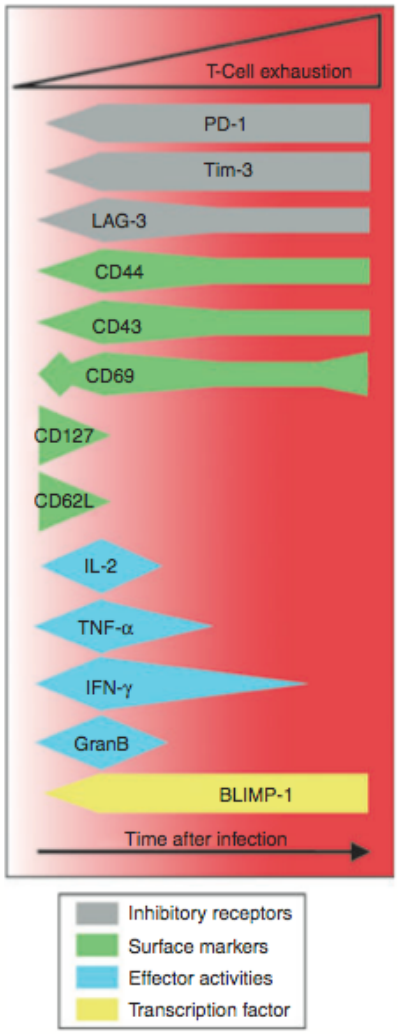


Figure 1.7. Sequential phenotypic and functional changes during the T-cell exhaustion process. Granzyme B (GranB); L-selectin (CD62L, cell adhesion molecule)[91].

Defects in NK cell activity have been described in patients with various metastatic malignancies, including melanoma[94,95,96,97]. Some hypotheses have been postulated to explain this dysfunctional phenotype including upregulation of MHC molecules, high expression of immunosuppressive cytokines (IL-10 and TGF-β), contact with myeloid-derived suppressor cell (MDSC), tumor-associated macrophages (TAM) or Tregs, or even chronic stimulation with soluble NKG2D ligands (MHC class I chain-related genes A and B - MICA and MICB)[3]. Nevertheless, there is little characterization of an "exhausted NK cell state" in metastatic disease, nor of the mechanisms that might underlie this phenotype.

Different strategies, including adoptive transfer of NK cells, have been attempted to control tumor growth, however they have been associated with mixed benefit. These disappointing results could be due to development of NK cell exhaustion. Indeed, adoptively transferred murine NK cells that trafficked to tumor sites, became rapidly dysfunctional and failed to control tumor growth[98]. NK cell "exhaustion," therefore, could explain the failure of NK cells to contain tumor growth in advanced cancers.

1.1.3.2 Exhaustion markers

Functional exhaustion is accompanied by a marked change in T cell phenotype, characterized by constitutive expression of inhibitory receptors (PD-1 and CTLA-4), which act synergistically to negatively regulate the functional and proliferative potential of the responding cells.

PD-1 is a type I transmembrane glycoprotein and is a member of immunoglobulin superfamily that contains a single V-set domain in its extracellular domain. The cytoplasmic domain contains one immunoreceptor tyrosine-based inhibitory motif (ITIM) domain and one immunoreceptor tyrosine-based switch motif (ITSM) domain, each of them containing a tyrosine residue. This receptor is expressed during thymic development primarily on $CD4^-$ $CD8^-$ T cells, and is induced not only on peripheral T cells but also on B cells, monocytes and NK cells upon activation[99]. The main role of PD-1 is to control the T cell response in peripheral tissues at the time of an inflammatory response. This results in a decrease of autoimmunity but also in an inefficacious anti-tumor response. PD-L1 and PD-L2 are upregulated in response to inflammation and have different patterns of expression, while PD-

L1 is expressed on many cell types, including hematopoietic, endothelial and epitelial cells, PD-L2 is upregulated on DCs and macrophages. When PD-1 binds to its ligands, ITSM becomes phosphorylated and recruits SHP-2 that inhibits kinases involved in T cell activation[100]. PD-1 crosslinking inhibits the TCR signal, decreasing the duration of T cell-APCs or T cell-target cell contact. Therefore, PD-1 plays a role in establishing peripheral tolerance and inhibits the proliferation and function of T cells, by repressing TCR signaling and inducing genes that impair T cell function such as basic leucine zipper transcription factor (BATF). *In vitro* studies show that blocking the PD-1 pathway allows T cell exhaustion to be partly overcome by enhancing the proliferation of $CD8^+$ and $CD4^+$ T cells and promoting cytokine production[101,102].

CTLA-4 is an inhibitory receptor structurally characterized by a type I transmembrane protein with a single V-set immunoglobulin extracellular domain, a transmembrane region and a cytoplasmic region that contains an ITIM domain. This receptor was firstly described on T cells, but recently it has also been shown to be present on monocytes and mature DCs[103]. In T cells the levels vary according to the cell subtype and activation status. Intracellular CTLA-4 is detectable only at low levels in naïve human T cells, whereas human T cell clones and memory T cells show significant amounts of this receptor. Upon activation, CTLA-4 is rapidly expressed on the cell surface, lasting longer on the membrane of memory cells compared with naïve cells. Tregs show high amounts of intracellular CTLA-4 and constitutively express this receptor at the cell surface[104]. The total amount of CTLA-4 in activated peripheral T cells is comparable to CD28; however its levels at the cell surface are significantly lower. Therefore, CTLA-4 is primarily localized in intracellular compartments [such as trans Golgi network (TGN), endosomes, secretory granules and lysosomal vesicles] whose surface expression is tightly regulated by restricted trafficking to the cell surface and rapid internalization. This is important because minor changes in surface expression levels could have major effects on the outcome of T-cell activation[105]. CTLA-4 is localized in the TGN, where it binds to the transmembrane adaptor T-cell receptor interacting molecule (TRIM) and/or the clathrin adaptor protein-1 (AP-1). Upon T-cell activation, CTLA-4 is translocated to the cell surface, promoted by the presence of TRIM. Alternatively, binding to clathrin AP-1 mediates direct transport to lysosomal compartments. In order to keep CTLA-4 on the cell surface, the Y201 residue must be phosphorylated by Src kinases. Subsequent dephosphorylation at Y201 allows the binding of the clathrin AP-2 and the rapid

internalization and transport of CTLA-4 to endosomal or lysosomal compartments. Internalised CTLA-4 might be degraded quickly or recycled to the cell surface via secretory lysosomes[106]. CTLA-4 has a key role in controlling and dampening T cells after they have received co-stimulatory signals. By binding to CD80/CD86, CTLA-4 diminishes T-cell responses and helps bring an inflammatory response back down to homeostatic levels, establishing the peripheral immune tolerance. This idea was even clearer when the CTLA-4 knockout mouse phenotype was described to develop a lymphoproliferative disease[107]. Moreover, some polymorphisms in the CTLA-4 gene have been associated with susceptibility to different autoimmune diseases (type 1 diabetes, multiple sclerosis, rheumatoid arthritis, Graves' disease, colitis and systemic lupus erythematosus). Several mechanisms have been reported for CTLA-4 inhibition of T-cell activation. First, by direct competition for CD28 binding to CD80 and CD86. Second, through the disruption of CD28 localization at the immunological synapse. Finally, by interference with the expression or composition of lipid rafts on the surface of T cells. When T cells become activated, CTLA-4 binds to CD80 and/or CD86, leading to decreased IL-2 production, inhibition of proliferation and negative modulation of TCR signalling. Besides $CD8^+$ T effector cells, CTLA-4 seems to have an important role on two major subsets of $CD4^+$ T cells, with distinct effects: downregulation of Th cells and enhancement of Tregs. Therefore, Treg cells constitutively express high levels of CTLA-4; in an *in vitro* experimental model of colitis blockade of CTLA-4 specifically affected the suppression capacity of Tregs[108]. Additionally, CTLA-4 has a key role in $CD4^+$ T-cell differentiation. CTLA-4-deficient mice and Balb/c mice treated with anti-CTLA-4 monoclonal antibody (mAb) showed an increased Th17 differentiation and IL-17 production[109]. Given the fact that CTLA-4 is an inhibitory receptor that regulates T cell responses, it has become a potential therapeutic target. Preclinical studies, using anti-CTLA-4 mAb, demonstrated significant antitumor responses *in vitro* and *in vivo* models, such as, lymphoma[110], prostate cancer[111], colon carcinoma and fibrosarcoma. Interestingly, only the animals that induced an endogenous anti-tumor response after tumor implantation, responded to the CTLA-4 treatment[112].

Although PD-1 and CTLA-4 are the best characterized inhibitory receptors associated with exhaustion, several other receptors have also been shown to impair T-cell responses during chronic infections, including CTLA-4, Tim-3, LAG-3, 2B4 (CD244, non-MHC binding natural killer cell receptor), among others. These inhibitory receptors act synergistically, but

not redundantly, to establish T cell exhaustion that is ultimately "tuned" by the availability of ligands in the environment[113]. Blockade of negative checkpoint receptors is a strategy to overcome the tumor-induced T cell dysfunction, and has emerged as a promising approach for treatment of cancers. As mentioned in the immunotherapy section, CTLA-4- and PD-1-blocking monoclonal antibodies are FDA approved for treatment of melanoma and lung cancer and are currently being tested in other malignancies.

1.1.4. Tim-3

Tim-3 was first described as a negative regulatory molecule important for dampening Th1 and Tc1 driven immune responses. This receptor is comprised of an N-terminal IgV domain followed by a mucin domain, a transmembrane domain and a cytoplasmic tail. In the IgV domain there are four non-canonical cysteines, which result in the formation of an unique binding cleft, that has been shown to be important for the binding of Tim-3 to phosphatidylserine and for the clearance of apoptotic bodies by macrophages and DCs. It is not expressed on the surfaces of naive T cells but emerges on the cell surface of fully differentiated $CD4^+$ Th1 cells, and also on $CD8^+$ T cells, Th17, Tregs, NK cells, macrophages, monocytes, DCs, mast cells and microglia. Tim-3, as one of the critical $CD4^+$ Th1 cell surface molecules, may have multiple functions in regulating T cell responses[114].

1.1.4.1 Ligands

Different molecules have been postulated as Tim-3 ligands, each with distinct roles. Galectin-9 (Gal-9) was the first one described; it is a soluble molecule that is widely expressed and is upregulated by IFN-γ. It binds to Tim-3 amino-linked carbohydrates on the IgV domain and when it activates Tim-3 on Th1 cells, induces cell death[115]. High mobility group box 1 (HMGB1) and phosphatidylserine (PtdSer) are two other ligands that bind to Tim-3 through a metal ion–dependent ligand-binding site in the hydrophobic FG-loop of the immunoglobulin variable domain. HMGB1 is a deoxyribonucleic acid (DNA)-binding protein that besides acting as a chromatin factor with roles in genome stability and the promotion of transcription in the nucleus, it has also key roles in inflammation, immune responses, DC differentiation, migration of inflammatory cell, and regulation of autophagy. In a recent study it was shown

that the regulation of nucleic acid–mediated innate immune responses by Tim-3 is dependent on HMGB1. HMGB1 consists of DNA-binding A and B boxes, as well as an acidic carboxyl terminus. Tim-3 actively competes with nucleic acids to bind the A box of HMGB1. As a receptor for HMGB1, Tim-3 impairs the HMGB1-mediated recruitment of nucleic acids into endosomes, a key step in the sensing of DNA by the innate immune system[116]. In addition, DCs are essential targets in efforts to generate therapeutic immunity to cancer; they interact with natural killer cells to promote their further activation and immunity to tumors through the release of HMGB1. Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) was recently described as a Tim-3 ligand, which facilitates the maturation and cell surface expression of Tim-3, and their interaction determines the tolerance-inducing function of Tim-3 and contributes to its inhibitory function. Interestingly, co-blockade of CEACAM1 and Tim-3 enhances the anti-tumor immune response in a tumor mouse model[117]. Finally, HLA-B-associated transcript 3 (Bat3) was shown to be a binding partner to the Tim-3 intracellular tail. However, contrary to the previous ligands, it functions as an inhibitory receptor for Tim-3 function, since it protects Th1 cells from galectin-9 mediated cell death, and promotes both proliferation and pro-inflammatory cytokine production. Therefore, Bat3 seems to represent an important novel repressor of Tim-3 signaling that protects Th1 responses from Tim-3-mediated inhibition[118].

1.1.4.2 Role of Tim-3 on T cells

It has been reported that Tim-3 negatively regulates Th1-mediated inflammatory diseases such as experimental autoimmune encephalomyelitis (EAE), type I diabetes, and acute graft-versus-host disease (aGVHD). The blockade of the Tim-3–Tim-3L pathway *in vivo* exacerbates EAE and Type-1 diabetes[119]. Similarly, abrogation of Tim-3 signaling either with blocking antibody or by RNA interference increases the secretion of IFN-γ by activated human T cells[120]. In addition, Tim-3 is also involved in the induction of peripheral tolerance, since its blockade abrogates the development of tolerance in Th1 cells and Tim-3-deficient mice are refractory to induction of tolerance after high dose antigen administration[121]. More recently Tim-3 has emerged as a marker of T cell exhaustion and is over-expressed in impaired $CD8^+$ T cells in various chronic viral infections and advanced cancer (**Figure 1.8**)[122]. This finding is clinically relevant since it is possible to restore the function of these impaired $CD8^+$ T cells by blocking the Tim-3-Tim-3L pathway[123]. Interestingly, upregulation

of Tim-3 has also been observed in PD-1$^+$ CD8$^+$ T cells in some tumor mice models, as well as, in blood of patients with advanced melanoma. In both cases, Tim-3$^+$PD-1$^+$ CD8$^+$ cells represent the most impaired population of CD8$^+$ T cells, exhibiting defects in proliferation and producing the least amount of IL-2, TNF, and IFN-γ. Furthermore, combined treatment with anti-Tim-3 and anti-PD-L1 or anti-PD-1 resulted in a much better response when compared with each blockade separately[124,125]. Interestingly, the majority of intratumoral FoxP3$^+$ Tregs express Tim-3 and PD-1, are highly suppressive and are rarely observed in the peripheral tissues or blood of tumor-bearing mice. Moreover, the co-blockade of the Tim-3 and PD-1 signaling pathways *in vivo* results in the downregulation of molecules associated with Tim-3$^+$ Treg suppressor functions, suggesting that the potent clinical efficacy of co-blocking Tim-3 and PD-1 signaling is a result of the reversal of T-cell exhaustion combined with the inhibition of regulatory T-cell function in tumor tissues[126,127].

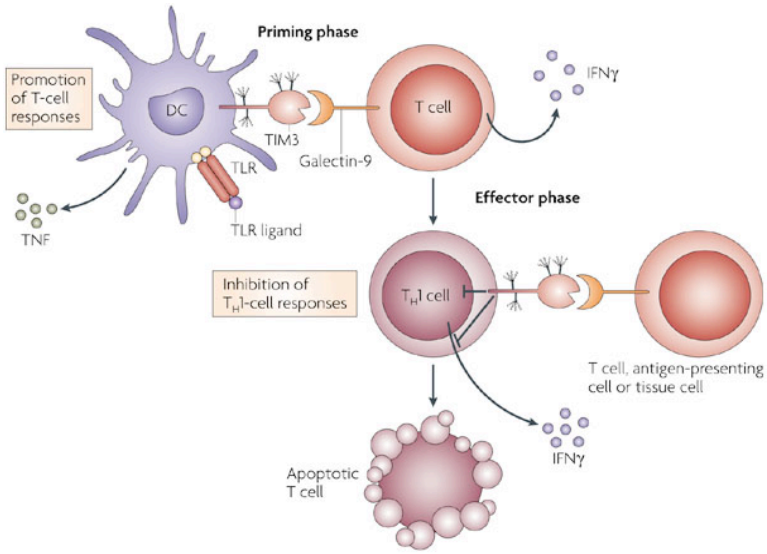


Figure 1.8. The role of Tim-3 on innate (DCs) and adaptive (T cells) immune cells[122]

1.1.4.3 Role of Tim-3 on innate cells

Tim-3 is constitutively expressed on innate immune cells including NK cells, macrophages, monocytes and DCs. On macrophages, Tim-3 is upregulated in response to stimuli, acting as an activation marker. However, blockade of the Tim-3 pathway during sepsis *in vivo* or downregulation of Tim-3 on macrophages *in vitro* led to enhanced macrophage activation,

thereby indicating that it is acting as an inhibitory receptor. Therefore, Tim-3 can be upregulated on macrophages in response to stimuli, but it may also act to suppress macrophage activity[128]. In regard to monocytes, stimulation with the toll-like receptor 4 (TLR4) ligand LPS or the toll-like receptor 7/8 (TLR7/8) ligand R848 downregulates Tim-3 expression, leading to an enhanced IL-12 expression[129]. Thus, increased Tim-3 expression on monocytes may act to suppress their activity, whereas reduced Tim-3 expression may be associated with monocyte activation. In such a scenario, increased Tim-3 expression and a failure in the downregulation of Tim-3 on macrophages/monocytes may be an indicator for dysfunctional macrophages/monocytes, as demonstrated previously for $CD4^+$ and $CD8^+$ T cells[130]. In addition, a recent study demonstrated that Tim-3 recognizes apoptotic cells through the FG loop in its IgV domain and is crucial for the clearance of apoptotic cells by phagocytes. Therefore, cross-presentation of dying cell-associated antigens was shown to be reduced by Tim-3 pathway blockade *in vitro* and *in vivo*[131]. Tim-3 was also studied in DCs and in the TME these cells up-regulate Tim-3 that interacts with the alarmin HMGB1, suppressing nucleic-acid-mediated innate immune responses. Remarkably, when Tim-3 signaling was blocked using anti-Tim-3 mAb, chemotherapy was found to be more effective, suggesting that Tim-3 plays a role in tumor pathogenesis[116].

Two studies on the role of Tim-3 on NK cells from healthy donors were published presenting different conclusions. One of the studies showed that many cytokines, such as interferon-alpha (IFN-α) and combinations of IL-12+IL-18 or IL-12+IL-15, could induce Tim-3 upregulation on NK cells. Furthermore, NK cell IFN-γ production and cytotoxic ability were associated with high Tim-3 levels. These authors therefore concluded that Tim-3 might act as a marker of fully mature and functional NK cells. Moreover, when Tim-3 was cross-linked on NK cells with antibodies or encountered target cells that expressed its ligand Gal-9, it suppressed NK cell-mediated cytotoxicity, showing that Tim-3 also suppresses NK cell functions[132]. On the contrary, the second study demonstrated that Tim-3 was upregulated on human NK cells following activation and promoted IFN-γ production in response to Gal-9[133]. A negative regulatory role of Tim-3 on the activity of NK cells has been demonstrated in chronic hepatitis B infection where Tim-3 expression is upregulated on NK cells. This receptor suppresses NK cell function as this inhibition could be reversed by blockade of the Tim-3 pathway[134]. These data suggest that Tim-3 is a marker of NK cell activation, however

its overexpression may also restrain NK cell function in certain situations.

1.1.4.4 Role of Tim-3 on nonhematologic cells

Tim-3 is also expressed on cancer cells and in other nonhematologic cells. Tim-3 is expressed in endothelial cells in lymphoma resulting in local immune suppression and, consequently, adverse clinical features of lymphoma[135]. Furthermore, Tim-3 is expressed at a higher levels in clinical cervical cancer cells compared to cervical intraepithelial neoplasia (CIN) and chronic cervicitis controls, and that this expression may promote metastatic potential in cervical cancers[136].

1.1.4.5 Tim-3 as a therapeutic target

To date, few studies have extensively assessed the mechanism of action of anti-TIM3 mAb against tumors. In a fibrosarcoma mice model, the combination of anti-TIM3 and anti-PD-1 mAbs significantly suppressed established tumor growth and even resulted in cures in a small proportion of these treated mice[123,124,137]. Interestingly, Tim-3 blockade in combination with a vaccine stimulates potent anti-tumor responses against established melanoma. This effect occurs via NK cell-dependent mechanisms and not through T-cell action[138]. Of importance, anti-TIM3 alone or in combination with anti-PD-1 was well tolerated, and no overt autoimmunity was observed in treated tumor-bearing mice.

1.1.5. NK cells

NK cells are innate immune cells with natural cytotoxicity against tumor cells. These cells are distributed throughout lymphoid and nonlymphoid tissues in different proportions, representing only 2% to 18% in human peripheral blood with a turnover of two weeks. NK cells are generally divided into two subsets according to the expression of CD56 e CD16 receptors and function. The cytotoxic subset, characterized by $CD56^{dim}CD16^{+}$, corresponds to 90% of NK cells present in the peripheral blood and spleen. On the contrary, the cytokine producer subset, $CD56^{bright}CD16^{-}$, is the majority of NK cells in the lymph nodes and tonsils, and comprises only 10% of the peripheral blood population. Different chemokines and their

respective receptors are responsible for NK cell mobilization and homing. Therefore, $CD56^{bright}CD16^{-}$ NK cells express C-C chemokine receptor type 7 (CCR7) that induces their homing to lymph nodes. Moreover, the $CD56^{dim}CD16^{+}$ human NK cell subset expresses CXC chemokine receptor 1 (CXCR1), also called interleukin 8 receptor alpha (IL-8Rα), which is important for recruitment of NK cells into peripheral inflammatory sites[139].

1.1.5.1 NK-cell differentiation and education

The precursor NK (pNK) cell is generated from multipotent haematopoietic precursors, including haematopoietic stem cells and early lymphoid precursors. NK cells are derived from $CD34^{+}$ pNK cells through discrete stages of development, such as pNK, immature NK (iNK) and mature NK, and are characterized by the sequential acquisition of surface receptors and effector function. As the cells differentiate from pNK to iNK cells, the expression of Fms-like tyrosine kinase 3 (Flt3) and interleukin-7 receptor alpha (IL-7Rα/CD127) decreases, whereas the expression of IL-2R beta chain (IL-2Rβ), CD2 (cell adhesion molecule) and 2B4 increases. The final maturation of NK cells is characterized by the acquisition of activating and inhibitory receptor expression. The acquisition of mature NK cell function is also dependent on the presence of EOMES and T-bet that induce the expression of the cytolytic machinery (for example, perforin and granzymes) and IFN-γ. The next step of NK cell maturation is called "education or licensing", when inhibitory NK-cell receptors engaged by self MHC molecules generate NK cells that are tolerant to self and have the capacity to "sense" MHC class I expression levels on target cells that may vary during infection, transformation or stress. Nevertheless, around 10-20% of NK cells do not express any known inhibitory receptors for self MHC class I molecules and consequently do not interact with self MHC molecules. These cells, could be potentially harmful as they fail the inhibition by self MHC class I molecules, however they have a reduced response to stimulation via activating receptors. Therefore, mature NK cells ($CD11b^{hi}CD4^{+}9b^{+}$) found in the periphery include educated, fully responsive NK cells stimulated via through activating receptors and self-tolerant NK cells, but also hyporesponsive NK cells[140].

After the maturation process NK cells leave the site where they are generated and traffick to other tissues, such as the secondary lymphoid organs, lungs, liver and gut. In these different sites, according to their activation status, they can be "resting" NK cells, where they lack

signs of recent activation or "activated" NK cells, that are highly efficient and bear markers of recent stimulation[140].

1.1.5.2 NK cell function

The activity of NK cells is a result of a dynamic balance between activating and inhibitory receptors that allows them to be ready to kill target cells or to be self-tolerant to avoid autoimmunity[141] (**Figure 1.9**). The activating receptors detect the presence of diferent types of ligands on cells in "distress" including, stress-induced self-ligands (MICA, MICB and UL16 binding protein - ULBP), infectious nonself ligands and TLR ligands. These activating receptors include NKG2D, natural cytotoxicity receptors (NCRs – such as natural killer p30, p44 and p46 - NKp30, NKp44 and NKp46, respectively) and DNAM-1. Furthermore, as NK cells also express the low-affinity Fc receptor CD16, they are able to exert antibody-dependent cell cytotoxicity. Some of these activating cell surface receptors stimulate protein tyrosine kinase-dependent pathways through reversible associations with transmembrane signaling adaptors. Others, like NKG2D, are not directly coupled to immunoreceptor tyrosine-based activation motifs and are well associated with transmembrane signaling adaptors (like DNAX-activating protein 10 and 12 - DAP10 and DAP12), as well as integrins and cytokine receptors. IL-2 is an important regulator of the expression of activating receptors, inducing some of them (NKp44 and CD69) and up-regulating others (NKp30, NKG2D and partially, NKp46). Inhibitory receptors detect the absence of constitutively expressed self-molecules on susceptible target cells (HLA class I molecules) allowing the NK cells to target specifically those cells ("missing-self theory"). In fact, tumor cells with low HLA class I expression (result of immunoediting), are the best targets for NK cells[142]. The inhibitory receptors belong to two main categories: the killer cell immunoglobulin-like receptors (KIRs) and lectin-like CD94-NKG2A. A common feature of these inhibitory receptors is the presence of ITIMs, that are phosphorylated on their tyrosine residues and recruit lipid or tyrosine phosphatases after ligand binding. The tyrosine phosphorylation status of several signaling components that are substrates for both protein tyrosine kinase and protein tyrosine phosphatases is thus key to the propagation of the NK cell effector pathways[142].

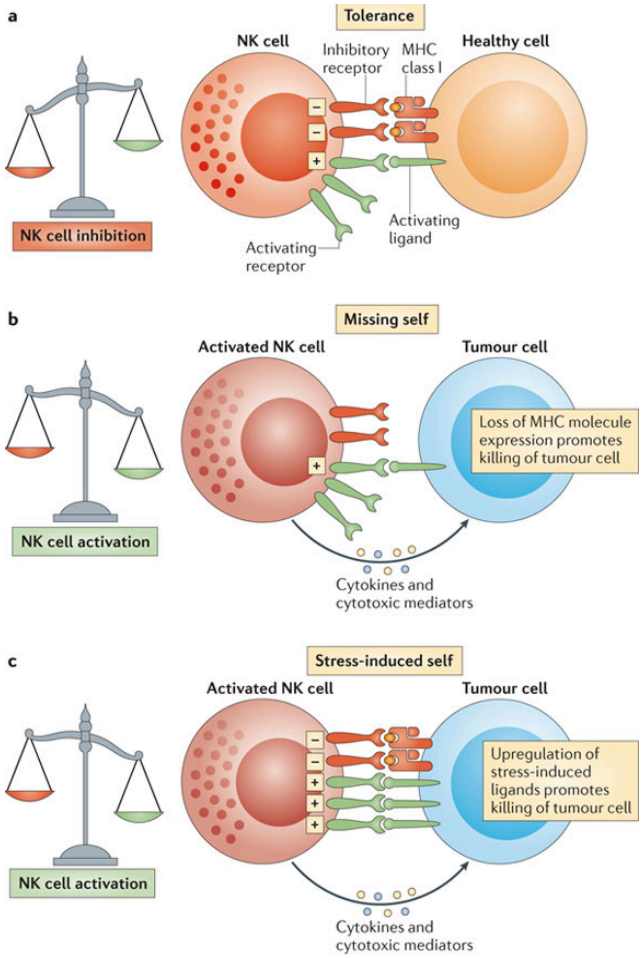


Figure 1.9. Different scenarios of NK cell activity according to the balance between activating and inhibitory receptors[141].

The intensity and the quality of NK cell cytotoxic and cytokine responses depend on the microenvironment, the presence/absence of cytokines and the interaction with some immune (DCs, $CD4^+$ T cells and Tregs) and non-immune (endothelial cells and fibroblasts) cells. NK cells need to be "primed" for full activation, as resting human peripheral blood NK cells are poorly effective. IL-2 is a key cytokine for NK cell activation, promoting their proliferation, cytotoxicity and cytokine secretion. NK cells are activated in the lymph nodes through T-cell-derived IL-2 production. Type I IFN, IL-12, IL-18 and IL-15 are other well known activators of NK cell effector function[139].

NK cells are mainly cytotoxic lymphocytes that act through direct cytotoxicity and/or by secreting some cytokine and chemokines. One of the mechanisms used by these cells is

exocytosis of granules containing perforin and various granzymes in the immunological synapse, leading to the perforation of target cells and subsequent apoptotic death. The NK cell immunological synapse is the dynamic interface formed between an NK cell and its target cell. The formation of the NK cell immunological synapse involves several distinct stages, from the initiation of contact with a target cell to the directed delivery of lytic granule contents for target cell lysis[143]. Progression through the individual stages is regulated, and this tight regulation underlies the precision with which NK cells select and kill susceptible target cells, including tumor cells that they encounter during their surveillance of the body. Another mechanism of cytotoxicity involves the engagement of TNF receptor superfamily (TNFRSF) members, such as Fas receptor (FasR), TNF-related apoptosis-inducing ligand (TRAIL) receptors and TNF receptor 1 (TNFR1), on tumor cells by the corresponding ligands (FasL, TRAIL and TNF) expressed on or secreted by NK cells. Furthermore, NK cells have a high capacity to secrete a variety of cytokines and chemokines, TNF, GM-CSF and macrophage inflammatory protein-1α (MIP-1α), and are considered as the major source of IFN-γ *in vivo*[144].

1.1.5.3 NK cell immunological memory

Adaptive immune cells, such as T cells, have the ability to learn from previous experience, such as a single encounter with many pathogens that exist, and to react with a specific and strong immune response. The result is immunological memory that confers long-lasting protection. Interestingly and unexpectedly, there is recent evidence showing that NK cells could be key players in the persistence of immunity, due to immunological memory. Therefore, a subset of liver NK cells were able to mediate an "adaptive-like" immune reaction of hapten-specific contact hypersensitivity in mice lacking T and B cells[145]. In a different study, mouse NK cells expressing the activating receptor lymphocyte antigen 49H (Ly49H), which recognizes the MCMV m157 protein, were shown to clonally expand and subsequently contract while leaving a few long-lived cells able to mount a "secondary" response. Furthermore, these cells were found to be active and protective when transferred to naive mice. In fact, following MCMV infection, NK cells can therefore give rise to a population of long-lived cells with an intrinsic ability to exhibit enhanced effector functions when restimulated[146]. Moreover, NK cells activated *in vitro* by cytokines (IL-12/IL-18) and

adoptively transferred *in vivo* also display some "memory-like" properties with an enhanced ability to secrete IFN-γ[147]. Altogether, these results prompt research into the mechanisms that allow the boosted effector function of NK cells to be maintained across cell divisions, in particular the epigenetic marks associated with various stages of these cells' activation.

1.1.5.4 NK cell surveillance

NK infiltration in tumor tissue is associated with better prognosis in squamous cell lung, gastric and kidney carcinomas[95,148,149], while low activity of peripheral blood NK cells is associated with increased risk of cancer[150]. Moreover, a remarkable increase in survival from relapse in myeloid leukaemia was reported for patients lacking HLA class I ligands for donor-KIR who received alloreactive NK cells in the course of allogeneic haematopoietic stem cell transplantation. This observation is clear evidence for the beneficial role of NK cells in the control of human malignancies[151]. Furthermore, it has been shown that high doses of IL-2 induces clinical responses in advanced melanoma patients, through T cell and also NK cell activation[54].

NK cells recognize tumor cells through the interaction with their activating and inhibitory receptors. The most important NK cell activating receptors that sense aberrant cells are NKG2D, NCRs (NKp30 and NKp46) and DNAM-1. NKG2D is a key receptor in the NK cell–mediated immune surveillance, as it has been shown to contribute to tumor progression in the context of impaired NKG2D function[152]. In addition, different NKG2D haplotypes are correlated with the natural cytotoxic activity of peripheral blood lymphocytes and the risk of cancer development in humans[153]. NKp30 is also important in NK-cell mediated antitumor activity. It mediates NK-DC crosstalk and promotes tumor cell recognition and lysis. Therefore, the loss of NKp30 expression correlates with impaired NK cell cytotoxicity and shorter survival in patients with acute myeloid leukemia (AML)[154]. Moreover, the lack of NKp46 failed to control effectively the growth of transplanted melanoma in a mice model[155]. DNAM-1 is an adhesion molecule with two well-known ligands, CD155 and C122 that are expressed by different tumors, including melanoma. Its relevance in the NK cell-mediated immuno-surveillance was shown in DNAM-1 knockout mice[156].

1.1.5.5 Immunosubversion

Immune surveillance is an important mechanism in controlling cancer progression; however it also induces immunoselection, where malignant cells with changes in NK cell-activating ligands are able to escape NK cells. Therefore, genetic instability of tumor cells might lead to mechanisms of immunosubversion including, among others, upregulation of MHC molecules that deliver strong inhibitory signals, and impaired expression of activating receptors and modulation of cellular ligands that engage activating immunoreceptors on cytotoxic cells, including NK cells and cytotoxic T lymphocytes and promote exhaustion. The shedding of NKG2DL from the surface of tumor cells is a good example of this last mechanism.

NKG2D is mainly expressed on NK and $CD8^+$ T cells and this expression is regulated by cytokines; IL-15 enhances NKG2D surface expression, while TGF-β downmodulates NKG2D. NKG2D-NKG2DL ligation causes phosphorylation of DAP10 that leads to activation of NK cells and costimulation of T cells. MICA/MICB and the ULBP constitute the two families of NKG2DL, that are induced by various forms of cellular stress such as viral infection, DNA damage or ultraviolet (UV) radiation. They are expressed in many tumors in humans[157], however MICA and/or MICB are also detected on non-tumoral tissues, such as the gastrointestinal ephitelium and tissues exposed to autoimmune attack. The expression of NKG2DL varies according to the type of tumor as well as to the stage of the tumor. Invasive tumors have elevated levels of serum sMICA because as disease progresses, tumor cells express more proteases, that are responsible for surface NKG2D ligands shedding. Interestingly, it has been shown that membrane-bound NKG2D ligands stimulate immunity whereas soluble NKG2DL impair host immunity, as recently demonstrated in the transgenic mice model[96]. Tumor cells release NKG2DL in a soluble form which critically affects tumor immunogenicity by downregulating the expression of activating receptors and dampening the activating signals for NK and T cells. Therefore, tumor shedding of MIC and sustained exposure of NK cells to NKG2DL systemically impairs the responses of $CD8^+$ T cells and NK cells[157,158].

Finally, an important phenomenon mentioned previously, is progressive NK cell exhaustion in the context of various tumors, as a consequence of continuous exposure to certain target antigens. Therefore, NK cells exhibited strong anti-tumor functions and cytokine production

early after adoptive transfer, however, starting at day 5 post-transfer, NK cells exhibited weak IFN-γ production and cytotoxicity, despite being present at the tumor sites[98].

1.1.5.6 NK cell-based therapies

NK cells preferentially target tumor cells with low HLA class I expression. This alternative recognition system of NK cells, which benefits from HLA downregulation on tumor cells, combined with their innate antitumor potential, offers a valid therapeutic option. In addition, some anti-tumor strategies, such as chemotherapy and radiotherapy, by forcing the expression of ligands for DNAM-1 and NKG2D on tumor cells, make them more prone to be targeted by NK cells. Therefore, NK cells are an attractive alternative, or potential adjuvant, to T cell-based immunotherapy[159]. There are several strategies to optimize the NK-cell anti-tumor activity including, activation of endogenous NK cell response, adoptive transfer of alloreactive NK cells, blocking inhibitory signals and blocking immunosuppressive mechanisms in tumors susceptible to NK lysis[142].

NK cell dysfunction has been reported in different types of tumors, thus exogenous NK activators, such as IL-2, IL-12, IL-15, IL-18, IL-21 and type I interferons, may be used to improve the anti-tumor NK cell response. High dose IL-2 has been shown to be able to activate and expand NK cell populations in some cancer patients, and is translated into clinical responses; however, this treatment is limited by severe side effects. IL-15 is a promising candidate for activating NK cells because IL-15 is indispensable for NK cell maturation. When combined with chemotherapy, the administration of IL-15 potentiated antitumor effects[160].

Adoptive transfer of alloreactive NK cells consists of NK cell isolation from the tumor (or their production *in vivo*), their expansion and introduction to patients. The first trial involving adoptive transfer of NK cells, using a combination of *ex vivo*-expanded autologous lymphokine-activated killer cells and exogenous IL-2, did not achieve better clinical results than high-dose IL-2 alone[161]. Unlike autologous cell transfers, allogeneic adoptive cell transfers have the benefit of mismatched KIR/KIR ligands between the host and donor NK cells, although these therapies run a great risk of GVHD that can cause severe damage to host tissues. An infusion of haploidentical allogeneic NK cells has also been applied to patients with poor prognosis in Hodgkin's lymphoma and various solid tumors, such as melanoma and

renal cancer, with interesting clinical results[162]. While NK cells may achieve clinical responses, certain issues need to be considered. First, the type of NK cells to be infused must be considered, as the use of distinct NK cell subsets or protocols for NK cell activation *ex vivo* might interfere with the outcome. Second, the criteria for donor selection are crucial. For this reason, KIR genotyping and phenotyping must be performed, the size of the alloreactive subset must be measured, and the NKG2D and/or NCR haplotypes must be determined. Third, the conditioning regimen of patients before the adoptive transfer, which may vary depending on the immune status of the disease being treated. Fourth, clinical indication must be appropriately selected on the basis of the susceptibility of target cells to lysis[163].

The selective blockade of inhibitory receptors (CD94/NKG2A and KIRs) and upregulation of activating receptors (NKG2D) has been developed to enhance the effect of NK cell immunotherapy. As an example, the human monoclonal antibody called 1-7F9, that prevents signaling via KIRs (KIR2DL1, KIR2DL2 and KIR2DL3) was developed to increase NK cell-mediated cytotoxicity against tumors expressing HLA-C[164]. Furthermore, It has become increasingly clear that the TME plays a crucial role in the impairment of the immune response and in the development of many overlapping mechanisms that create an immunosuppressive microenvironment. It has been reported that tumor-associated NK cells display a modified phenotype, thereby supporting the notion that tumor-induced alterations of activating NK cell receptor expression may hamper immune surveillance and promote tumor progression. Given the important immunomodulatory effects of the TME, it stands to reason that it may represent a therapeutic target that can be manipulated to improve the anti-tumor immune response. Thus, the first clinical interventions that aim to target the microenvironment to enhance tumor immunity are under active evaluation[165].

In summary, NK cells are “innate killers” but they are not *just* “innate killers”. The scientific community is aware of that, and now the focus is to try to unveil the processes orchestrating NK cell diversity.

1.2. Rationale, Hypothesis and Aims

1.2.1. Rationale

Despite the anti-tumor response, particularly the presence of anti-tumor immune cells (T cells, NK cells and NKT cells) infiltrating into the tumor stroma, many tumors continue to grow, including melanoma. Two main interrelated phenomena have been described to justify that fact: the tumor cells can create an immune tolerant microenvironment and the anti-tumor cells can be dysfunctional. The underlying mechanism for this anti-tumor immune cell dysfunction or "exhaustion" is not well understood; however It is postulated that the TME, enriched in immunosuppressive molecules (TGF-β, IL-10, sMICA) and cells (TAM, MSDC, Tregs) may contribute to a weakened anti-tumor immune response. This exhausted phenotype was firstly described for T cells in the context of chronic infectious diseases and now also in different types of cancer, particularly in melanoma. This phenotype is characterized by early loss of proliferative capacity, cytotoxic potential, and the ability to produce IL-2, as well as, upregulation of inhibitory receptors, including CTLA-4, PD-1, Tim-3, among others. Besides T cells, studies are starting to evaluate this phenotype in NKT cells, however nothing is known about exhaustion in NK cells.

NK cells are large granular lymphocytes with natural cytotoxicity against tumor cells. Their activity is a result of the balance between activating and inhibitory receptors. Activating receptors (NKG2D, NKp30, NKp44, NKp46) detect the presence of ligands on cells in 'distress'. Inhibitory receptors (NKG2A and KIRs) gauge the absence of constitutively expressed self-molecules on susceptible target cells (HLA class I molecules), targeting specifically those cells ("missing-self theory"). Therefore, tumor cells with low HLA class I expression (as a result of immunoediting), are the best targets for NK cells.

TIM-3 is not expressed on the surface of naive T cells but emerges on the cell surface of fully differentiated $CD4^+$ Th1 cells, and also in $CD8^+$ T cells, Th17, Tregs, NK cells, macrophages and DCs. It negatively regulates Th1-mediated inflammatory diseases such as experimental autoimmune encephalomyelitis and acute graft-versus-host disease, and is also a marker of T cell exhaustion. However, nothing is known about the role of Tim-3 in NK cells, specifically whether it is a marker of exhaustion.

1.2.2. Hypothesis

We first hypothezise that, as melanoma progresses, NK cell gradually develop a functional profile of exhaustion, similar to T cells, as a consequence of a more immunossupressive microenvironment. TGF-β, MICA or HMGB1 are present in the TME and are potential players in the mechanism of induction of NK cell exhaustion. We predict that high expression of any of these three molecules in the plasma of melanoma patients is associated with worse prognosis. Tim-3 has been described as an inhibitory receptor in T cells in the context of advanced cancers; however its role in NK cells from HD is still controversial, and it has not been studied in the context of melanoma. We believe Tim-3 negatively regulates NK cell function in melanoma patients, and that its blockade reverses NK cell exhaustion in this context. Lastly, ipilimumab is able to reverse T cell exhaustion, that translates into clinical responses. Even though CTLA-4 has little expression on NK cells, we believe that ipilimumab has, directly or indirectly, an impact on the NK cells' exhaustion phenotype.

1.2.3. Specific aims

1. To characterize the phenotype and function of NK cells from melanoma patients.

   - To characterize the phenotype and function of NK cells from advanced melanoma patients in comparison with healthy donors (**CHAPTER 2**).
   - To analyze the phenotype and function of NK cells from early stage melanoma patients (stage I, II and III) and determine how they relate with well-known prognostic factors including thickness, ulceration and mitotic index (**CHAPTER 3**).

2. To identify potential inducers of NK cell exhaustion as prognostic markers.

   - To measure the expression of sMICA, TGF-β and HMGB1 in the plasma of melamona patients and determine how it relates with prognosis (**CHAPTER 3**).

3. To define the role of Tim-3 expression in NK cells from advanced melanoma patients.

   - To crosslink Tim-3 on NK cells from advanced melanoma patients in comparison with healthy donors to assess its role in modulating NK cell function (**CHAPTER 4**).

4. To study the effect of checkpoint blockade on the NK cell phenotype and function in the context of advanced melanoma patients.

- To inhibit Tim-3 in order to modulate NK cells function in advanced melanoma patients (**CHAPTER 4**).
- To analyze the effect of ipilimumab treatment on NK cells (**CHAPTER 5**).

CHAPTER 2:

NK CELLS FROM ADVANCED MELANOMA PATIENTS DISPLAY AN EXHAUSTED PHENOTYPE

2.1. Introduction

Melanoma is the most aggressive type of skin cancer; it accounts for less than 2% of all skin cancers, but corresponds to more than 70% of their deaths[8]. In fact, melanoma responds poorly to systemic chemotherapy, and the 2-year OS was until recently around 15-20%. However, new therapeutic options are improving response rates and OS[35,36].

One such intervention is ipilimumab[35], a monoclonal antibody against CTLA-4, a negative regulator that is upregulated on exhausted T cells in cancers and chronic infections[166]. The exhaustion phenotype has been described for T cells as a state of cellular dysfunction that arises as a consequence of continuous and chronic stimulation by viral or tumor antigens, as well as by immunosuppressive cytokines. It is characterized by a progressive and stepwise process of cell dysfunction, where IL-2 production and proliferative ability are the first effector activities to be extinguished, followed by cytotoxic activity (decreased production of perforin and granzymes), whereas the ability to produce IFN-γ is more resistant to inactivation[90]. In addition, as T cells acquire this exhausted phenotype, they start expressing some inhibitory receptors[91,92], including CTLA-4[167,168] , PD-1[169] and Tim-3[93], while losing some activating receptors, such as CD44 and CD69. Furthermore, lower levels of the transcription factors T-bet and Eomes are also observed[91]. Besides T cells, new studies are starting to check this phenotype in NKT cells, however nothing is known about exhaustion in NK cells.

NK cells are large granular lymphocytes with natural cytotoxicity against tumor cells. Their activity is a result of the balance between activating and inhibitory receptors. Activating receptors (NKG2D, NKp30, NKp44, NKp46) detect the presence of ligands on cells in 'distress'. While inhibitory receptors (NKG2A and KIRs) gauge the absence of constitutively expressed self molecules on susceptible target cells ("missing self theory")[139]. Accordingly,

NK cell infiltration in tumor tissue is associated with better prognosis[95,148,149], while low activity of peripheral blood NK cells is associated with increased risk of cancer[150]. These findings have prompted interest in the development of cancer therapies based on NK cells, such as NK cell adoptive transfer or targeting of NK cell inhibitory receptors[170].

In this study, we aimed to characterize the phenotype and function of NK cells from advanced melanoma patients. For this purpose we characterized the phenotype and function of NK cells from advanced melanoma patients in comparison with healthy donors. We found that NK cells from these subjects were functionally impaired/exhausted, opening exciting avenues for new therapies targeting NK cells in tumor immunotherapy.

2.2. Material and Methods

Human samples: Blood samples from healthy donors (HD) were purchased from the New York Blood Center. Blood samples were obtained under the Interdisciplinary Melanoma Cooperative Group Institutional Research Board (IRB) approved protocols (#H10362) from 50 untreated melanoma patients (melanoma donors – MD; stages III/IV). The study was approved by the Institutional Research Board at NYU in accord with a Federalwide Assurance approved by the Department of Health and Human Services, and all patients provided informed written consent at the time of enrollment.

Reagents: For staining - Tim-3 antibody used for staining experiments was purchased from R&D. Anti CD279 (PD-1) was purchased from BD Bioscience and anti-CTLA-4 was purchased from LifeSpan Biosciences. To check the purity of selected NK cells they were stained with antibodies to CD56, CD16, CD3, CD14 and CD19 purchased from Biolegend. Anti-CD25 was obtained from Miltenyi, and anti-CD122, anti-CD132, anti-CD107a-FITC (fluorescein isothiocyanate) and anti-IFN-γ-FITC antibodies were purchased from Biolegend. Anti-NKG2D, anti-NKp46, anti-DNAM-1, anti-KIR3DL1 and anti-KIR2DL3 antibodies (BD Bioscience) were used as indicated by the manufacturer. Anti-Eomes and anti-T-bet were purchased from eBioscience.

For stimulation - the cytokines rhIL-2, rhIL-12, rhIL-15 and rhIL-18 were purchased from R&D systems.

Peripheral blood mononuclear cells (PBMCs) purification: Blood was slowly layered on top of Ficoll-Paque and then spun at 2200 rpm, for 20 min, with low brake and slow acceleration. After spinning, the white blood cells (WBC) layer was removed and added to RPMI media (50mL total). It was spun again at 1600 rpm, for 8min, with normal brake and acceleration. The supernatant was aspirated, the pellet was resuspended in 50mL of RPMI (Roswell Park Memorial Institute) media and it was spun again. These two steps were repeated twice.

NK cell purification: Peripheral blood mononuclear cells (PBMCs) NK-cell enrichment was performed by negative selection using Easy-Sep Human NK cell Enrichment Kit (StemCell Technologies) according to the manufacturer's recommendations, obtaining more than 95% $CD3^{-}CD56^{+}$ populations. Cell suspension was prepared at a concentration of 5 x 107 cells/mL in phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 2 mM ethylenediamine tetraacetic acid (EDTA) - MACS Buffer. EasySepTM Human NK Cell Enrichment Cocktail at 50 µL/mL cells was added to the cell suspension, mixed and incubated at room temperature (15 - 25°C) for 10 minutes. The mix was vortexed for 30 seconds. EasySep^{TM} D Magnetic Particles at 100 µL/mL cells was added to the cell suspension, mixed and incubated at room temperature (15 - 25°C) for 5 minutes. MACS buffer was added to the cell suspension up to 2.5 mL, mixed and placed in the tube in the magnet. The desired fraction (cells in suspension) was then aspirated.

Cell lines: K562 cells were cultured in complete media (RPMI-1640 - Life Technologies) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. K562 is a chronic myelogenous leukaemia cell line used as target cells in the cytotoxicity assay.

Cell Staining and Flow Cytometry Analysis: Before staining, cells were washed twice with MACS Buffer. For surface staining, cells were incubated with specific antibodies for 30min at 40ºC. For intracellular staining, cells were first fixed with 4% paraformaldehyde (PFA) for 10min at room temperature, permeabilized with MACS buffer supplemented with 0.1% saponin and stained with specific antibodies for 30min at room temperature. Cells were then washed twice with MACS buffer and analyzed by fluorescence-activated cell sorting (FACS). Data analysis was performed using FlowJo software.

NK cell stimulation: For short term functional assays (cytotoxicity and IFN-γ production), purified NK cells were activated overnight in the presence of 1000U/mL of IL-2 complete media (RPMI-1640 - Life Technologies, Carlsbad, CA) supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, and 100µg/ml streptomycin. For the long-term experiments and proliferation assays, NK cells were cultured in the same media and stimulated with 200U/ml of IL-2.

Cytotoxicity Assay: Purified NK cells were co-cultured with target cells at a 5:1 effector/target ratio in combination with anti-CD107a (lamp-1) antibody (0.5µg/ml) and monensin 1000X. After a 4h incubation, CD107a expression on $CD56^+$ cells was quantified by flow cytometry.

IFN-γ production assay: Purified NK cells were cultured for 4h in the presence of 1µg/ml of IL-12 and brefeldin A (BFA, 10µg/ml). After 4h cells were fixed with 4% paraformaldehyde (PFA) and permeabilized (0.1% saponin). Permeabilized cells were stained for intracellular IFN-γ and analyzed by flow cytometry.

Proliferation assay: Purified NK cells were loaded with 2µM carboxyfluorescein succinimidyl ester (CFSE) and cultured in complete media supplemented with 200U/ml of IL-2. After 6 days of culture, CFSE dilution was analyzed by flow cytometry as a measure of cell proliferation.

Statistical analyses: Two tailed unpaired t test was used to compare parameters between healthy and melanoma NK cells. Each experiment was performed in duplicate.

2.3. Results

Advanced tumors are characterized by an environment that promotes T cell exhaustion[92]. Exhausted T cells are characterized by: 1) over-expression of inhibitory receptors such as CTLA-4 and PD-1; 2) downregulation of cytokine receptors, rendering them refractory to cytokine stimulation; 3) loss of function (cytotoxicity, cytokine production and proliferation); and 4) downregulation of T-bet and Eomes[171]. In metastatic melanoma, peripheral blood $CD8^+$ T cells are functionally exhausted[93]; however, NK cell phenotype and function have not been evaluated in this context.

To determine whether peripheral NK cells from advanced melanoma patients display an exhausted phenotype, we examined whether they expressed features associated with T cell exhaustion. NK cells were purified from peripheral blood of advanced melanoma patients and healthy donors **(Figure 2.1)**.

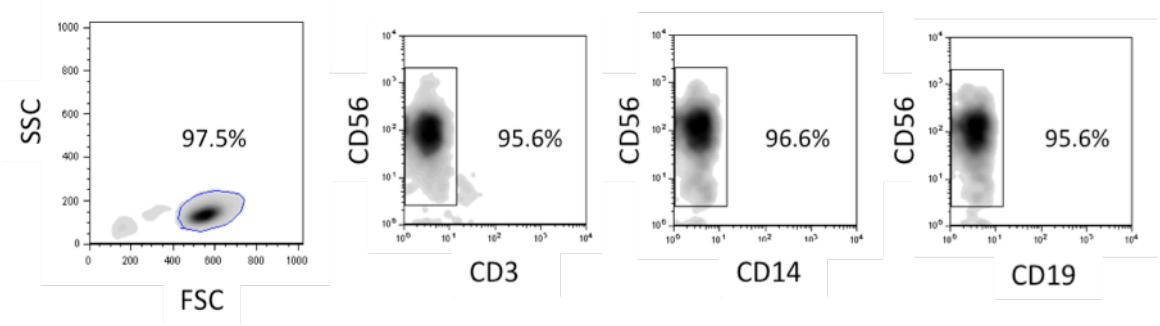


Figure 2.1. NK cells were purified from peripheral blood. The plot on the left represents the gate on NK cells purified from peripheral blood from a melanoma patient. The other three plots show >95% of purity in the NK cell negative selection (> 95% are $CD56^{+}$ $CD3^{-}$ $CD14^{-}$ $CD19^{-}$ cells).

Interestingly, melanoma donor NK cells downregulate the NK cell markers CD16 and CD56 **(Figure 2.2.A)**, and consequently display a decrease in the percentage of both $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets **(Figure 2.2.B)**.

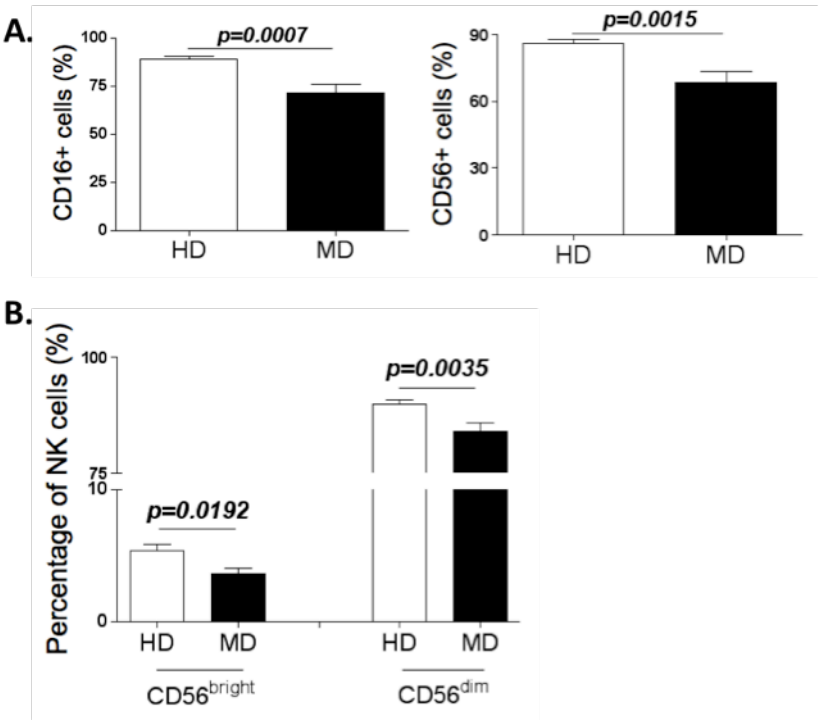


Figure 2.2. NK cells from advanced melanoma patients downregulate CD56 and CD16 receptors. (**A**) The graph represents the percentage of $CD16^{+}$ (**left panel**) and $CD56^{+}$ NK (**right panel**) cells in healthy donors (n=13) and melanoma patients (n=11). **(B)** The plot shows the percentage of each NK cells' subset ($CD56^{dim}$ and $CD56^{bright}$) in total NK cells from healthy donors (n=34) and melanoma patients (n=24).

We then measured the expression of activating and inhibitory receptors in NK cells from MD and HD. Clearly, NK cells from MD expressed higher levels of inhibitory receptors (KIR3DL1 and KIR2DL3) **(Figure 2.3.A)** and lower levels of activating receptors (NKG2D, NKp46 and DNAM-1) **(Figure 2.3.B)** when compared with HD.

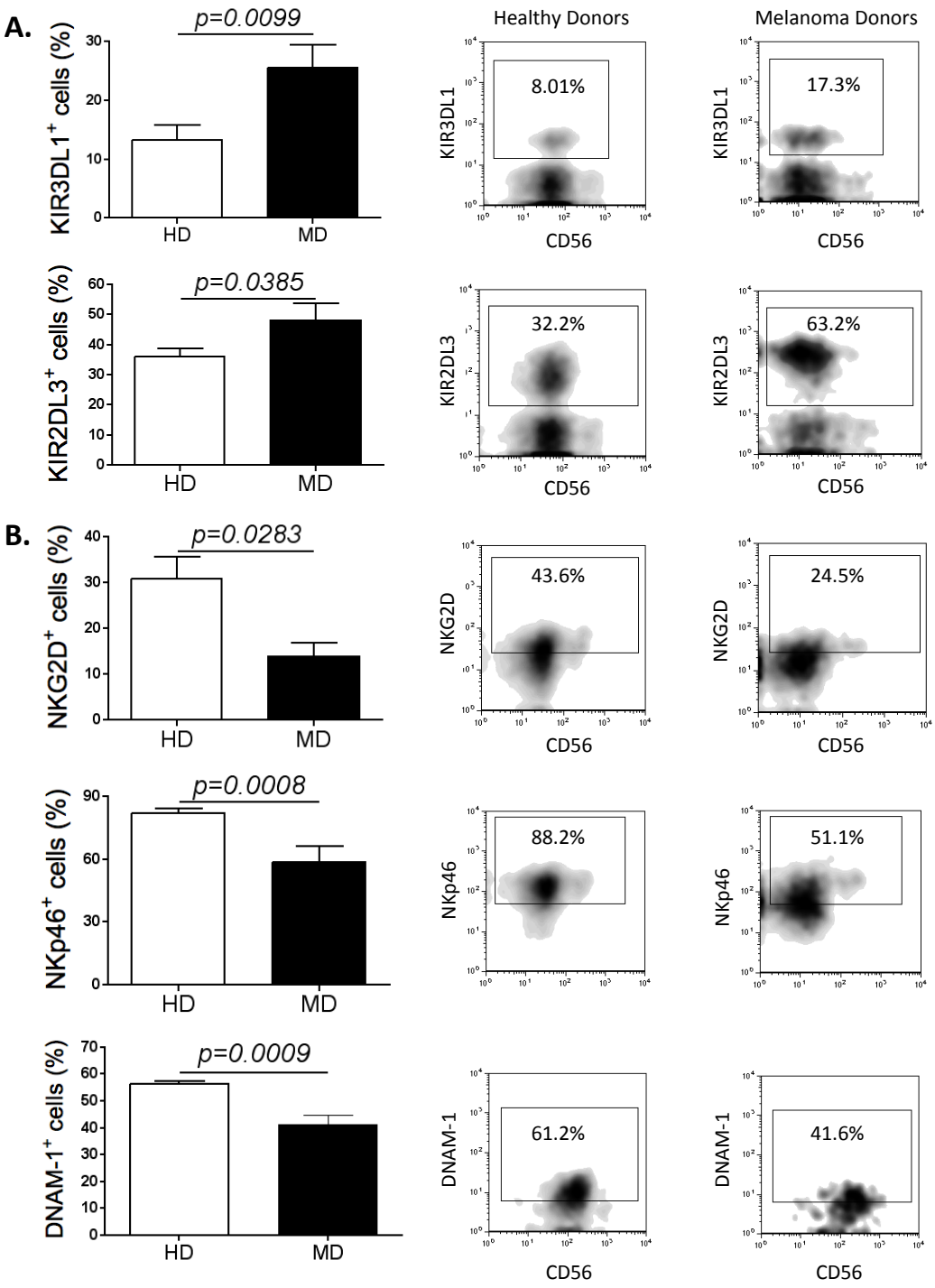


Figure 2.3. MD NK cells up-regulate inhibitory receptors and down-regulate activating receptors. (**A**) Graphs compare the expression of the inhibitory receptors KIR3DL1 (HD n=18; MD n=8) and KIR2DL3 (HD n=26, MD n=12); **(B)** and the activating receptors NKG2D (HD n=25; MD n=12), NKp46 (HD n=20, MD n=11) and DNAM-1 (HD n=10; MD n=10), in peripheral NK cells purified from healthy donors and from melanoma patients. On the **right panel**, plots depicting the expression of NKG2D, NKp46, DNAM-1, KIR3DL1 and KIR2DL3 according to CD56 expression, in NK cells from a representative healthy donor and a representative melanoma patient. All experiments were performed in duplicate.

We also evaluated PD-1 and CTLA-4 expression, however the levels were very low (1-3% of PD-1$^+$ and CTLA-4$^+$ NK cells) and there were no significant differences between MD and HD **(Figure 2.4.A)**. Tim-3 is another immune checkpoint expressed by CD8$^+$ T cells in advanced tumors, namely melanoma. However, the role of Tim-3 in NK cells in the same context remains unknown. Therefore, we first measured Tim-3 expression in MD NK cells. Compared to HD NK cells, MD NK cells expressed significantly higher levels of surface Tim-3 (*p=0.0001*) **(Figure 2.4.B)**.

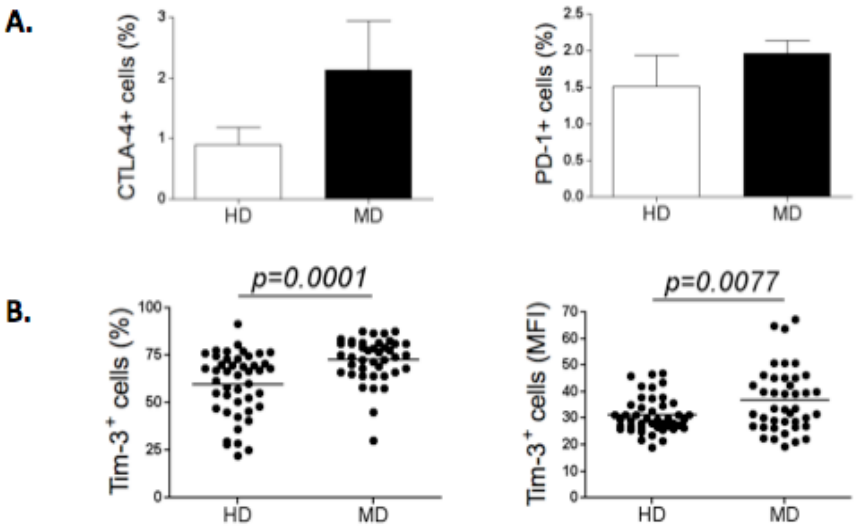


Figure 2.4. MD NK cells up-regulate the immune checkpoints CTLA-4, PD-1 and Tim-3. **(A)** The plots show the percentage of CTLA-4 (**left panel**) and PD-1 (**right panel**) expression in NK cells from healthy donors (n=10) and melanoma patients (n=5); (**B**) Graphs comparing Tim-3 expression in NK cells from healthy donors (HD; n=45) and melanoma patients (MD; n=41), represented as the percentage of Tim-3$^+$ cells (**left panel**) and the MFI of the Tim-3$^+$ population (**right panel**). All experiments were performed in duplicate.

Secondly, we measured the levels of the three IL-2R chains [α chain (CD25; IL-2Rα), β chain (CD122; IL-2Rβ) and γ chain (CD132; IL-2Rγ)]. The expression of each subunit was measured in steady state conditions (day 0) and on days 2, 4 and 6 after IL-2 stimulation. HD or MD resting NK cells did not express significant levels of CD25 at rest. Interestingly, after IL-2 stimulation, HD NK cells upregulated CD25 to very high levels, whereas MD NK cells failed to do so **(Figure 2.5.A)**. At rest, CD122 expression was similar in NK cells from healthy and melanoma subjects and decreased after IL-2 addition **(Figure 2.5.A)**, while

CD132 expression was slightly higher in resting HD NK cells compared to MD NK cells. After IL-2 stimulation, both sources of NK cells showed an increase of this receptor, followed by a plateau phase and then a decrease, in expression of the CD132 receptor. However, the overall expression of CD132 on HD NK cells was substantially higher than that of MD NK cells **(Figure 2.5.A).** We next tested whether altered IL-2R expression translated into a differential response to IL-2 stimulation. While cytotoxicity was induced in both sources of NK cells, the levels increased more significantly over time in HD NK cells **(Figure 2.5.B)**. Whereas IFN-γ production was induced by and increased in HD NK cells in response to IL-2, it did not change from baseline in the case of MD NK cells **(Figure 2.5.B)**. Moreover, HD NK cells upregulated both KIR3DL1 and NKG2D, while MD NK cells did not **(Figure 2.5.C)**. We also measured the levels of the IL-15 receptor (IL-15R), another significant NK cell stimulatory molecule. Interestingly, IL-15R levels were significantly lower in MD NK cells when compared with HD NK cells **(Figure 2.5.D).**

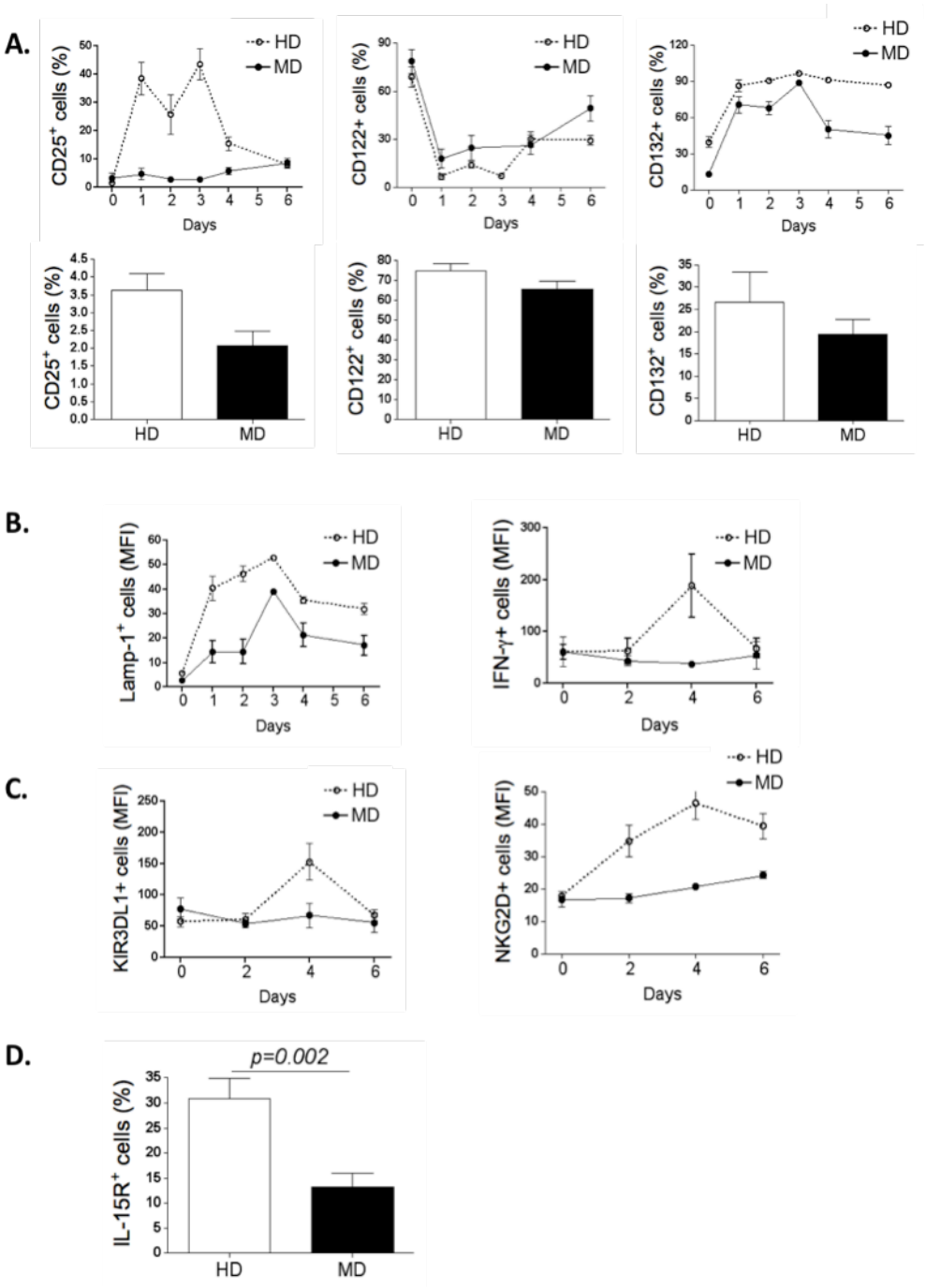


Figure 2.5. NK cells from melanoma patients downregulate the expression of cytokine receptors, rendering them refractory to cytokine stimulation. (**A, B and C**) Freshly purified NK cells (HD n=12; MD n=5) were stimulated with 200U/ml of IL-2: (**A**) expression of CD25 (IL-2Rα; **left panel**), CD122 (IL-2Rβ; **middle panel**) and CD132 (IL-2Rγ; **right panel**); (**B**) expression of Lamp-1 (CD107a; **left panel**) and IFN-γ (**right panel**); and (**C**) expression of KIR3DL1 (**left panel**) and NKG2D (**right panel**), were monitored every two days over 6 days (day 0, 2, 4 and 6) by flow cytometry. (**D**) The plot shows the percentage of IL-15R^{+} cells on NK cells from healthy donors (n=10) and melanoma patients (n=10). (**A, lower panels**) Baseline expression of CD25 (IL-2Rα; **left panel**), CD122 (IL-2Rβ; **middle panel**) and CD132 (IL-2Rγ; **right panel**). All experiments were performed in duplicate.

Finally, we determined whether NK cells from melanoma patients were functionally impaired, by assessing cytotoxicity, IFN-γ production and cell proliferation. We found that, in comparison with HD NK cells, MD NK cells failed to efficiently kill target cells as assessed by LAMP-1 expression, after stimulation by either IL-2 (*p=0.0158*) or IL-15 (*p=0.04*) **(Figures 2.6A and 2.6B).**

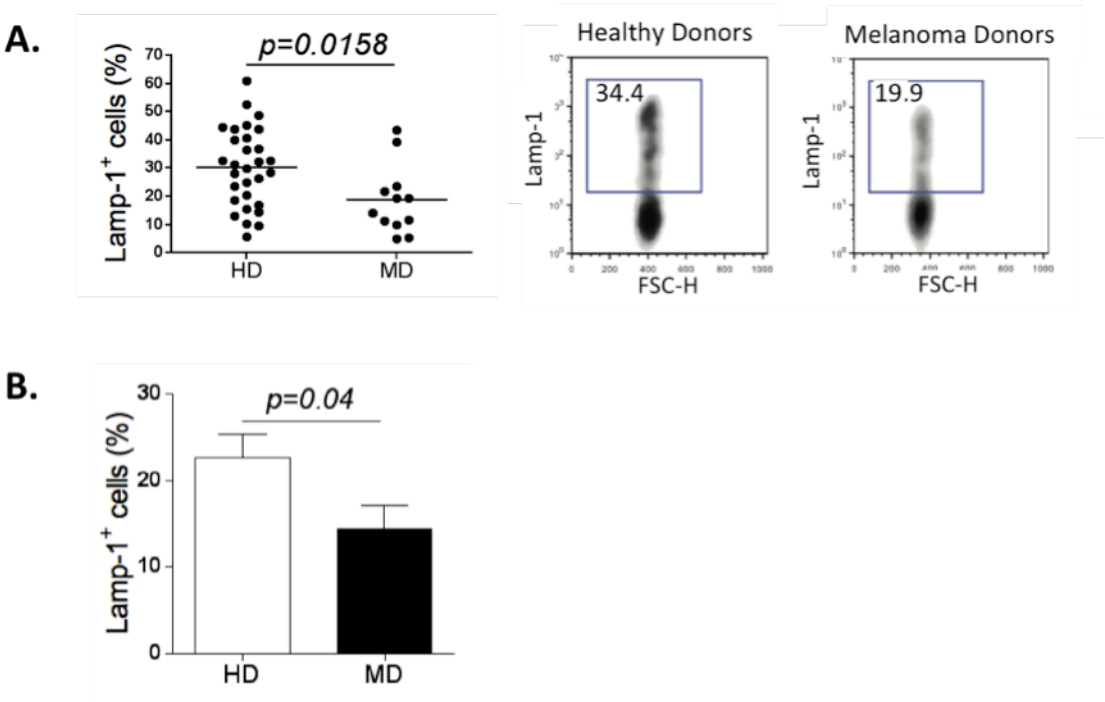


Figure 2.6. MD NK cells are functionally impaired/exhausted – display a lower cytotoxic ability. (A) The percentage of Lamp-1$^+$ NK cells from healthy (n=30) and melanoma donors (n=12) after a cytotoxic assay, using K562 cells as target cells, is shown. On the right panel, plot depicts the expression of Lamp-1 in NK cells purified from a representative healthy donor and a representative melanoma patient. **(B)** The plot shows the percentage of Lamp-1$^+$ NK cells from healthy (n=11) and melanoma donors (n=10), stimulated overnight with IL-15 and co-cultured with K562 cells for 4h. All experiments were performed in duplicate.

In addition, MD NK cells produced less IFN-γ in response to IL-12 (*p=0.0312*) **(Figure 2.7.A)**, the combination of IL-12 and IL-18 (*p=0.001*), IL-15 (p=0.05) **(Figure 2.7.B)**, or after coculture with K562 cells (*p=0.0173*) **(Figure 2.7.C)**.

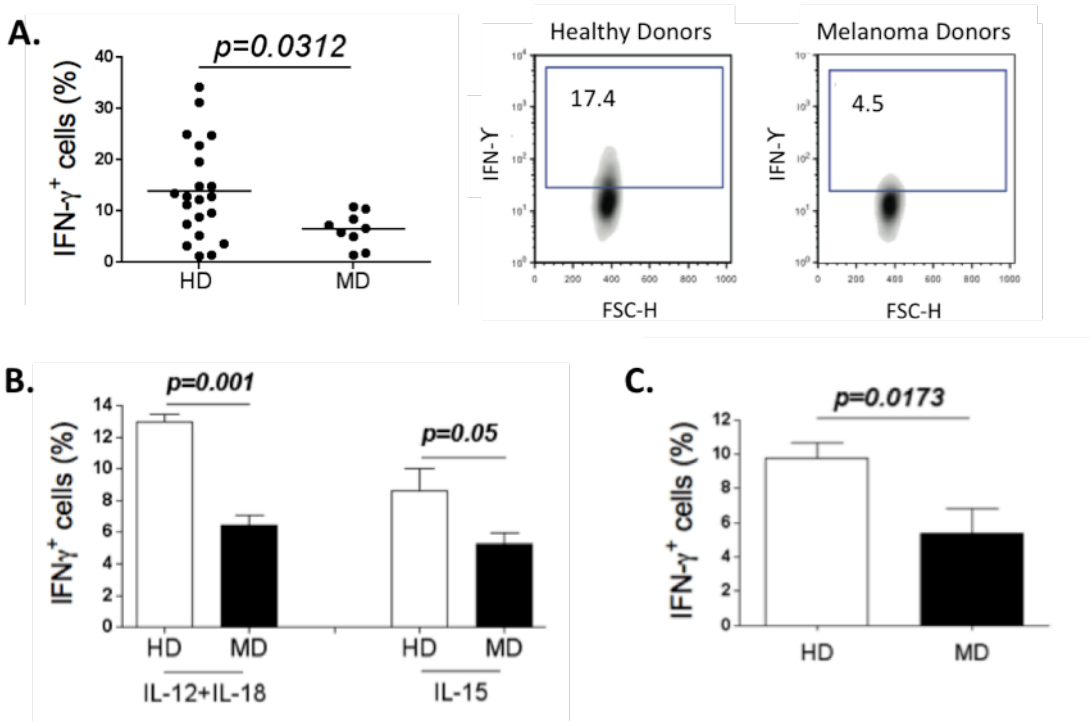


Figure 2.7. MD NK cells are functionally impaired/exhausted – display a lower cytokine production ability. The percentage of IFN-γ^{+} NK cells stimulated with: **(A)** IL-12 cytokine (HD n=22; MD n=9); plot depicts the expression of IFN-γ in NK cells purified from a representative healthy donor (middle panel) and a representative melanoma patient (right panel); **(B)** IL-12+IL-18 or IL-15 cytokines (HD n=11; MD n=10); or **(C)** K562 cells (HD n=9; MD n=6). All experiments were performed in duplicate.

Finally, NK cells from melanoma patients lost their ability to proliferate when cultured with IL-2 (*p=0.0024*) (**Figure 2.8.A**), IL-12 (*p=0.04*), IL-15 (*p=0.05*) or IL-18 (*p=0.01*) **(Figure 2.8.B)**.

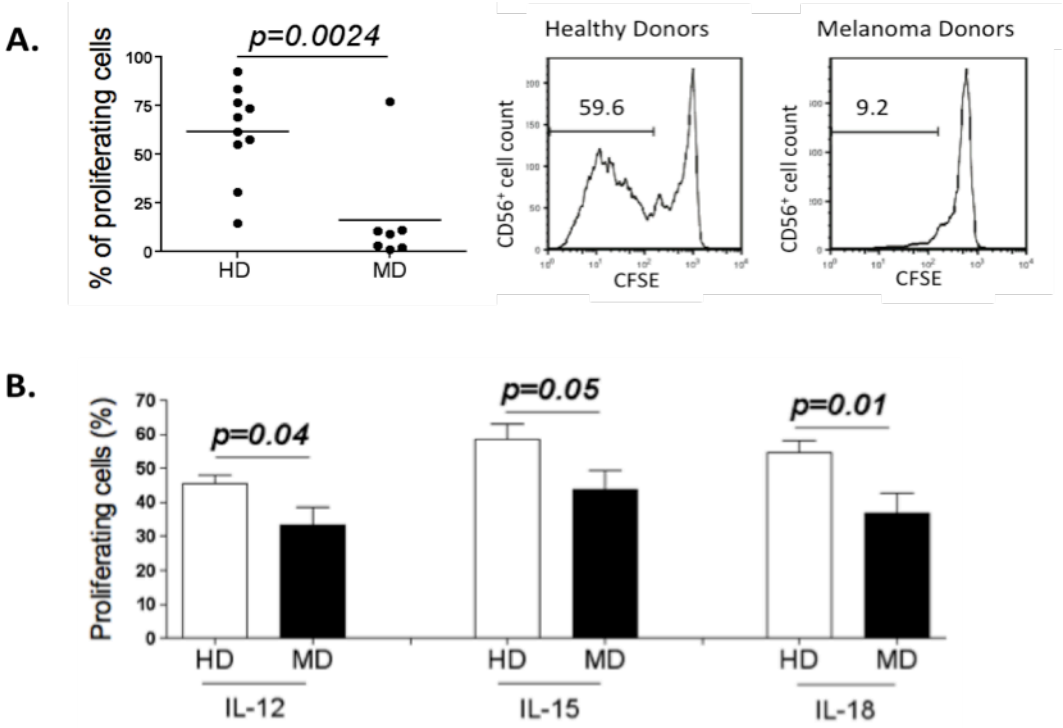


Figure 2.8. MD NK cells are functionally impaired/exhausted – display a lower proliferative ability. The percentage of proliferating NK cells after 6 days of culture in presence of: **(A)** IL-2 cytokine (HD n=10; MD n=7); plot depicts the expression of proliferating NK cells purified from a representative healthy donor (middle panel) and a representative melanoma patient (right panel); **(B)** IL-12, IL-15 or IL-18 cytokines (HD n=11; MD n=10). All experiments were performed in duplicate.

The transcription factors T-bet and Eomes regulate the function of NK cells and a recent study showed that downmodulation of these transcription factors is a feature of NK cell exhaustion[98]. As expected, the expression levels of Eomes and T-bet are lower in NK cells from melanoma donors when compared with NK cells from healthy donors **(Figure 2.9)**.

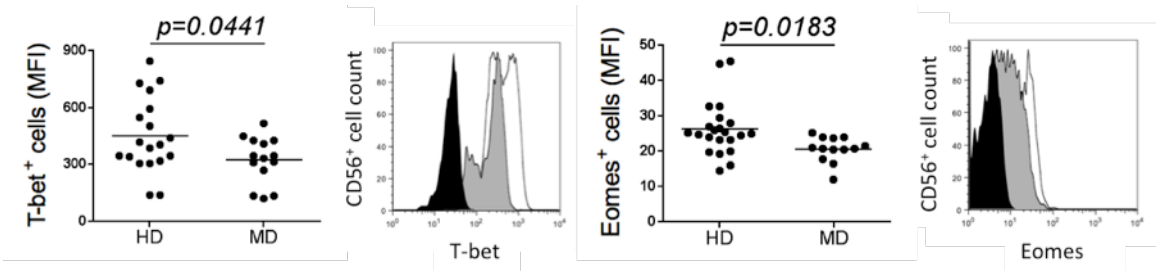


Figure 2.9. MD NK cells downregulate the expression of T-bet and Eomes transcription factors. Graphs representing the mean fluorescence intensity (MFI) of T-bet and Eomes in NK cells purified from healthy (n=19) and melanoma donors (n=14). Representative plots are shown (Isotype control: black; HD: unfilled; MD: gray). All experiments were performed in duplicate.

These results clearly demonstrate that NK cells from advanced melanoma patients are functionally exhausted as shown by overexpression of inhibitory receptors and downregulation of activating receptors, an impaired response to IL-2 stimulation (possibly due to IL-2R downmodulation), defects in cytokine production, in proliferation and cytotoxicity, and a downmodulation of the transcription factors T-bet and Eomes.

2.4. Discussion

T cell exhaustion has been extensively studied in the context of chronic infectious diseases and different types of cancer, however, little is known about exhaustion of NK cells. Mamessier E. and colleagues have recently shown that NK cells from breast cancer patients depict some dysfunctional phenotype[97]; however our results provide the first demonstration that NK cells from advanced melanoma patients display the four main characteristics that define T cell exhaustion. Therefore, similarly to T cells, exhausted NK cells upregulate inhibitory receptors while downregulating IL-2R and consequently are unable to respond appropriately to IL-2 stimulation. In addition, they are functionally impaired (reduced

cytotoxicity, cytokine production and proliferation) and express reduced levels of activating receptors and the transcription factors Eomes and T-bet. Interestingly, this exhaustion phenotype is associated with a higher expression of the inhibitory receptor Tim-3, while there is almost no expression of CTLA-4 or PD-1.

Nevertheless, since the late 1980s several studies have demonstrated the reversal of this tumor-associated NK cell phenotype after IL-2 stimulation, suggesting the absence of any inherent NK cell defect *per se*[172,173]. Recent studies have shown that different strengths of the activating stimuli are required for specific NK cell responses. Therefore, a low threshold for activation is needed for adhesion and release of chemokines, while degranulation and the release of other cytokines such as TNF-α require stronger activating stimuli, and IFN-γ displays the highest activation threshold for NK cell receptor cooperation. Consistently, some studies argue against an inherent NK cell defect in cancer patients and suggest the absence of sufficient activating signals for full NK cell effector function in the TME[174]. In our study, we have shown that even after IL-2 stimulation, NK cells from melanoma patients have a dysfunctional phenotype compared with healthy donors. Therefore, both theories may contribute to the explanation of the NK cell dysfunctional phenotype. On the one hand, TME does not provide the stimuli needed for a full NK cell activation, and hence it is possible to partially improve their function through cytokine activation. On the other hand, even with this *ex vivo* activation, it is not possible to reverse completely the NK cell exhausted phenotype.

The exact mechanism of NK cell exhaustion is still unclear, however it is likely different from that seen in T cells, where there is a chronic stimulation and proliferation of a T cell clone against a specific antigen. It is possible that NK cells become exhausted due to systemic production of cytokines, or within the TME in response to specific ligands. Indeed, chronic stimulation with IL-2 and IL-15[175,176] and shed MICA, a tumor-derived NKG2DL[157,177], has been described to induce NK cell exhaustion. Exhausted T cells also upregulate CTLA-4 and PD-1, however we found no significant expression of these receptors in the membrane of MD NK cells.

In conclusion, this study demonstrated for the first time that NK cells from advanced melanoma patients display an exhausted phenotype, similarly to T cells, including an upregulation of the exhaustion marker Tim-3. NK cells, therefore, represent promising therapeutic target that could enhance antitumor immunity with the potential to produce durable clinical responses.

CHAPTER 3:

NK CELL PHENOTYPE AND SMICA ARE POTENTIAL PROGNOSTIC MARKERS IN MELANOMA PATIENTS

3.1. Introduction

Defects in NK cell activity have been described in patients with various metastatic malignancies, including melanoma[94,95,96,97]. Moreover, adoptive transfer of NK cells as an attempt to control tumor growth control has been associated with disappointing results. These results can be explained by a rapid acquisition of an exhausted phenotype, as it was shown in a mice model[98]. NK cell "exhaustion," therefore, could explain the NK cell failure to contain tumor growth.

Several factors can contribute to this NK cell exhausted phenotype, including cross-talk with immune cells (tumor-associated macrophages, myeloid-derived suppressor cells and T regulatory cells) and tumor cell-derived factors (i.e. TGF-β, VEGF, MICA and HMGB1)[163]. TGF-β is a cytokine that is produced by tumor cells that inhibit NK cells following chronic contact-dependent interactions between NK and tumor cells[178]. MICA is a MHC class I related molecule and is the NKG2DL, a NK cell activating receptor. It is expressed in the membrane of several types of cancer cells, and activates NK cells, when it binds to NKG2D. However, in the context of hypoxia, MICA is shed by these tumor cells and sMICA, on the contrary, leads to a downregulation of NKG2D on NK cells decreasing their cytotoxic ability[157,158,165]. HMGB1 is an endogenous dangerous signal that activates the immune system through different mechanisms. HMGB1 induces DC maturation while inhibiting NK cell cytotoxicity against DCs, thus playing key role in the crosstalk between NK cells and DCs. However, HMGB1 is a ligand of Tim-3, and the interaction between HMGB1 and Tim-3 may regulate tumor escape from the immune system[116,179]. We hypothesize that TGF-β, MICA and HMGB1 could be involved in that process and therefore would be highly expressed in advanced melanoma.

In this study, we aimed to evaluate NK cell phenotype and function as a consequence of progressive melanoma. For this purpose, we characterized the phenotype of NK cells in different phases of tumor progression and related it to prognostic factors. In addition, we quantified the expression of TGF-β, MICA and HMGB1 in the plasma/sera of our main cohort and of an independent validation cohort. These data demonstrated that NK cells become progressively exhausted in the context of melanoma progression and that targeting NK cells earlier in the disease may present some benefits on innate immune function. Finally, our data suggest that soluble MICA is a potential prognostic marker predictive of relapse in early stages.

3.2. Material and Methods

Human samples: Blood samples from healthy donors were purchased from the New York Blood Center. Blood samples from 175 untreated melanoma patients (stages I, II and III/IV) and plasma/sera from an additional cohort of 280 untreated melanoma patients (all stages) were obtained under the Interdisciplinary Melanoma Cooperative Group IRB approved protocols (#H10362). Patient demographics and primary tumor pathological characteristics were obtained for each patient at the time of enrollment. Recurrence and survival information were prospectively obtained via active follow-up every six months for all patients. The study was approved by the IRB at NYU in accord with a Federalwide Assurance approved by the Department of Health and Human Services, and all patients provided informed written consent at the time of enrollment.

Reagents, cell lines, and methods used for NK cell purification and stimulation, Cell Staining and flow cytometry analysis, cytotoxicity, IFN-γ production and proliferation assays are refered in CHAPTER 2.

MICA, HMGB1 and TGF-β expression: enzyme-linked immunosorbent (ELISA) assays for MICA, HMGB1 and TGF-β were performed on patients' plasma/sera with commercially available kits (R&D systems and antibodies-online, respectively).

Statistical analyses: Separate analyses were performed for each experiment individually. We compared the continuous measurements among patients based on the baseline characteristics by two tailed t-test (for two groups) or ANOVA (three groups). The continuous measurements

among patients with different tumor stage levels were compared using ANOVA. We compared the baseline tumor characteristics among patients with different MICA levels by using the Chi-square test. We also compared the distributions of Day.to.Recurrence and Day.to.Death survival among patients with different MICA levels with the Kaplan-Meier curves and log-rank test. We further evaluated the association between the MICA levels and Day.to.Recurrence, Day.to.Death, and Day.to.Death given recurrence survival by using the simple Cox regression model. In addition, we also applied multivariate cox regression models to assess the independent association between MICA levels and survivals.

3.3. Results

We have previously shown that NK cells from advanced melanoma patients (Stage III and IV) display an exhausted phenotype. However, the phenotype of NK cells in different phases of tumor progression (Stage I-IV) and how it can be related with prognostic factors is unknown.

We studied a prospectively-enrolled cohort of patients *(exploratory cohort*) presenting with stage I (n=56), stage II (n=21) and stages III/IV (n=23) melanoma; and of 25 healthy donors. NK cells were purified from the peripheral blood and were characterized acccording to the expression of activating (NKG2D) (**Figure 3.1.A**) and inhibitory (KIR3DL1, Tim-3) receptors (**Figure 3.1.B and 3.1.C**), function (cytotoxicity, IFN-γ production and proliferation) and the intracellular expression of the transcription factors (T-bet and Eomes) (**Figure 3.1.D**).

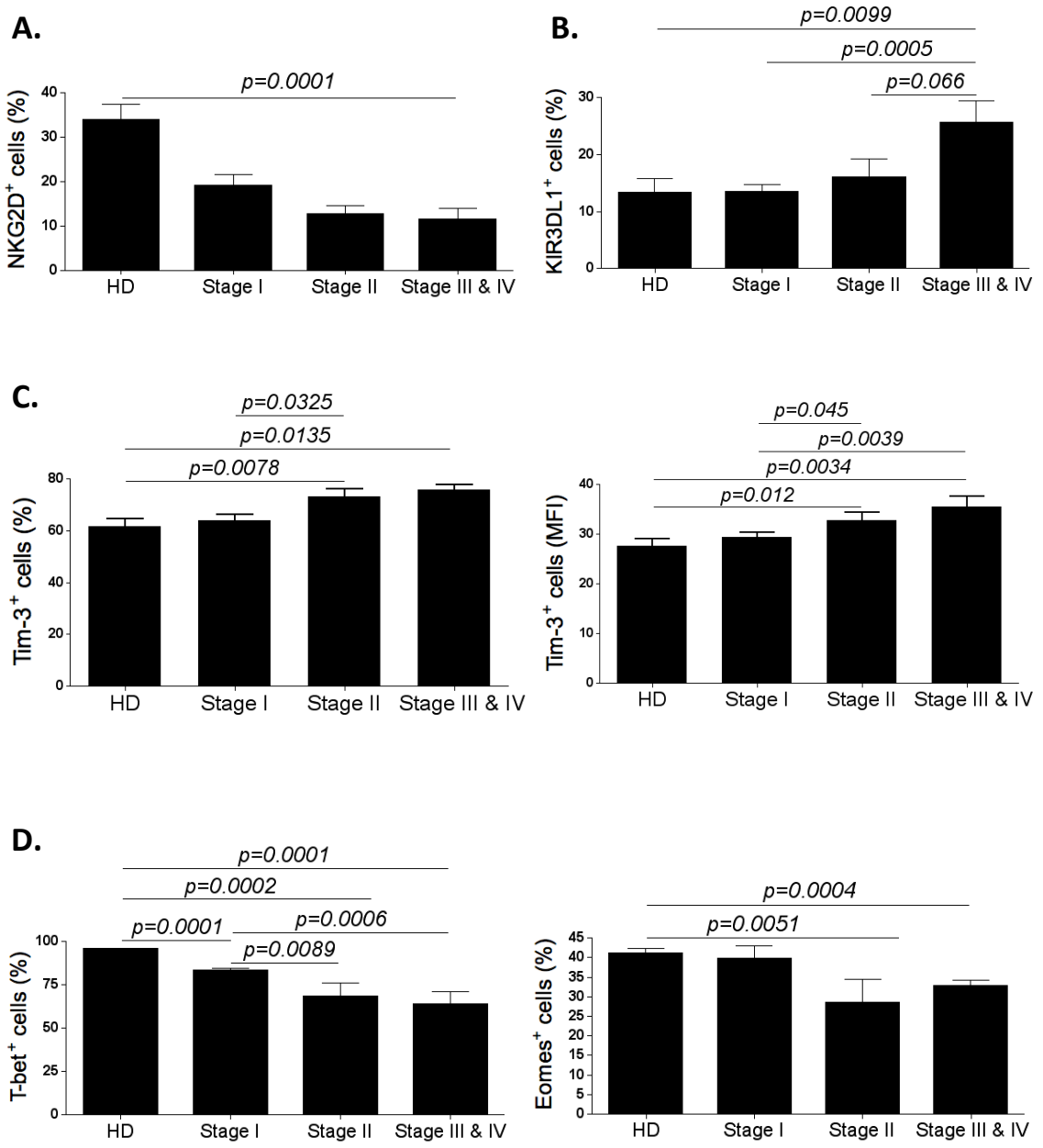
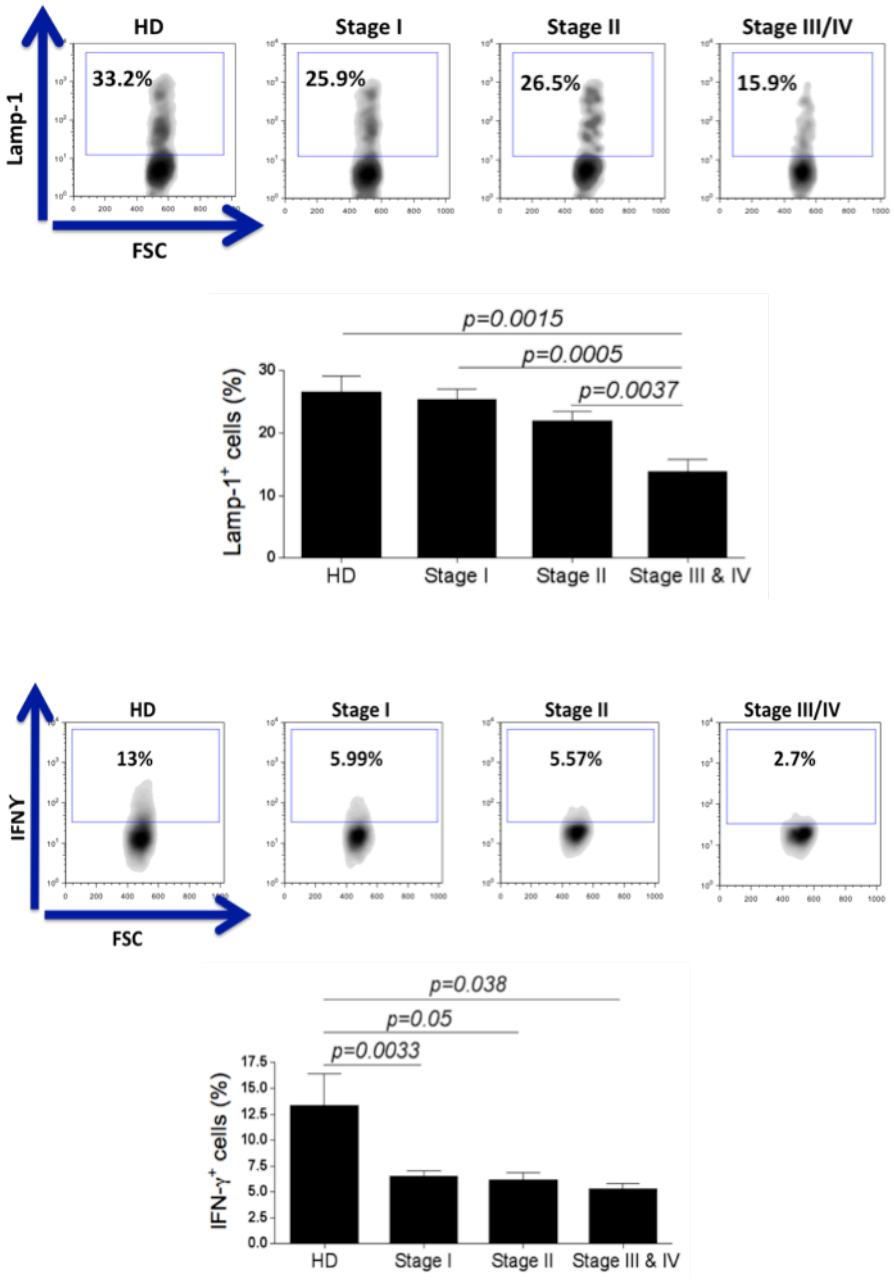


Figure 3.1. MD NK cells gradually develop a phenotypic profile of exhaustion. An exploratory cohort of 100 patients, who presented with melanoma stage I (n=56), stage II (n=21) and stage III/IV (n=23), plus 25 HD were studied using different criteria: expression of the activating receptor NKG2D **(A);** inhibitory receptors KIR3DL1 **(B)** and Tim-3 **(C)**; and T-bet and Eomes transcription factor expression **(D)**. All experiments were performed in duplicate.

Our data show that NK cells gradually develop a phenotypic and functional profile consistent with exhaustion, as the melanoma stage advances. The development of this exhaustion phenotype along the different stages is characterized by: 1) progressive increase in expression

of inhibitory receptors, concomitant with a progressive decrease of activating receptors (**Figure 3.1.A**, **3.1.B and 3.1.C**); 2) progressive decrease in expression of the transcription factors, T-bet and Eomes (**Figure 3.1.D**); 3) impaired cytotoxicity, IFN-γ production and proliferation (**Figure 3.2**).



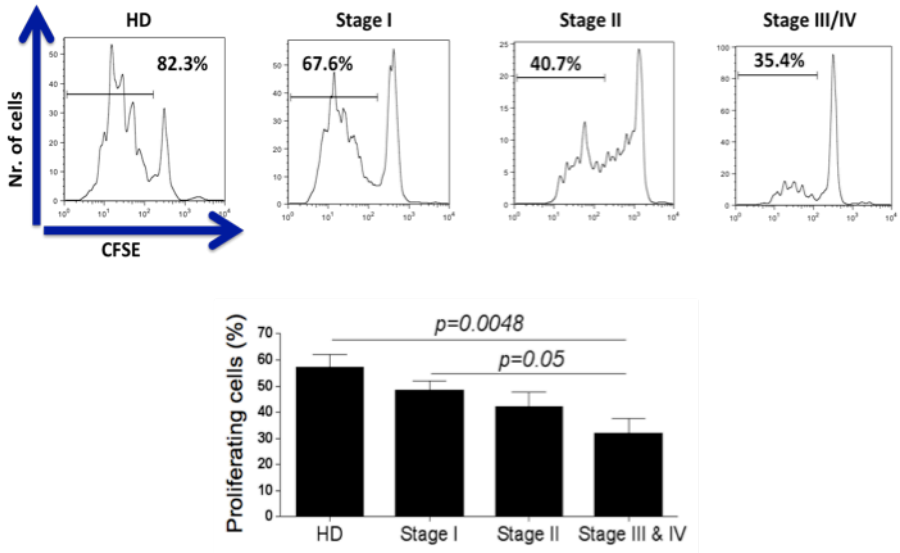


Figure 3.2. MD NK cells gradually develop a functional profile of exhaustion. An exploratory cohort of 100 patients, who presented with melanoma stage I (n=56), stage II (n=21) and stage III/IV (n=23), plus 25 HD were studied according to: cytotoxicity **(upper panel)**, IFN-γ^+ production **(middle panel)** and proliferating **(lower panel)**. Upper plots show a representatiive melanoma patient from each stage group. All experiments were performed in duplicate.

We then evaluated the association between each NK parameter (expression of inhibitory/activating receptors, function and expression of transcription factors) with demographic and clinical parameters (age, gender, stage, thickness, mitotic index, ulceration, metastasis). Interestingly, the expression of inhibitory receptors (KIR3DL1 and Tim-3), two main functions of NK cells (cytotoxicity and IFN-γ production) and T-bet expression are associated with important prognostic factors, such as thickness and presence of metastases. Thicker melanomas (> 1mm) were found to be associated with a higher expression of Tim-3 (*p=0.040*), lower cytotoxicity (*p=0.027*) and lower cytokine production ability (*p=0.03*). Moreover, local or distant metastases were found to be associated with a higher expression of Tim-3 (*p=0.004*) and KIR3DL1 (*p=0.0009*), and a lower cytotoxicity (*p=0.0004*) and lower T-bet expression (*p=0.02*). These results were confirmed in an independent validation cohort of 50 patients presenting with stage I (n=37), stage II (n=9) and stage III/IV (n=4) (**Tables 3.1, 3.2 and 3.3**).

Demographic parameters	Number of patients (N=100(%))	Tim-3+ cells (MFI) Mean (SD)	*p*-value	KIR3DL1+ cells (%) Mean (SD)	*p*-value	T-bet+ cells (%) Mean (SD)	*p*-value
Age Groups			***0.2231***		***0.1648***		***0.0796***
<= 45y	26 (26)	33.62 (8.790)		13.51 (10.20)		79.70 (21.20)	
46-70y	38 (38)	31.41 (7.356)		16.29 (12.59)		81.81 (18.45)	
>=71y	36 (36)	30.16 (7.283)		20.48 (17.60)		69.37 (25.62)	
Gender			***0.5189***		***0.4541***		***0.4448***
Female	36 (36)	30.86 (7.410)		16.88 (15.18)		75.25 (24.86)	
Male	64 (64)	31.91 (7.993)		14.66 (11.24)		79.24 (17.80)	
Stage			***0.0008****		***0.0044****		***0.0001****
I	56 (56)	29.44 (6.372)		13.35 (10.93)		83.10 (13.21)	
II	21 (21)	32.72 (7.275)		16.01 (14.66)		68.37 (31.13)	
III/IV	23 (23)	33.52 (9.798)		25.60 (16.86)		63.59 (29.22)	
Demographic parameters	**Number of patients (N=100(%))**	**Cytotoxicity (%) Mean (SD)**	***p*-value**	**IFN-γ (%) Mean (SD)**	***p*-value**	**Proliferation (%) Mean (SD)**	***p*-value**
Age Groups			***0.0413***		***0.1648***		***0.5496***
<= 45y	26 (26)	23.90 (10.66)		7.675 (5.847)		40.68(24.56)	
46-70y	38 (38)	25.58 (14.75)		6.545 (3.931)		41.26(27.67)	
>=71y	36 (36)	18.39 (8.440)		6.572 (3.035)		48.23 (30.16)	
Gender			***0.8042***		***0.4519***		***0.7710***
Female	36 (36)	22.40 (12.23)		6.594 (3.893)		44.06 (28.36)	
Male	64 (64)	23.06 (12.14)		7.284 (4.802)		42.35 (25.44)	
Stage			***0.0018****		***0.0016****		***0.0223****
I	56 (56)	25.31 (12.48)		6.459 (4.187)		35.35 (20.01)	
II	21 (21)	21.81 (7.153)		6.082 (3.617)		25.23 (18.56)	
III/IV	23 (23)	13.72 (8.603)		5.189 (2.824)		11.07 (9.332)	

Table 3.1. Higher expression of Tim-3 and KIR3DL1, and a lower cytotoxity, cytokine production, proliferation and T-bet expression are associated with more advanced melanoma stages. The association of NK cell phenotype (expression of inhibitory/activating receptors, function and expression of transcription factors) with demographic and clinical parameters (age, gender and stage) was evaluated in an exploratory cohort of 100 patients and validated in an independent cohort of 50 patients (*).

Clinical parameters	Number of patients (N=100(%))	Tim-3+ cells (MFI) Mean (SD)	*p*-value	KIR3DL1+ cells (%) Mean (SD)	*p*-value	T-bet+ cells (%) Mean (SD)	*p*-value
Thickness			***0.0406****		***0.0153***		***0.0005***
<= 1 mm	58 (66)	29.36 (5.297)		12.83 (10.58)		83.10 (13.21)	
>1 mm	30 (34)	32.24 (5.464)		19.30 (13.57)		64.91 (29.85)	
Mitotic Index			***0.0176***		***0.5935***		***0.6164***
<1/mm^2	43 (49)	29.05 (5.246)		14.46 (12.34)		77.75 (20.68)	
>=1/mm^2	40 (45)	32.21 (5.914)		15.89 (12.02)		75.07 (24.33)	
Unclassified	5 (6)						
Ulceration			***0.0277***		***0.9155***		***0.7915***
Absent	65 (74)	29.45 (5.721)		14.58 (11.07)		78.19 (19.14)	
Present	15 (17)	33.61 (5.843)		14.23 (13.96)		80.00 (27.25)	
Unclassified	8 (9)						
Clinical parameters	**Number of patients (N=100(%))**	**Cytotoxicity (%) Mean (SD)**	***p*-value**	**IFN-γ (%) Mean (SD)**	***p*-value**	**Proliferation (%) Mean (SD)**	***p*-value**
Thickness			***0.0266****		***0.0295****		***0.2602***
<= 1 mm	58 (66)	25.17 (12.28)		7.591 (4.584)		45.69 (27.71)	
>1 mm	30 (34)	19.44 (8.184)		5.531 (3.292)		39.00 (25.39)	
Mitotic Index			***0.1701***		***0.6104***		***0.6579***
<1/mm^2	43 (49)	25.45 (13.12)		7.214 (4.556)		43.77 (26.95)	
>=1/mm^2	40 (45)	21.78 (10.94)		6.720 (4.230)		46.44(27.07)	
Unclassified	5 (6)						
Ulceration^2			***0.9341***		***0.2820***		***0.5553***
Absent	65 (74)	23.77 (12.26)		7.020 (4.620)		43.57 (27.20)	
Present	15 (17)	23.45 (12.62)		5.586 (3.670)		38.87 (24.20)	
Unclassified	8 (9)						

Table 3.2. Higher expression of Tim-3 and a lower cytotoxity and cytokine production ability are associated with thicker melanomas. The association of NK cell phenotype (expression of inhibitory/activating receptors, function and expression of transcription factors) with demographic and clinical parameters (thickness, mitotic index and ulceration) was evaluated in an exploratory cohort of 100 patients and validated in an independent cohort of 50 patients (*).

Clinical parameters	Number of patients (N=100(%))	Tim-3+ cells (MFI) Mean (SD)	*p*-value	KIR3DL1+ cells (%) Mean (SD)	*p*-value	T-bet+ cells (%) Mean (SD)	*p*-value
Metastasis			***0.0042****		***0.0009****		***0.0157****
Absent	77 (77)	30.33 (6.744)		14.06 (11.99)		79.38 (19.88)	
Present	23 (23)	35.55 (9.614)		25.60 (16.86)		63.59 (29.22)	
Clinical parameters	**Number of patients (N=100(%))**	**Cytotoxicity (%) Mean (SD)**	***p*-value**	**IFNg (%) Mean (SD)**	***p*-value**	**Proliferation (%)**	***p*-value**
Metastasis			***0.0004****		***0.0625***		***0.0904***
Absent	26 (26)	24.83 (11.91)		7.243 (4.402)		45.39 (27.52)	
Present	38 (38)	13.72 (8.603)		5.189 (2.824)		31.62 (21.71)	

Table 3.3. Higher expression of Tim-3 and KIR3DL1, lower cytotoxity and lower T-bet expression are associated with local or distant metastases. The association of NK phenotype (expression of inhibitory/activating receptors, function and expression of transcription factors) with the presence of regional/distant metastases was evaluated in an exploratory cohort of 100 patients and validated in an independent cohort of 50 patients (*).

In addition we investigated whether there was any correlation between the expression of the inhibitory/activating receptors and NK cell function. Although some statistically significant correlations ($p<0.05$) were found, namely between Tim-3 and KIR3DL1 or NKp46 and cytotoxicity, the correlation coefficient was low ($r^2<0.3$).

These data demonstrate that NK cells become progressively exhausted in the context of melanoma progression. Moreover, the exhausted NK cell phenotype was found to be associated with clinical parameters such as thickness and metastases, well-known prognostic factors in melanoma.

We next measured the expression of TGF-β (cytokine), MICA (NKG2DL) and HMGB1 (Tim-3 ligand) in the plasma of melanoma patients from the same exploratory and validation cohort as described in Figure 3.2 using an ELISA kit. MICA (n=111) and HMGB1 (n=61) expression was found to increase from stage I to stage IV ($p=0.02$ and $p=0.045$, respectively) **(Figures 3.3.A and 3.3.B)**. TGF-β (n=65) levels increased from stage II to stage III/IV, however this increase is not statistically significant **(Figure 3.3.C)**. In addition we investigated whether there was any correlation between the expression of MICA, HMGB1 or

TGF-β and NK cell phenotype (receptors expression and function), however no statistically significant association was found.

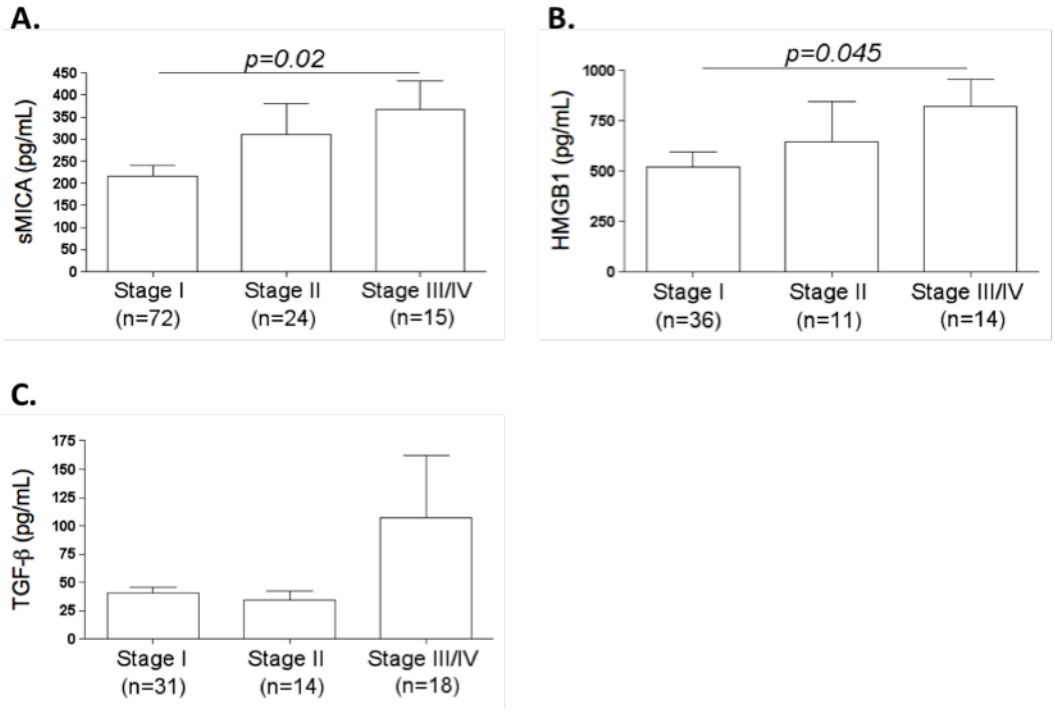


Figure 3.3. MICA and HMGB1 expression in the plasma/serum from melanoma patients increases from stage I to stage IV. Expression of **(A)** MICA, **(B)** HMGB1, **(C)** TGF-β in the plasma of melanoma patients at different stages of the disease (by ELISA). All experiments were performed in duplicate.

We then studied the expression of MICA and HMGB1 in an independent validation cohort (n=280). We divided the cohort in three groups, according to MICA expression: 1) level 1, no MICA expression (n=106); 2) level 2, <500pg/mL (n=129) and 3) level 3, >= 500pg/mL (n=45) (**Table 3.4**). Of note, higher levels of sMICA in the plasma of melanoma patients are associated with thicker melanomas, advanced stages, higher recurrence and death rate. Interestingly, we observed that higher expression of MICA in the plasma was associated with shorter recurrence-free survival (*p=0.0058*) **(Figure 3.4.A)**, with a hazard ratio for recurrence of 2.4 between level 1 and level 3 of MICA expression (*p=0.002*) **(Table 3.5)**. Remarkably, we also observed an association between higher MICA expression and shorter overall survival (*p=0.0056*) **(Figure 3.4.B)**, with a hazard ratio for death of 2.6 between levels 1 and 3 **(Table 3.5)**. We did not observe any association between HMGB1 expression and prognosis.

		Level=1		Level=2		Level=3		
		106	**37.9%**	**130**	**46.4%**	**44**	**15.7%**	
		values	**percent**	**values**	**percent**	**values**	**percent**	
Gender	F	53	50.0%	52	40.0%	17	38.6%	0.236
	M	53	50.0%	78	60.0%	27	61.4%	
	NA	0		0		0		
Age at Diagnosis	median	63.0		65.0		64.5		0.867
	mean	62.1	(16.7)	61.8	(17.2)	63.4	(16.2)	
Histologic.subtype	NM	50.0	0.5	45.0	0.4	21.0	0.5	0.213
	SSM	33	32.7%	53	44.5%	12	27.9%	
	OTHER	18	17.8%	21	17.6%	10	23.3%	
	NA	5		11		1		
Thickness	median	2.4		2.0		2.1		0.012
	mean	3.7	(4.7)	2.4	(2.1)	3.2	(3.0)	
Mitoses	NONE	16	15.8%	29	24.6%	9	20.9%	0.281
	PRESENT	85	84.2%	89	75.4%	34	79.1%	
	NA	5		12		1		
Ulceration	ABSENT	58	55.8%	84	68.3%	24	54.5%	0.094
	PRESENT	46	44.2%	39	31.7%	20	45.5%	
	NA	2		7		0		
TILs	ABSENT	32	33.0%	23	20.4%	7	17.5%	0.054
	PRESENT	65	67.0%	90	79.6%	33	82.5%	
	NA	9		17		4		
Stage.and.substage	I	17	16.0%	48	36.9%	14	31.8%	0.000
	II	80	75.5%	49	37.7%	16	36.4%	
	III	8	7.5%	27	20.8%	14	31.8%	
	IV	1	0.9%	6	4.6%	0	0.0%	
	NA	0		0		0		
Recurrence	No	83	79.0%	86	69.4%	21	47.7%	0.001
	Yes	22	21.0%	38	30.6%	23	52.3%	
	NA	1		6		0		
Outcome_index	0	86	81.1%	92	70.8%	23	52.3%	0.002
	1	20	18.9%	38	29.2%	21	47.7%	
	NA	0		0		0		

Table 3.4. Higher levels of sMICA in the plasma of melanoma patients are associated with thicker melanomas, advanced stages, higher recurrence and death rate. Demographic (gender and age) and tumor (histologic subtype, thickness, mitosis, ulceration, TILs, stage) characteristics, and outcomes (recurrence and death), for different melanoma patient groups defined according to plasma sMICA levels (level 1, no expression; level 2, <500pg/mL; level 3, >= 500pg/mL). All experiments were performed in duplicate.

	Baseline	Level	Coeff	HR	*p*-value	<.1*,<.05**
Day.to.Recurrence	Level=1	Level=2	0.31	1.363	0.209	
	Level=1	Level=3	0.885	2.423	0.002	**
Day.to.Death	Level=1	Level=2	0.364	1.439	0.189	
	Level=1	Level=3	0.968	2.632	0.002	**
Day.to.Death Given Recurrence	Level=1	Level=2	0.683	1.98	0.061	*
	Level=1	Level=3	0.772	2.163	0.054	*

Table 3.5. Higher levels of sMICA in the plasma of melanoma patients are associated with shorter recurrence free survival and overall survival. Recurrence free survival and overall survival for different melanoma patient groups defined according to plasma sMICA levels (level 1, no expression; level 2, <500pg/mL; level 3, >= 500pg/mL). All experiments were performed in duplicate.

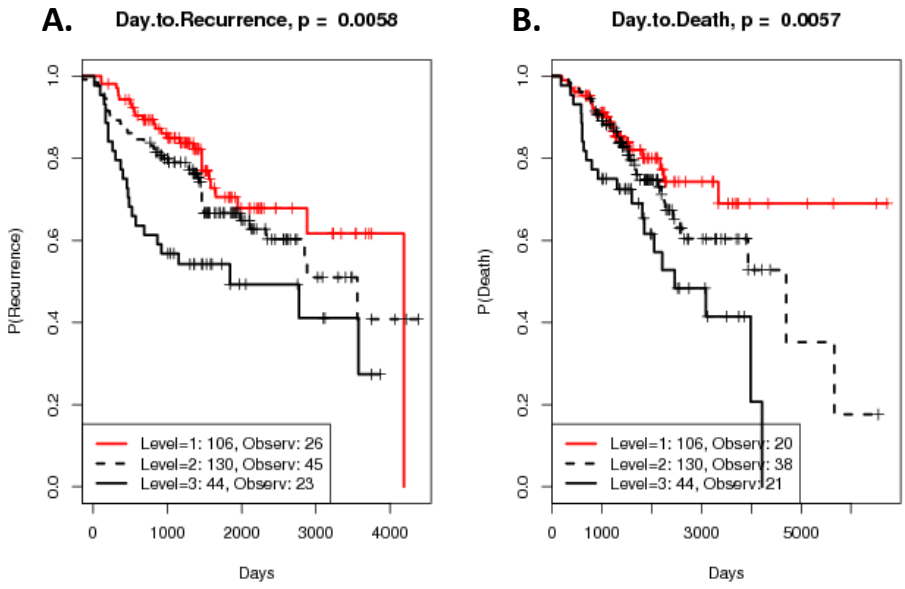


Figure 3.4. Higher levels of sMICA in the plasma of melanoma patients are associated with shorter recurrence free survival and overall survival. (A) Recurrence free survival and **(B)** overall survival for different melanoma patient groups defined according to plasma sMICA levels (level 1, no expression; level 2, <500pg/mL; level 3, >= 500pg/mL). All experiments were performed in duplicate.

AJCC staging is the best prognostic classification in melanoma thus far. However, if we look at the 5 year overall survival at stage II (primary melanoma lesion with thickness > 1mm, without lymph node or distant metastases) for instance, this ranges from 50-90%, which is still considered too broad[17]. Therefore, new prognostic markers are needed to improve the prognostic classification. We hypothesized that MICA expression in the plasma/serum may function as a prognostic factor, independent of AJCC staging. Our results show that higher levels of MICA in the plasma are associated with shorter recurrence-free survival (HR=1.8; *p=0.046*) and shorter overall survival (HR=2.5; *p=0.005*), even after adjustment for some potencial confounders, including age and staging **(Table 3.6)**.

Response	Baseline	Level	Coeff	HR	p-value	<.1*,<.05**
Day.to.Recurrence	Level=1	Level=2	0.042	1.043	0.871	
	Level=1	Level=3	0.604	1.829	0.046	**
	Age	Age	0.013	1.013	0.067	*
	Stage=I	Stage=II	0.19	1.209	0.533	
	Stage=I	Stage=III	1.385	3.994	0	**
	Stage=I	Stage=IV	2.056	7.817	0	**
	Gender=F	Gender=M	0.376	1.456	0.086	*
Day.to.Death	Level=1	Level=2	0.258	1.294	0.365	
	Level=1	Level=3	0.905	2.471	0.005	**
	Age	Age	0.035	1.035	0	**
	Stage=I	Stage=II	0.613	1.846	0.075	*
	Stage=I	Stage=III	1.458	4.296	0	**
	Stage=I	Stage=IV	2.39	10.919	0	**
	Gender=F	Gender=M	0.238	1.268	0.316	
Day.to.Death Given Recurrence	Level=1	Level=2	0.557	1.746	0.139	
	Level=1	Level=3	0.826	2.284	0.042	**
	Age	Age	0.013	1.013	0.172	
	Stage=I	Stage=II	0.542	1.719	0.191	
	Stage=I	Stage=III	1.023	2.781	0.013	**
	Stage=I	Stage=IV	1.553	4.725	0.007	**
	Gender=F	Gender=M	0.069	1.071	0.814	

Table 3.6. Higher levels of sMICA in the plasma of melanoma patients are associated with shorter recurrence free survival and overall survival, adjusting for age, gender and stage. Recurrence free survival and overall survival for different melanoma patient groups defined according to plasma sMICA levels (level 1, no expression; level 2, <500pg/mL; level 3, >= 500pg/mL), adjusted for age and stage. All experiments were performed in duplicate.

Taken together, our data suggest that sMICA is a potential prognostic factor and a potential biomarker predictive of recurrence.

3.4. Discussion

Our data suggest that NK cells become exhausted in the context of melanoma, making them an interesting population to target therapeutically. Moreover, as the exhaustion phenotype is acquired progressively through the stages, it may be possible to intervene early in the course of tumor progression.

NK cells, together with T cells, are the most efficacious anti-tumor cell types. However, the downregulation of HLA class I in melanoma cells might play a significant role in the pathogenesis and clinical course of the disease, and it represents a considerable problem for T cell-based immunotherapy. On the contrary, NK cells preferentially target tumor cells with low HLA class I expression, through IFN-γ production and direct cytotoxicity, making them a potential and interesting therapeutic option. Furthermore, NK cells have the ability to recognize and lyse a broad range of tumor cells without prior priming[180]. Three sets of immunotherapeutic NK-cell based strategies have been studied thus far. Firstly, cytokines that boost the NK cell response, such as IL-2, IL-21 or IL-15. In early experiments, NK cells were activated by IL-2 *ex vivo* and adoptively transfered to the patients (called LAK therapy) with advanced metastatic renal cancer and melanoma[181]. Therefore, NK cell activity can be boosted in melanoma patients treated with high doses of IL-2, although IL-2 alone has yielded limited beneficial effects and is associated with considerable side effects[182]. Secondly, NK cells are an attractive alternative, or potential adjuvant, to T cell-based ACT in the context of MHC-I-deficient tumor cells[170]. Thirdly, inhibitory receptor blockade or the crosslinking of activating receptors. The blockade of inhibitory receptors, such as anti-KIRs, promotes tumor rejection in preclinical models of leukemia and melanoma. Phase I trials are currently investigating the effects of a fully human IgG4 mAb that blocks the interaction between KIR2DL1,2,3 inhibitory receptors and HLA-C ligands in patients with AML and multiple myeloma, in remission, after chemotherapy[183]. In addition to the activating and inhibitory receptors, NK cells express several members of the signalling lymphocyte activation molecule (SLAM/CD150) family that play an important role in the regulation of NK cell function. 2B4 and CS1 (CD319, CRACC) are two members of the SLAM family that promises targeting

NK mediated cytolytic function against tumor cells[184,185,186] Another important point to be taken into account is the potential of NK cells to execute antibody-dependent cellular cytotoxicity through the CD16 (FCRγIII) receptor on their surface. It has been shown that treatment with monoclonal antibodies like rituximab (anti-CD20), cetuximab (anti-EGFR) and trastuzumab (anti-HER2)[187] can trigger effector functions of NK cells against tumor targets.

Our results demonstrate that NK cell exhaustion is a progressive phenomena that follows tumor progression. The exact mechanism underlying this process is still unclear; however it is possible that NK cells become exhausted due to systemic production of cytokines or within the TME in response to specific inhibitory molecules, or due to contact with immunossuppressive cells (regulatory T cells, tumor associated macrophages and myeloid derived suppressor cells). In fact, chronic stimulation with the activating cytokines IL-2 and IL-15 has been previously shown to induce NK cell exhaustion[175,176]. Moreover, molecules such as TGF-β, IL-10 and IDO, were shown to inhibit NK cells' function[165]. Furthermore, under certain conditions, such as hypoxia, MICA is shed from tumor cells by metalloproteases. Sera of patients with epithelial and hematopoetic malignancies contain elevated levels of sMICA. Soluble MICA has been shown to systemically downregulate NKG2D and inhibit NK cells' cytotoxicity[157]. Moreover, reduced NKG2D expression, and consequent impaired cytotoxicity on tumor-infiltrating and peripheral blood T and NK cells of tumor patients correlates with elevated sMICA levels in the sera[157].

It is important to point out that many tumor cells, including those in the lung, breast, kidney and colon, express different NKG2DL on their membrane, and that, higher expression of MICA is associated with a good prognosis[188]. As mentioned before, MICA has an opposite role depending on whether it is expressed on the membrane of the tumor cells, activating the immune system, or in the plasma, inhibiting it. In this study we have shown that levels of MICA in the plasma/serum are higher in advanced melanoma patients when compared with early stages. Moreover, we have observed, in an independent validation cohort, that higher levels of MICA are associated with a shorter median survival in the context of melanoma.

The AJCC staging system is the best and most commonly used prognostic classification system. It stratifies solid tumors according to prognosis based on size of the tumor and presence/absence of lymph node/distant metastases. Nevertheless, the range of 5 year overall

survival is still too broad in some stages, mainly stage II (50-90%). MICA, whose levels can be easily measured in the plasma/serum of melanoma patients by ELISA, is a potential prognostic marker. However, its quantitation needs to be standardized and validated in different cohorts of patients from different centers. In addition, we did not see any correlation between the NKG2D levels in the membrane of NK cells and MICA expression in the plasma of the same patients. This result can be explained by the fact that plasma from the patients contains factors that can regulate NKG2D expression independently of MICA expression[189]. In fact, TGF-β has also been shown to decrease the transcription of MICA, UL16 binding protein 2 (ULBP2), and UL16 binding protein 4 (ULBP4) in human gliomas[190]. Moreover, the presence of TGF-β can also downregulate NKG2D receptor expression on effector cells, and blocking TGF-β can lead to an increased expression of NKG2D regardless of sMICA present in the serum[191].

In conclusion, this study suggests that NK cells from melanoma patients acquire an exhausted phenotype as they advance in stage. Moreover, higher expression of inhibitory receptors and impaired function are associated with well-known worse prognostic parameters (melanomas that are thicker than 1 mm and the presence of metastasis). Interestingly, higher levels of MICA in the plasma, that may be involved in the mechanism of NK cell exhaustion, are associated with patients with worse prognosis.

CHAPTER 4:

REVERSAL OF NK CELL EXHAUSTION IN ADVANCED MELANOMA BY TIM-3 BLOCKADE

4.1. Introduction

Immune checkpoints play a key role in the maintenance of immune tolerance, avoiding autoimmune reactions. However, they are also responsible for an innefficient anti-tumor response, leading to tumor progression. Tim-3, together with CTLA-4 and PD-1, among others, belong to this family of immune checkpoints[45].

Tim-3 plays a crucial role in mediating T cell exhaustion in both viral infections and tumors[119,121,124] , and its blockade reverses the exhausted phenotype of $CD4^+$ and $CD8^+$ T cells in several chronic diseases including melanoma[93,123,192]. Whereas in resting T cells Tim-3 is minimally expressed and upregulated only after chronic stimulation, it is constitutively expressed at considerably higher levels in activated NK cells[132]. Recent publications, however, have reported conflicting data regarding Tim-3 function in NK cells[132,133]. Ndhlovu and colleagues showed that Tim-3 inhibits NK cell-mediated cytotoxicity[132] while another study suggested that Tim-3 may instead enhance IFN-γ production[133]. Both studies only evaluated healthy donors and not patients with chronic diseases such as cancer, where Tim-3 expression on NK cells may have a more significant immunomodulatory role.

In this study, we aimed to study the Tim-3 function in NK cells from advanced melanoma patients. We found that Tim-3 is an inhibitory receptor in NK cells from advanced melanoma patients, and that is upregulated on functionally impaired/exhausted NK cells. Most importantly, we showed that Tim-3 blockade reversed this NK cell exhausted phenotype. These data open exciting avenues for new therapies targeting Tim-3 in tumor immunotherapy.

4.2. Material and Methods

Human samples: Blood samples from healthy donors were purchased from the New York Blood Center. Blood samples were obtained under the Interdisciplinary Melanoma Cooperative Group IRB approved protocols (#H10362) from 45 untreated melanoma patients (stages I, II and III/IV). The study was approved by the Institutional Research Board at NYU in accord with a Federalwide Assurance approved by the Department of Health and Human Services, with all patients providing informed written consent at the time of enrollment.

Reagents: For blocking experiments - 10 or 20μg/ml of Tim-3 blocking antibody (Biolegend, clone 2E2; R&D #AF2365), galectin-9 blocking antibody (BioLegend), or IL-2R (α, β and γ chains – R&D Biosystems) blocking antibody was added to the culture 1 hour before initiating the functional assays. For crosslinking experiments - the same anti-Tim-3 antibody (Biolegend) was used. Anti-CD16 and anti-CD94 antibodies from Biolegend were also used to coat beads and in the reverse-antibody-dependent cell-mediated cytotoxicity (ADCC) assay. For both experiments, crosslinking and blocking, we used the IgG1 isotype control from BD. 7-aminoactinomycin D (7AAD), purchased from Biolegend, was used. For stimulation - rh-Gal-9, purchased from R&D systems, was added to NK cells 1h before the cytotoxic assay. Reagents for the staining assay were described in CHAPTER 1.

Cell lines: Gmel, FM29, WM1552, WM3248, WM793b are melanoma cell lines used as target cell in cytotoxic assays in diferent experiments. Gmel and FM29 cells were cultured in complete media (RPMI-1640 - Life Technologies) supplemented with 10% FBS, 2mM L-glutamine, 100U/ml penicillin, and 100μg/ml streptomycin. Gmel is a melanoma cell line that expresses galectin-9 intracellularly and with 5% of these cells expressing galectin-9 on the membrane. Melanoma cell lines WM1552, WM3248, WM793b were purchased from the Wistar Institute. The P815 cell line was purchased from ATCC. This cell line is FcR^+ and was used for the reverse ADCC-assay. The K562 cell line was described in CHAPTER 2.

NK cell purification and stimulation, cell Staining and flow cytometry analysis, Cytotoxicity, IFN-γ production and proliferation assays were described in CHAPTER 2.

Blocking experiment: 10 or 20μg/ml of Tim-3 blocking antibodies (Biolegend, clone 2E2 or R&D #AF2365) was added to the NK cell culture 1 hour before initiating the functional assays (i.e. before adding K562 cells in the cytotoxicity assay, IL-12 in the cytokine

production assay and IL-2 in the proliferation assay). An IgG1 antibody from BD Biosciences was used as isotype control.

Cell sorting: Gmel cells were first stained for Gal-9, following the protocol described above. These cells were then sorted as Gal-9$^-$-Gmel and Gal-9$^+$-Gmel by flow cytometry. The same steps were followed to sort NK cells into Tim-3$^+$ and Tim-3$^-$ NK cells.

Crosslinking experiment: (A) Coated–Beads: Dynabeads® Pan Mouse IgG (Invitrogen) were labeled with purified mouse IgG_1, κ isotype control (BD Biosciences) or purified human anti-Tim-3 (Biolegend) according to the manufacturer's instructions. Antibody labeled beads were added to the NK cell suspension at a 1:1 ratio for 30 min at 4^0C, then incubated at 37^0C for 90 min. The functional assays were then performed as previously described. (**B**) **Reverse-ADCC**: NK cells were co-cultured with Fc receptor$^+$ (FcR$^+$) P815 cells and different antibodies were added to the reaction: anti-Tim-3, anti-CD94 (negative control) or anti-CD16 (positive control) antibody.

Endocytosis: To assess whether Tim-3 was endocytosed after Tim-3 blockade, IL-2-stimulated NK cells from healthy donors were incubated with the Tim-3 blocking mAb or IgG1 isotype control, as previously described, for 15 min, 30 min and 1 hour. Cells were then fixed, permeabilized or not, and stained with a Phycoerythrin (PE)-conjugated anti-mouse mAb in order to detect Tim-3 blocking antibody on the cell surface and/or in the cytoplasm.

Statistical analyses: Separate analyses were performed for each experiment individually. Two tailed t tests were used. Analyses took into account paired observations within donors when appropriate, or unpaired observations when we comparing parameters between healthy and melanoma NK cells.

4.3. Results

Tim-3 is upregulated in NK cells from advanced melanoma patients; however the role of this receptor in this context remains unknown. In order to determine the role of Tim-3 in NK cell exhaustion, we developed a system to engage Tim-3 through crosslinking, by using anti-Tim-3 antibody-coated beads. We observed that Tim-3 activation in MD NK cells reduced cytotoxicity by approximately 20% (*p=0.0361*) **(Figure 4.1.A)** and IFN-γ production by 25%

(*p=0.0176*) **(Figure 4.1.B)**, as compared to controls (IgG coated beads). Moreover, we performed a reverse-ADCC assay using FcR^+ P815 cells that bind the Fc portion of antibodies, thus allowing antibodies to crosslink their relevant target ligands on other cells. We co-cultured NK cells from melanoma donors with P815 cells and added the following antibodies to the co-culture: anti-Tim-3, anti-CD94 (negative control) or anti-CD16 (positive control). As expected, CD16 crosslinking activated NK cells rendering them more cytotoxic, while CD94 crosslinking reduced NK cell cytotoxicity. As shown below, when Tim-3 was crosslinked, we observed a decrease of NK cell cytotoxicity (*p=0.0002*) **(Figure 4.1.C)**.

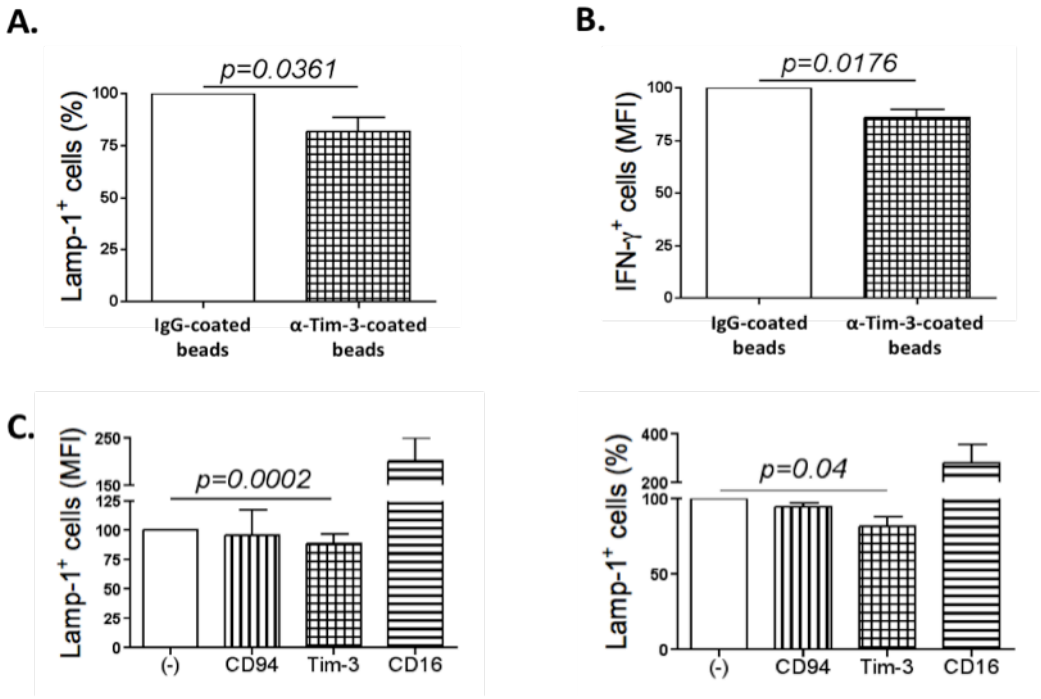


Figure 4.1. Tim-3 engagement inhibits NK cell functions. (**A**) The percentage of $LAMP\text{-}1^+$ (n=8) and (**B**) the MFI of $IFN\text{-}\gamma^+$ cells (n=6), of melanoma donors' NK cells pre-incubated with IgG-coated beads or anti-Tim-3 antibody-coated beads for 2 hours prior to evaluating cytotoxic function or IFN-γ production. (**C**) Reverse-ADCC assay using FcR^+ P815 cells. NK cells from melanoma donors were co-cultured with P815 cells and different antibodies were added to the reaction: anti-Tim-3, anti-CD94 (negative control) or anti-CD16 (positive control) antibodies. Data were normalized to values obtained for the condition without any antibody: (**A and B**) with IgG-coated beads (100%); (**C**) with no antibody. All experiments were performed in duplicate.

We used the same two systems to crosslink Tim-3 on HD NK cells, anti-Tim-3 antibody-coated beads **(Figure 4.2.A)** and the reverse-ADCC **(Figure 4.2.B)**, as well as, anti-Tim-3-coated K562 cells **(Figure 4.2.C)**. Our study showed that Lamp-1 expression was inhibited by NK cells (first two systems), and that there was a decrease in K562 cell apoptosis (third system). This finding confirms that Tim-3 acts as an inhibitory receptor in NK cells and that upon binding it downmodulates NK cell function in the context of advanced melanoma. It is important to point out that, as NK cells from melanoma patients may have already undergone significant Tim-3-mediated inhibition *in vivo*, it is not surprising that Tim-3 binding did not result in greater inhibition of NK cell function. In HD, Tim-3 binding also resulted in a significant decrease of cytotoxicity as previously described[132]. Tim-3 also acts as an inhibitory receptor on T cells, that when chronically activated, induces T cell apoptosis. However, in our system chronic Tim-3 stimulation did not induce NK cell apoptosis **(Figure 4.2.D).**

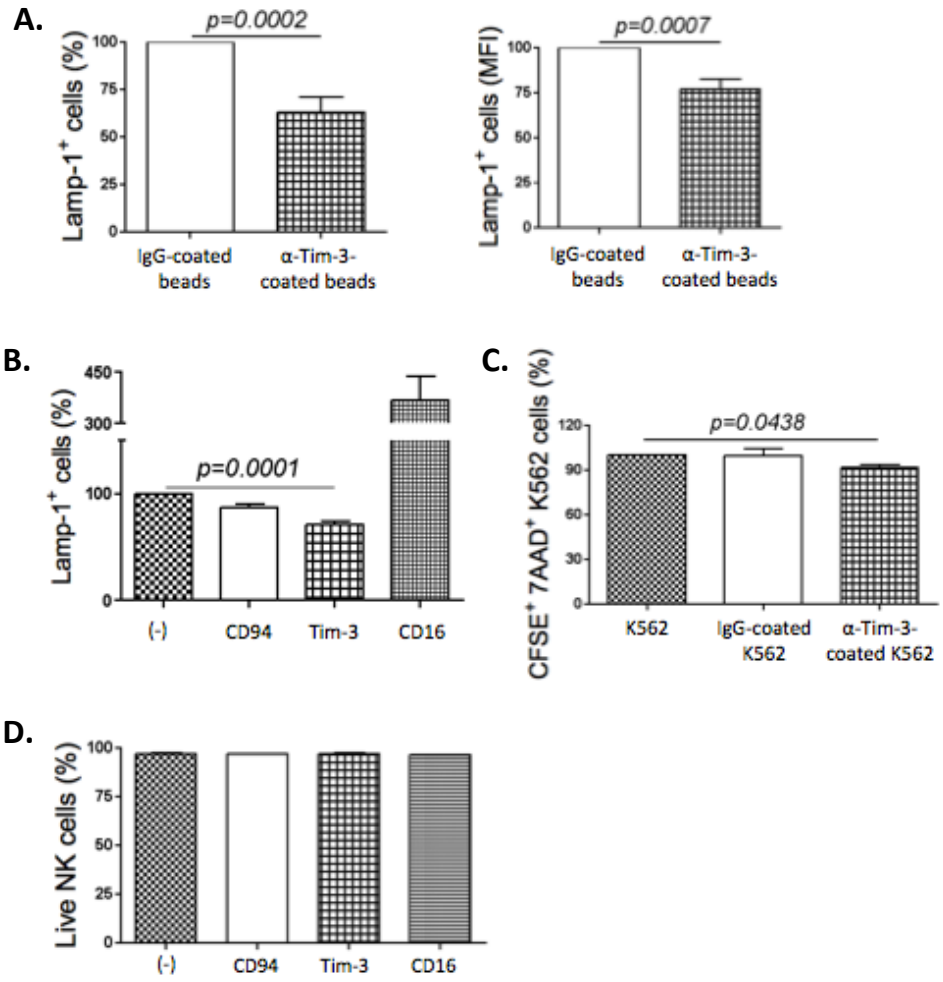


Figure 4.2. Tim-3 engagement on NK cells from healthy donors inhibits their cytotoxicity. (A) Graphs represent the percentage (**left panel**) and the MFI (**right panel**) of LAMP-1^{+} healthy donors' NK cells incubated with IgG-coated beads or anti-Tim-3 antibody-coated beads 2 hours previous to performing the cytotoxicity assay (n=16). Data were normalized to values obtained for the condition with IgG-coated beads. **(B)** Reverse-ADCC assay. Graph represents the % of LAMP-1^{+} healthy donors' NK cells co-cultured with P815 cells, in the presence of anti-Tim-3, anti-CD94 or anti-CD16 antibodies for 4 hours (n=5). **(C)** K562 cells were stained with CFSE, coated with IgG or anti-Tim-3, and co-cultured with NK cells from healthy donors for 4 hours (n=6). Data were normalized to values obtained for the condition with K562 cells only. The percentage of CFSE^{+}7AAD^{+} K562 cells ("killed cells") following a killing assay with NK cells from healthy donors is shown. **(D)** We performed a viability assay using the reverse-ADCC assay. MD NK cells were co-cultured with P815 cells, and anti-Tim-3, anti-CD94 or anti-CD16 antibodies were added to the reaction for 4 hours (n=5). The graph shows the percentage of Annexin V^{-}7AAD^{-} NK cells (live NK cells) for each condition. All experiments were performed in duplicate.

Gal-9 is the most well-studied Tim-3 ligand identified thus far. For this reason we chose to use recombinant and surface-bound Gal-9 as Tim-3 agonists. NK cells from HD were incubated with 25 and 50nM of rh-Gal-9 1 hour before assessing cytotoxicity, and displayed a significant decrease in Lamp-1 expression, but without significant toxicity **(Figure 4.3.A)**. We next were able to abrogate this effect by blocking Gal-9 with a specifc blocking antibody or β-lactose **(Figure 4.3.B)**. We then used a more physiological approach by using the melanoma cell line Gmel as source of Gal-9. Gmel cells were sorted according to their surface expression of Gal-9 into Gal-9^{+}-Gmel and Gal-9^{-}-Gmel cells and were then used as target cells. We found that NK cell-mediated cytotoxicity from both HD or MD was lower in the presence of Gal-9^{+}-Gmel cells compared with Gal-9^{-}-Gmel cells **(Figure 4.3.C)**. We then sorted NK cells according to Tim-3 expression into Tim-3^{+} vs Tim-3^{-} NK cells, and repeated the same experiment. The suppressive effect of Gal-9 was only evident in the presence of Tim-3^{+} NK cells **(Figure 4.3.D)**.

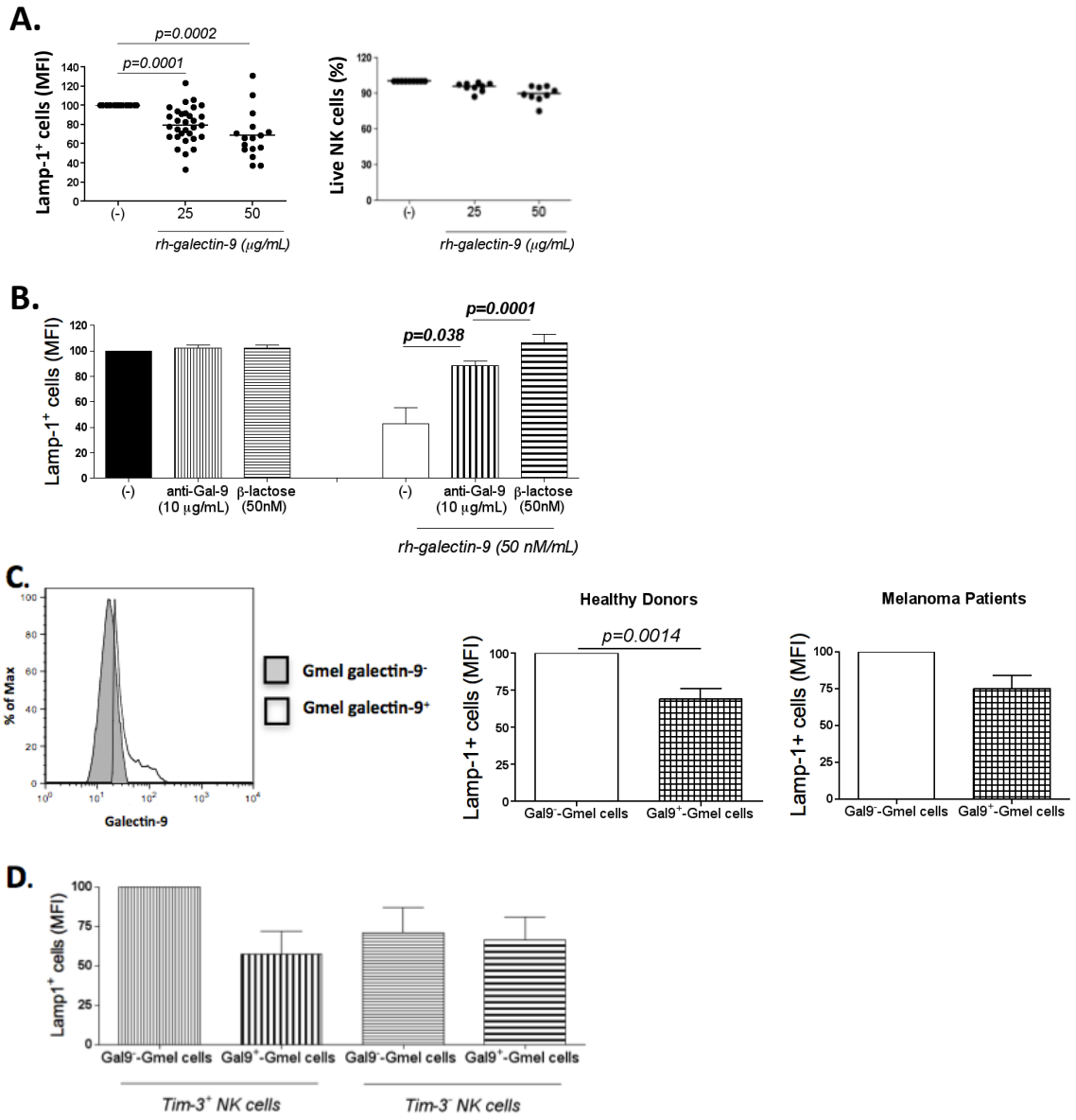
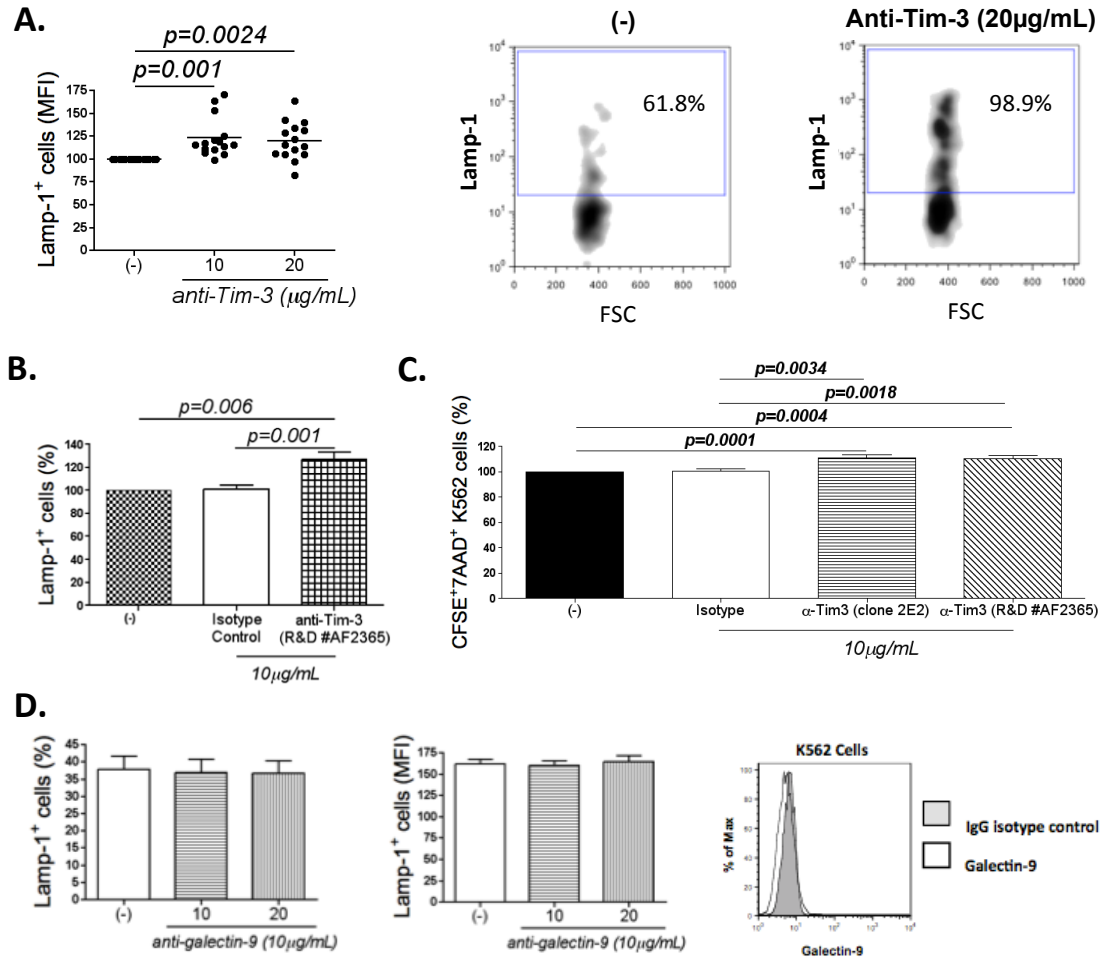


Figure 4.3. Tim-3 engagement by Galectin-9 on NK cells from healthy donors and melanoma patients inhibits their cytotoxicity. (**A**) Expression of LAMP-1 (MFI, n=20) by healthy donors' NK cells incubated with 25 or 50nM/ml of soluble rh-Gal-9 1 hour before assessing cytotoxicity (**left panel**) and NK cells' viability (**right panel**) using K562 cells as targets. (**B**) Lamp-1 expression (MFI) by healthy donors' NK cells (n=6) incubated with 50nM/ml of soluble rh-Gal-9 alone or in the presence of anti-Gal-9 antibody (10μg/mL) or β-lactose (50nM), to block the effect of soluble rh-Gal-9. (**C**) We used another approach to crosslink Tim-3 using Gal-9. The melanoma Gmel cells were sorted into Gal-9^{+}-Gmel and Gal-9^{-}-Gmel. **Left panel** shows galectin-9 expression in Gal-9^{+}-Gmel vs Gal-9^{-}Gmel. Graphs represent the expression of LAMP-1 (MFI) by NK cells from HD (**middle panel**; n=11) and by NK cells from MD (**right panel**; n=4), using Gal-9^{+}-Gmel or Gal-9^{-}Gmel as target cells. Data were normalized to values obtained for the condition without rh-Gal-9 (**Figure 4.A and 4.B**) or for the condition with Gal-9^{-}-Gmel cells as target cells (**Figure. 4.C**). (**D**) The graph shows Lamp-1 expression (MFI) in Tim-3^{+} vs Tim-3^{-} NK cells, in the presence of Gal-9^{-}-Gmel or Gal-9^{+}-Gmel as target cells.

In T cells, Tim-3 has been described as a marker of T cell exhaustion that, when blocked, can reverse the function of these cells[93,115,123]. In order to study whether Tim-3 blockade could also reverse the function of MD NK cells, we blocked the Tim-3 receptor by adding a soluble Tim-3 blocking antibody (10 and 20μg/mL; clone 2E2) 1 hour before assessing NK cell functions in various assays. Blocking Tim-3 significantly improved cytotoxicity by 20-25% (*p=0.002*) **(Figure 4.4.A)**. We obtained the same results with a different Tim-3 blocking antibody (R&D #AF2365; **Figure 4.4.B**). Both blocking antibodies were validated in a killing assay **(Figure 4.4.C**). Interestingly, addition of Galectin-9-blocking antibody did not affect cytotoxicity, suggesting that other Tim-3 natural ligands may participate instead, since K562 cells do not express galectin-9 **(Figure 4.4.D).** Importantly, Tim-3 blockade improved NK cell cytotoxicity against four different melanoma cell lines (**Figure 4.4.E**). All of the four melanoma cell lines express galectin-9 (**Figure 4.4.F**), however the expression levels did not correlate with the increase of cytotoxicity that is observed with the Tim-3 blocking antibody.



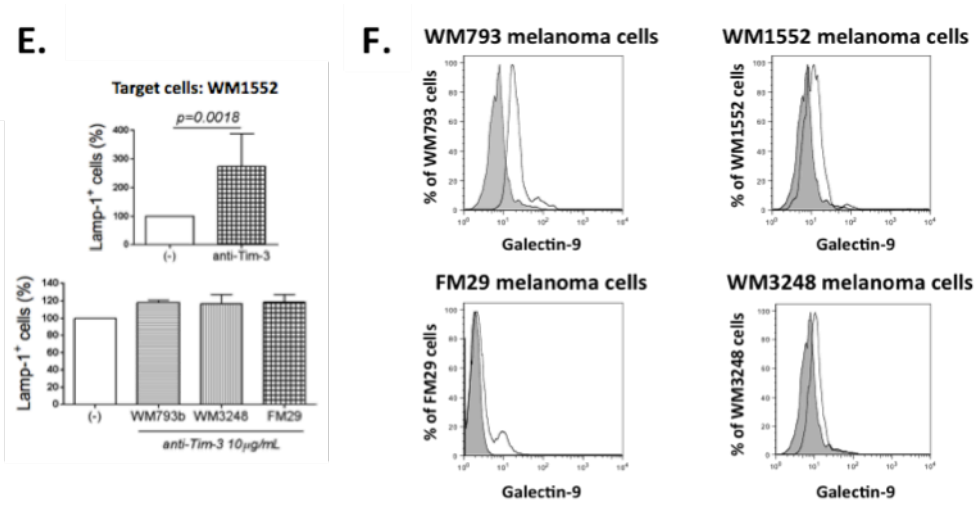


Figure 4.4. NK cell exhaustion can be reversed by Tim-3 blockade - Cytotoxicity. (A) Graph representing the expression of LAMP-1 (n=15; MFI) in melanoma donors' NK cells incubated with 10 or 20µg/ml of soluble Tim-3 blocking antibody 1 hour before the cytotoxic assay. **(B)** The plot shows the percentage of melanoma donors' Lamp-1^+ NK cell subset (n=8) incubated with 10µg/ml of a different soluble Tim-3 blocking antibody (R&D #AF2365) or IgG isotype control 1 hour before the cytotoxic assay. **(C)** Plot shows the percentage of $CFSE^+7AAD^+$ K562 cells (% of killed K562 cells) in the presence of 10µg/ml of two different Tim-3 blocking antibodies (clone 2E2 or R&D #AF2365) 1h before the killing assay (n=9 MD). **(D)** To determine if Galectin-9 was involved in our system, we used a blocking antibody for Galectin-9 for 1h (10 and 20ug/ml) and assessed cytotoxicity in NK cells from healthy donors (n=6) against K562 cells. Graphs represent percentage of Lamp-1^+ cells **(left panel)** and MFI **(middle panel)** of Lamp-1^+ cells. **(Right panel)** The plot shows galectin-9 expression in K562 cells. **(E)** Graphs depict the percentage of lamp-1^+ NK cells (n=5) incubated with 10µg/ml of soluble Tim-3 blocking antibody 1h before the cytotoxicity assay using melanoma cell lines as target cells: WM1552 (**upper panel**); WM793b, WM3248 or FM29 (**lower panel**). **(F)** The graphs show galectin-9 expression in the melanoma cell lines: FM29, WM793, WM1552 and WM3248. Data were normalized to values obtained for each condition without blocking antibody. All experiments were performed in duplicate.

IFN-γ production also increased in the presence of Tim-3 blocking antibody by 15-20% (*p=0.007*) **(Figures 4.5.A and 4.5.B)**.

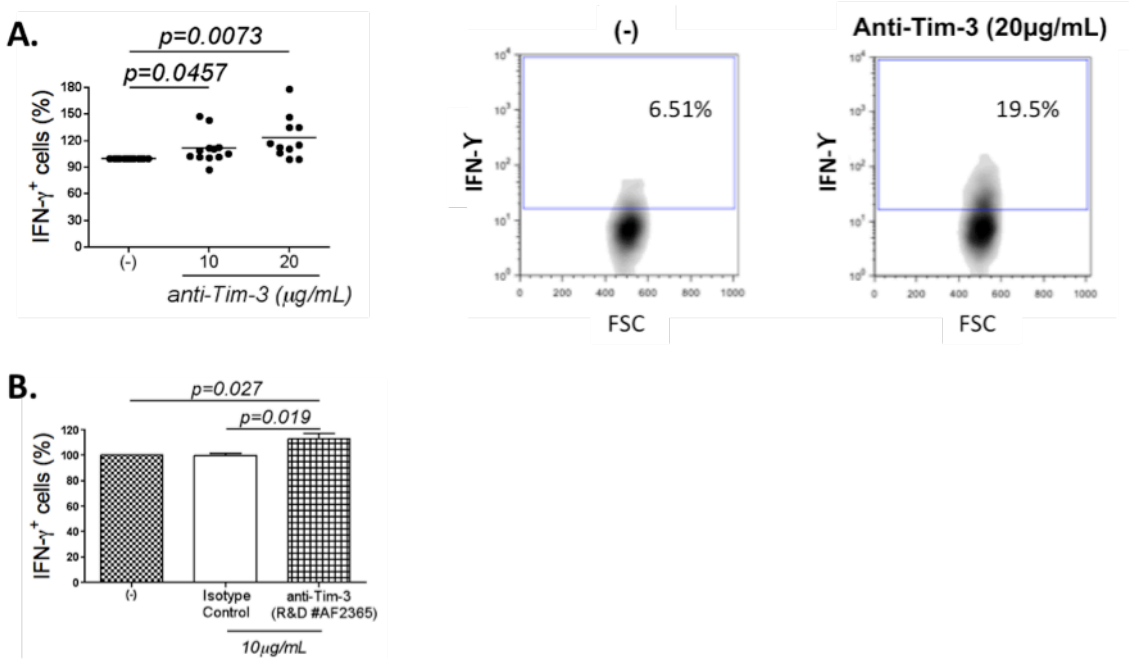


Figure 4.5. NK cell exhaustion can be reversed by Tim-3 blockade – IFN-γ production. **(A)** Graph represents the percentage of IFN-γ^+ cells (n=12) in melanoma donors' NK cells incubated with 10 or 20µg/ml of soluble Tim-3 blocking antibody 1 hour before the functional assay. Plots depicting the percentage of IFN-γ^+ with (**right panel**) and without (**middle panel**) Tim-3 blockade, from a representative melanoma patient. **(B)** The plot shows the percentage of melanoma donors' IFN-γ^+ NK cells subset (n=8) incubated with 10µg/ml of a different soluble Tim-3 blocking antibody (R&D #AF2365) or IgG isotype control 1h before performing the IFN-γ production assay. Data were normalized to values obtained for each condition without blocking antibody. All experiments were performed in duplicate.

Finally, the percentage of proliferating cells dramatically increased by 30% and 60% with the addition of 10 and 20µg/mL anti-Tim-3 antibody (*p=0.009*) **(Figures 4.6.A and 4.6.B)**. The isotype control did not produce any effect on NK cell cytotoxicity, IFN-γ production or proliferation capacity (**Figure 4.6.C**).

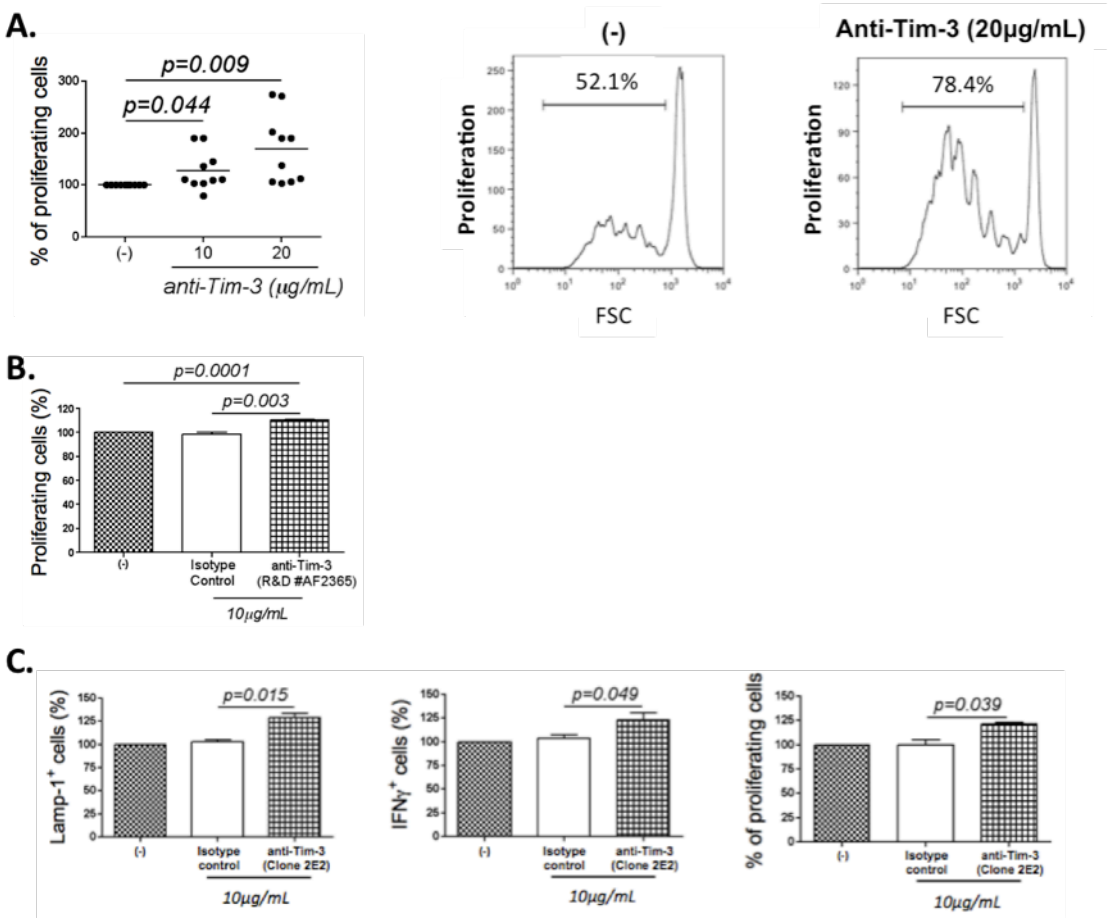


Figure 4.6. NK cell exhaustion can be reversed by Tim-3 blockade – proliferation. **(A)** Graph represents the percentage of proliferating cells (n=10; %) in melanoma donors' NK cells incubated with 10 or 20µg/ml of soluble Tim-3 blocking antibody 1 hour before performing the functional assay. Plots depicting the percentage of proliferating cells with (**right panel**) and without (**middle panel**) Tim-3 blockade from a representative melanoma patient. **(B)** Plot showing the percentage of proliferating NK cells from melanoma donors (n=8) incubated with 10µg/ml of a different soluble Tim-3 blocking antibody (R&D #AF2365) or IgG isotype control 1 hour before the proliferation assay was performed. **(C)** Graphs represent the expression of LAMP-1 (n=5; **left panel**), IFN-γ (n=5; **middle panel**) and the percentage of proliferating cells (n=5; **right panel**) in NK cells from melanoma donors incubated with 10 µg/ml of soluble Tim-3 blocking antibody (clone 2E2) or IgG1 isotype control 1 hour before the functional assays. Data were normalized to values obtained for the condition without blocking antibody. All experiments were performed in duplicate.

We next investigated the mechanism underlying the reversal of NK cell exhaustion by blockade of Tim-3. NK cells treated with Tim-3 blocking antibody showed a clear decrease of surface membrane Tim-3 levels **(Figure 4.7.A)**. We hypothesized that Tim-3 expression at the surface membrane decreased due to internalization. To address this possibility, NK cells from HD were first incubated with Tim-3 blocking antibody or isotype control, then fixed, permeabilized and stained with a PE anti-mouse IgG antibody in order to detect Tim-3 antibody, both on the cell surface and in the cytoplasm. As control, we also assessed antibody levels in cells that were fixed but not permeabilized. These experiments demonstrated that soluble Tim-3 blocking antibody induced internalization of Tim-3 **(Figures 4.7.B and 4.7.C**). To demonstrate that reduced Tim-3 levels on the membrane account for reversal of NK cell exhaustion, NK cells were pre-treated with soluble Tim-3 blocking antibody and residual Tim-3 was cross-linked using anti-Tim-3-coated beads. As expected, in this context, the beads had no effect on cytotoxicity, due to prior internalization of Tim-3 by the soluble blocking antibody **(Figure 4.7.D)**. Moreover, we confirmed that this effect is indeed due to Tim-3 blockade and not consequent to the engagement of the Fc portion of the Tim-3 blocking antibody with CD16 receptor (FcγRIII) in NK cells. Indeed, before adding the Tim-3 blocking antibody, we incubated MD NK cells with a CD16 (FcγRIII) blocking antibody for 1 hour. This anti-CD16 antibody prevents the possibility that the Fc portion of other antibodies added to the reaction (in this case Tim-3 blocking antibody) binds to CD16 receptor (FcγRIII). **Figure 4.7.E** shows an increase of cytotoxicity when using Tim-3 blocking antibody, even following CD16 blockade.

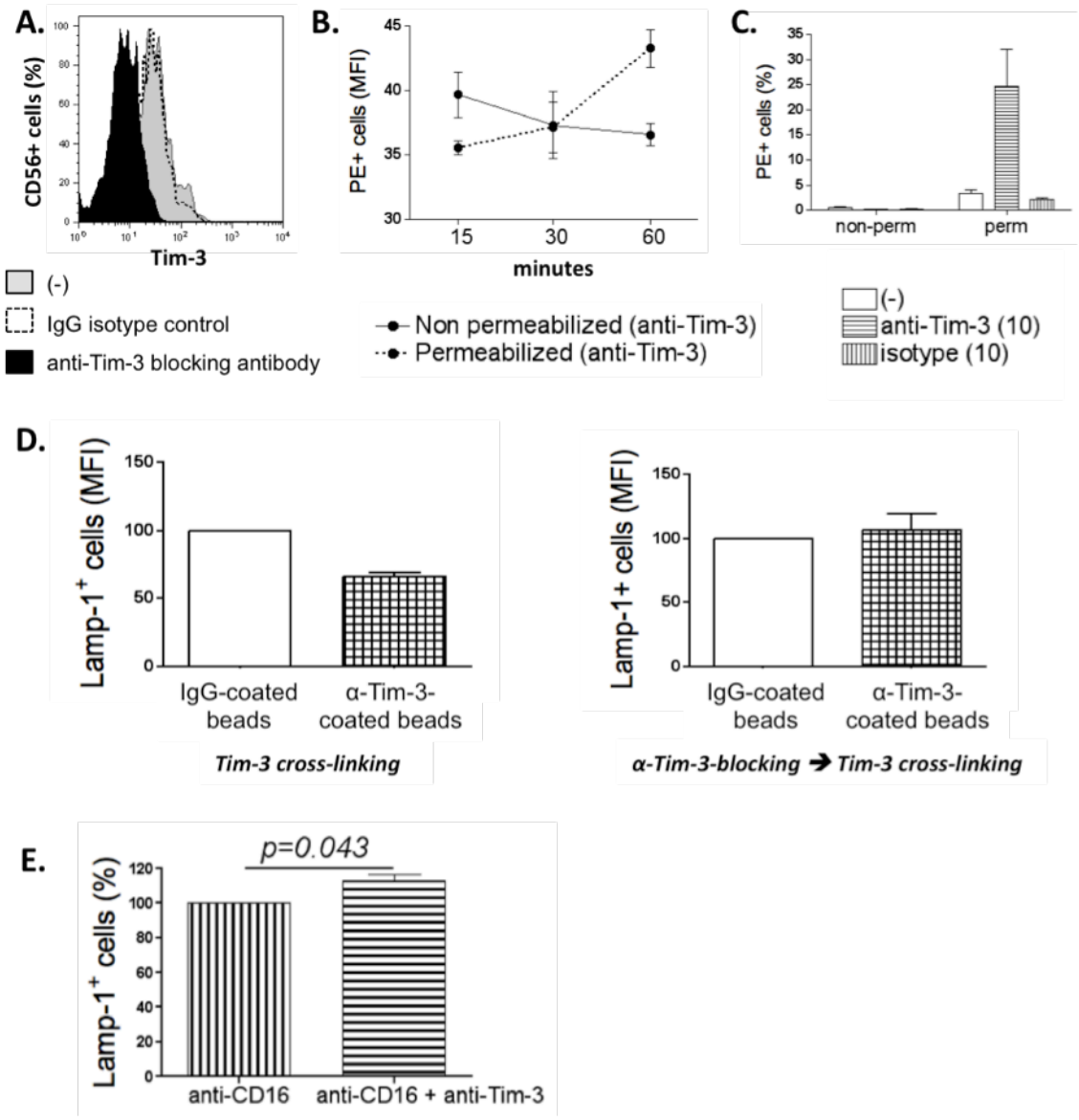


Figure 4.7. Soluble Tim-3 blocking antibody induces internalization of Tim-3. (**A**) Plot depicting the decrease of Tim-3 expression in the membrane of NK cells after 1 hour treatment with soluble Tim-3 blocking antibody. (**B**) Graph representing the MFI of NK cells positive for the PE anti-mouse IgG antibody, with or without permeabilization (n=6). (**C**) The previous experiment was repeated also including an IgG1 isotype control. Graph showing the percentage of cells positive for the PE anti-mouse IgG antibody after 1 hour of incubation with antibodies, soluble Tim-3 blocking antibody and IgG1 isotype control (n=6). (**D**) Graph representing the MFI of Lamp-1$^+$ NK cells from healthy donors (n=6) after performing a cytotoxic assay with K562 cells. (**Left panel**) NK cells were pre-incubated (2 hours) with anti-Tim-3-coated beads or IgG-coated beads. (**Right panel**) Soluble Tim-3 blocking antibody was added 1 hour before crosslinking with anti-Tim-3-coated beads as described previously. Data were normalized to values obtained for the condition with beads alone. **(E)** The graph shows the percentage of Lamp-1$^+$ NK cells after 1 hour treatment with 10μg/ml CD16 blocking antibody alone or after 1 hour treatment with 10μg/ml CD16 blocking antibody followed by 1h treatment with 10μg/ml soluble Tim-3 blocking antibody. All experiments were performed in duplicate.

Levels of activating and inhibitory receptors on the membrane of NK cells treated with Tim-3 blocking antibody were also measured. Interestingly, CD16 expression increased by 10% after Tim-3 blockade (**Figure 4.8.A)**. Likewise, there was a significant increase in expression of α and γ chains of the IL-2R (**Figure 4.8.B)**. NK cells respond to IL-2 stimulation with increased expression of activating and inhibitory receptors, cytotoxicity and IFN-γ production. In order to define the role of each chain of IL-2R in the NK cell response, we cultured purified NK cells from HD with 200UL/mL of IL-2 and separately blocked each chain of the IL-2R (α, β and γ). After two days, expression of activating and inhibitory receptors, and cytotoxicity were assessed. Our data demonstrate that both the α chain and mainly the γ chain play a key role in the expression of activating and inhibitory receptors (**Figure 4.8.C)**, and in enhancing NK cell-mediated cytotoxicity after IL-2 stimulation (**Figure 4.8.D)**.

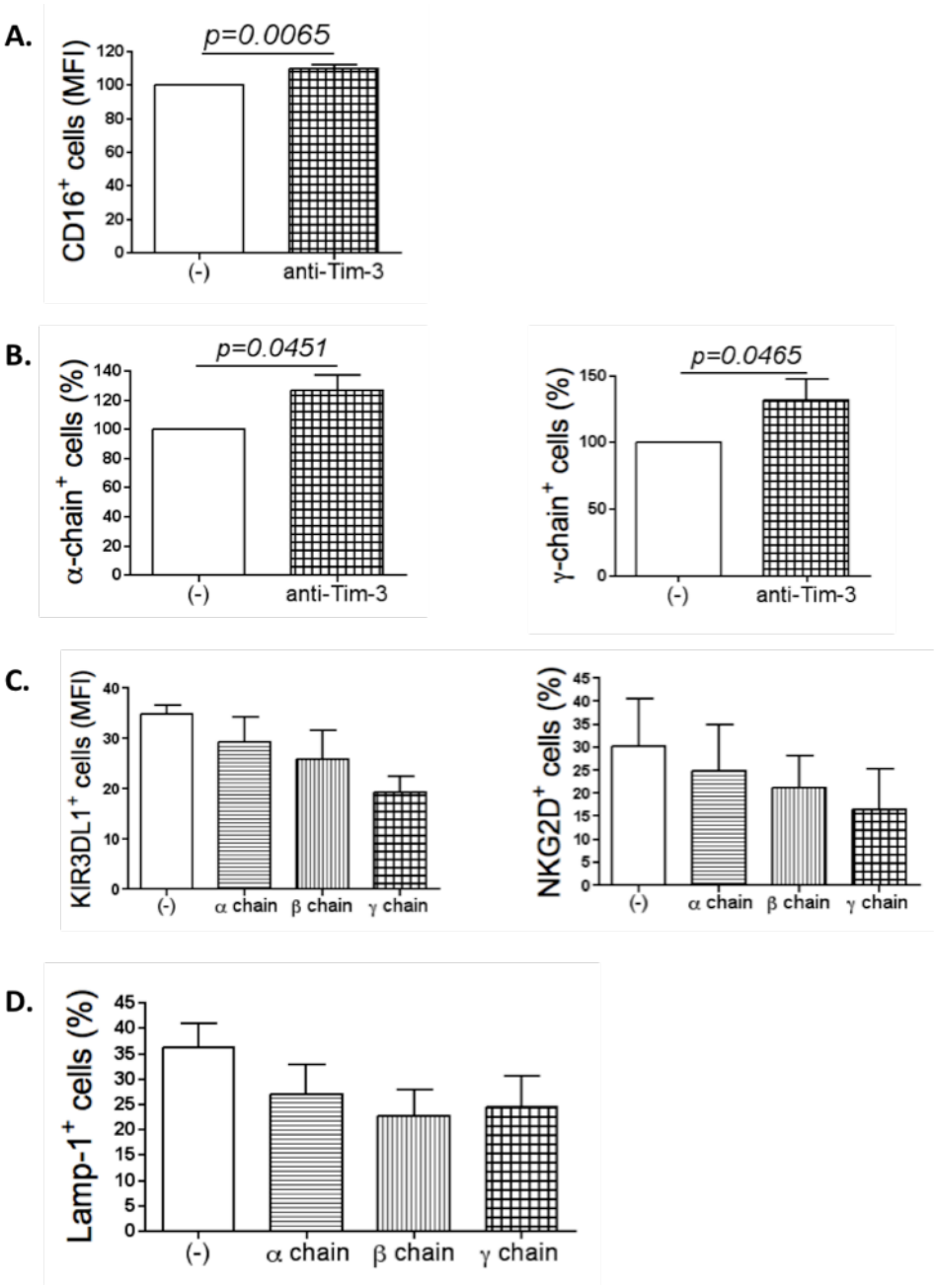


Figure 4.8. Soluble Tim-3 blocking antibody upregulates IL-2R, rendering them more responsive to IL-2 stimulation. (**A**) Plot showing the expression of CD16 receptor (MFI) in NK cells untreated or after 1 hour treatment with 10μg/ml of soluble Tim-3 blocking antibody. (**B**) Plots showing the expression of IL-2R α and γ chains (% of positive cells) in NK cells untreated or after 1 hour of treatment with 10μg/ml of soluble Tim-3 blocking antibody (n=7). (**C**) Graphs depict the NK cell expression of KIR3DL1 (left panel - MFI) and NKG2D (right panel - % $NKG2D^+$ cells) after two days of culture with 200U/mL untreated or treated with anti-α chain, anti-β chain or anti-γ chain blocking antibodies. (**D**) Graph depicting the percentage of $Lamp\text{-}1^+$ NK cells after two days of culture with 200U/mL of IL-2, untreated or treated with blocking antibody for α, β or γ chain (n=5). (**A and B**) Data were normalized to values obtained in the absence of Tim-3 blocking antibody. All experiments were performed in duplicate.

Our data indicate that following activation, Tim-3 functions as an inhibitory receptor in melanoma patients' NK cells by reducing their cytotoxicity and cytokine secretion potential. More importantly, these results demonstrate that Tim-3 blockade increases the expression of IL-2R, making NK cells more responsive to IL-2 stimulation, and consequently can enhance the function of NK cells from advanced melanoma patients.

4.4. Discussion

Our results demonstrate for the first time that exhausted NK cells upregulate Tim-3 which behaves as an inhibitory receptor/exhaustion marker, similarly to its role described for T cells. Furthermore, the levels of this receptor seem to be of key importance, since NK cells express Tim-3 in unstimulated conditions and upregulate this receptor when they become exhausted.

Contradictory roles have been described for Tim-3 in NK cells from healthy donors. While one study showed that Tim-3 inhibits normal donor NK cell-mediated cytotoxicity[132], the other suggested that Tim-3 may instead enhance IFN-γ production[133]. While the two studies are difficult to align, different model systems and contexts could explain these contradictory outcomes, as has been reported for other NK cell receptors such as 2B4 and KIR2DL4[193,194]. Our data are consistent with Tim-3 acting as an inhibitory receptor on NK cells from healthy, and most importantly, from melanoma donors. When triggered, we also show that it does not promote NK cell death. Rather, Tim-3 negatively regulates NK cell function in a Gal-9-independent manner, suggesting a role of other Tim-3-ligands. PtdSer, a Tim-3 ligand, is exposed on the surface of apoptotic cells and may be a candidate for causing NK cell exhaustion *in vivo* after tumor cell death.

Interestingly, when we blocked Tim-3 receptor with a soluble antibody, we were able to partially recover NK cell function. This reversal is comparable to that seen in T cells after in vitro blockade of another immune checkpoint such as PD-1 blockade[92,195], that is now being used in clinical trials with impressive clinical responses[61,62]. The Tim-3 blocking antibody binds and internalizes the Tim-3 receptor, decreasing its expression on the membrane of NK cells and the possibility of binding to the natural ligands. Another possibility is that we are blocking the intrinsic inhibitory pathway of Tim-3, independently of any ligand. We also showed that Tim-3 blockade induces a 10% increase of CD16 expression (mean fluorescence

intensity - MFI), which could provide another explanation for the increase in NK cell function. Thus CD16, an activating receptor that is directly involved in the lysis of tumor cells, may function not only through ADCC but also independently of antibody binding. Finally, we demonstrated that Tim-3 blockade increases expression of the IL-2R on the membrane of MD NK cells, making them more responsive to IL-2 stimulation. The enhanced responsiveness may contribute towards the partial reversal of MD NK cell function after Tim-3 blockade.

Similarly to CTLA-4 and PD-1, Tim-3 belongs to the group of immune checkpoint molecules and is a potential therapeutic target. Although there is no clinical data available yet, it has been reported that Tim-3 is co-expressed with PD-1 on human tumor-specific $CD8^+$ T cells, and that dual blockade of both molecules significantly enhances the *in vitro* proliferation and cytokine production of human T cells[93]. Furthermore, *in vivo* studies have shown that Tim-3 blockade alone, or in combination with PD-1 blockade, is able to control tumor growth in four different tumor models, including melanoma[123,124]. A recent study showed that Tim-3 blockade stimulates potent anti-tumor responses against established melanoma via NK cell-dependent mechanisms when associated with a vaccine[138]. However, in those studies it was not clear if Tim-3 had a direct effect on NK cells. Our findings provide the first evidence that Tim-3 blockade can directly reverse NK cell exhaustion and improve the function of NK cells from melanoma patients. Even though the recovery of melanoma NK cell function is significant, it is not complete. Possibly, Tim-3 works along with other receptors to regulate NK cell exhaustion, although we could not detect a role for either CTLA-4 or PD-1. Nevertheless, combinatorial strategies that also target other inhibitory NK cell receptors could possibly recover NK cell phenotype more completely. Our study has direct clinical relevance since as shows for the first time that, by blocking Tim-3, it is possible to improve, *ex vivo*, the function of NK cells which could be used for NK cell adoptive transfer therapy. Moreover, our studies support the concept that systemic Tim-3 blockade could improve anti-tumor response in the context of melanoma, as is the case with systemic CTLA-4 and PD-1 blockade. Less adverse events should be expected with Tim-3 blockade since Tim-3-deficient mice are viable and do not develop autoimmune or lymphoproliferative diseases[119], as opposed to CTLA-4 deficient mice[196].

In conclusion, this study shows for the first time that Tim-3 is an exhaustion marker expressed on NK cells from advanced melanoma patients and that its blockade reverses their exhausted

phenotype. Tim-3, therefore, represents a promising therapeutic target that could enhance antitumor immunity that is dependent not only upon T cells but also the innate immune system.

CHAPTER 5:

THE EFFECT OF IPILIMUMAB ON NK CELLS IDENTIFIES THE SUBSET OF ADVANCED MELANOMA PATIENTS WITH CLINICAL RESPONSE

5.1. Introduction

Metastatic melanoma is the deadliest and most aggressive form of skin cancer[197]. Current treatment strategies including surgery, chemotherapy, oncogenic kinase inhibitors, immunotherapy (ipilimumab), and/or radiation therapy have improved response rates and increased survival, however a broadly applicable curative therapy does not yet exist for this disease. Therefore, a better understanding of the current strategies for the treatment of melanoma is needed, in order to make them safer and more effective.

Ipilimumab is a monoclonal antibody against CTLA-4 that prolongs overall survival in advanced melanoma patients, as was demonstrated in a randomized phase III study[35], leading to its approval by the FDA. More impressive than the mean survival benefit was the effect of ipilimumab on long-term survival (3 years) in a subset of patients (≈20%)[35,68]. However, it has yet to be determined why this select group of patients respond, and what confers resistance[198].

CTLA-4 is an inhibitory receptor initially described in T cells, but that has recently also been shown to be present in monocytes and mature DCs[103]. Intracellular CTLA-4 is detectable only at low levels in naïve human T cells and, upon activation, this receptor is rapidly expressed on the cell surface. Tregs show high amounts of intracellular CTLA-4 and constitutively express this receptor at the cell surface[104]. CTLA-4 plays key role in controlling and dampening $CD8^+$ T cells after they have received co-stimulatory signals. Besides playing a role in $CD8^+$ T effector cells, CTLA-4 also seems to have an important role in the two major subsets of $CD4^+$ T cells, though with distinct effects: downregulation of Th cells and enhancement of Tregs. The expression of CTLA-4 on NK cells is very low, and its role in these cells remains still unknown. Moreover, the effect of ipilimumab on NK cells, and its association with clinical

response to this treatment has not yet been evaluated.

In this study we aimed to evaluate the effect of ipilimumab on the phenotype/function of NK cells from advanced melanoma patients, and to study how the effect of ipilimumab on NK cells from these patients relates to clinical response.

5.2. Material and Methods

Human samples: Blood samples were obtained under the Interdisciplinary Melanoma Cooperative Group IRB approved protocols (#H10362) from 10 advanced melanoma patients (stages III/IV) before and after treatment with ipilimumab (3mg/kg q3w). The study was approved by the Institutional Research Board at NYU in accord with a Federalwide Assurance approved by the Department of Health and Human Services. All patients provided informed written consent at the time of enrollment.

Reagents, cell lines, NK cell purification and stimulation, cell staining and flow cytometry analysis, cytotoxicity, IFN-γ production and proliferation assays were referenced in CHAPTER 2.

Study design: NK cells were purified from the peripheral blood of 10 advanced melanoma patients treated with ipilimumab. Blood samples were collected at baseline, after cycle #2 and after cycle #4 of ipilimumab treatment. NK cells were characterized according to the expression of activating (NKG2D) and inhibitory (KIR3DL1) receptors, function (cytotoxicity and IFN-γ production), levels of IL-2Rα and response to IL-2 stimulation. We then analyzed the effect of ipilimumab on NK cells as it relates to clinical response (**Figure 5.1**).

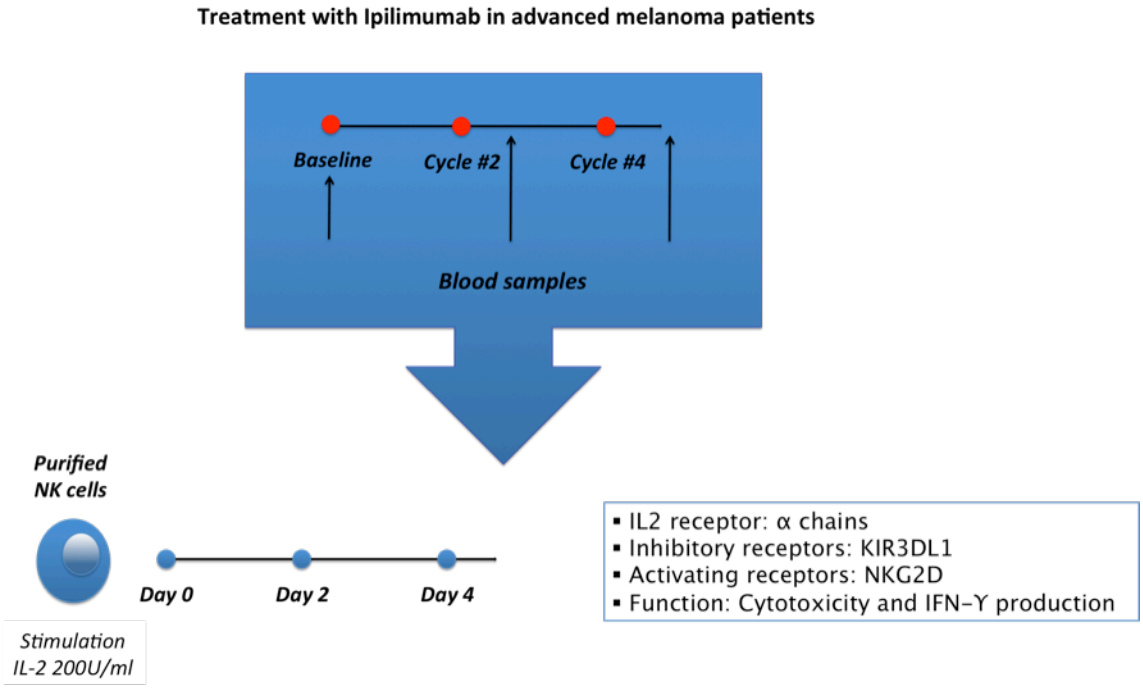


Figure 5.1. Scheme of the study design.

Statistical analyses: Separate analyses were performed for each experiment individually. Two tailed t tests were used. Analyses took into account paired observations within donors when appropriate, or unpaired observations when we compared parameters between responders and non-responders' NK cells.

5.3. Results

The effect of ipilimumab on NK cells was studied in 10 advanced melanoma patients (**Table 5.1**). We compared the phenotypic and functional profile from NK cells from these 10 melanoma patients before treatment with ipilimumab (baseline) and after each cycle (cycles #2 and #4). Interestingly, ipilimumab induces an upregulation of IL-2R α chain in NK cells, with an increase in approximately 50% of the IL-2Rα levels (*p=0.03*) (**Figure 5.2.A and 5.2.B**). There was no significant difference in the expression levels of other receptors or difference in function, including cytotoxicity and IFN-γ production (p>0.05).

10 Advanced Melanoma Patients	*N(%)*
Age (mean)	59
Gender	
Female	4 (40)
Male	6 (60)
Performance Status	
0	9 (90)
1	1 (10)
Metastases	
skin and soft tissue	2 (20)
lymph nodes	1 (10)
Bone	2 (20)
Lung	6 (60)
Liver	2 (20)
Brain	2 (20)
Other visceral	1 (10)
LDH (mean)	446
Clinical response	
CR/PR	4 (40)
NR	6 (60)

Table 5.1. Demographic and clinical characteristics of 10 advanced melanoma patients treated with ipilimumab.

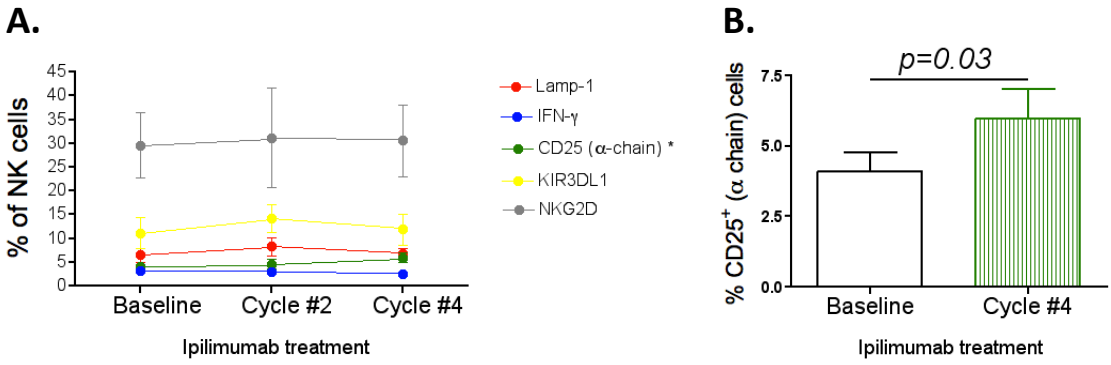


Figure 5.2. Ipilimumab induces an increase of IL-2R expression. (A) Plot showing the percentage of Lamp-1^{+}, IFN-γ^{+}, CD25^{+} (α-chain), KIR3DL1^{+} and NKG2D^{+} NK cells before (baseline) and after treatment with ipilimumab (cycles #2 and #4). The (*) means $p<0.05$. **(B)** Graph showing the difference in percentage of CD25^{+} (α-chain) NK cells between before treatment and after the #4 cycle of ipilimumab. All experiments were performed in duplicate.

We then assessed the phenotype and function of these NK cells after 48 hours of IL-2 stimulation and observed that in 4 out of 10 patients there was an increase of cytotoxicity and

an increase in levels of IL-2Rα. Remarkably, this improved response to IL-2 was observed in patients with clinical response to ipilimumab, with partial (decrease of more than 30% of the tumor volume) or complete (disappearance of all target lesions) response, compared with non-responders (progressive disease, with at least a 20% increase in the sum of diameters of target lesions, taking as reference the smallest sum on study over the treatment). Therefore, partial/complete responders (PR/CR) display higher cytotoxic ability (*p=0.05*) (**Figure 5.3.A**) and higher levels of IL-2Rα (*p=0.03*) (**Figure 5.3.B**).

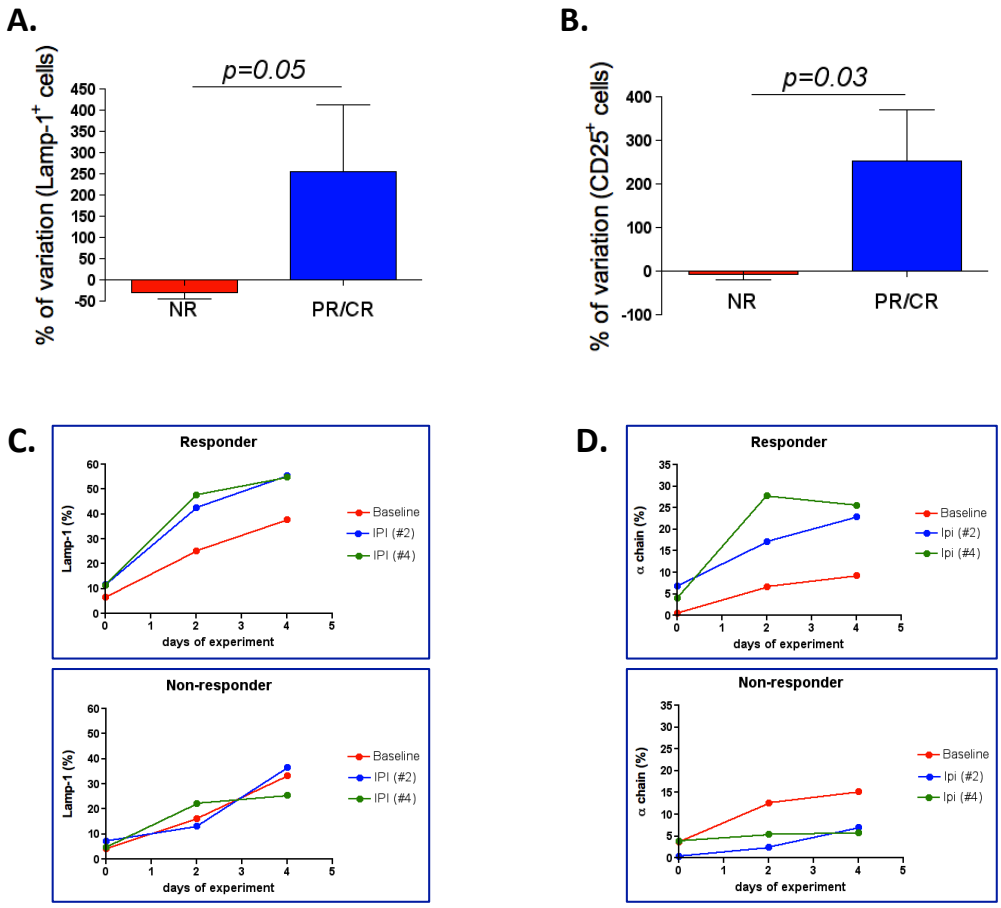


Figure 5.3. Responders to ipilimumab treatment have a better response to IL-2 stimulation. The plots display the difference between responders (PR/CR) and non-responders (NR) in the variation (unstimulated vs 2-days of IL-2 stimulation) of the percentage of lamp-1$^+$ **(A)** and CD25$^+$ **(B)** cells. **(C)** Lamp-1 and **(D)** CD25 expression on unstimulated and stimulated NK cells (from baseline and after #2 or #4 cycles of ipilimumab), from one representative responder (**upper panel**) and non-responder (**lower panel**) to the ipilimumab treatment. All experiments were performed in duplicate.

5.4. Discussion

We have shown that ipilimumab induces an increase of IL-2R expression in NK cells, and consequently a better response to IL-2 stimulation and enhanced cytotoxicity. More important, this effect of ipilimumab on NK cells is associated with a good clinical response.

The mechanism behind this effect is not clear yet; however some hypotheses can be considered. Even though the expression of CTLA-4 on NK cells is considerably low[45,104], this effect could be a consequence of the direct blockade of the 1-3% of CTLA-4 positive cells. Another hypothesis is that CTLA-4 blockade on Tregs would inhibit their suppressive effect on NK cells. Indeed, there is new evidence showing that the mechanism of action of ipilimumab takes place through the inhibition of the immunossupressive effect of Tregs, that constitutively express CTLA-4 in higher levels[199,200]. Finally, patients treated with anti-CTLA-4 antibody blockade generated high-titer anti-MICA antibodies that were associated with enhanced therapeutic efficacy by relieving immunosuppression and stimulating anti-tumor cytotoxicity[201]. Unfortunately it was not possible to test this hypothesis in our cohort, and further investigation is needed.

These intriguing and interesting results need to be confirmed in an independent cohort and more translational studies are needed to clarify the mechanism underlying this effect. Nevertheless, these data support the development of clinical trials to test the treatment combination of anti-CTLA-4 and IL-2 (NCT01856023)[202].

CHAPTER 6:

DISCUSSION

T cell exhaustion has been described in different contexts, including chronic infectious diseases and different types of cancer[92,93]; however, our results provide the first demonstration that NK cells from advanced melanoma patients can also display the four main characteristics that define T cell exhaustion. Our study demonstrates that NK cells in the context of metastatic melanoma upregulate inhibitory receptors, while downregulating activating receptors and IL-2R, are less responsive to IL-2 stimulation, are functionally impaired (reduced cytotoxicity, cytokine production and proliferation) and express reduced levels of the transcription factors Eomes and T-bet. Differently from T cells, NK cells do not upregulate significantly the expression of CTLA-4 or PD-1. Interestingly, even though NK cells express Tim-3 in the steady state, the NK cell exhausted phenotype is characterized by an upregulation of this receptor. Therefore, the levels of Tim-3 in association with functional defects seem to be of key importance in defining Tim-3's role in NK cell function.

The concept of immune cell exhaustion originated from a T cell functional hyporesponsiveness associated with $CD8^+$ T cells in the context of chronic viral infections. However, as previously mentioned, there are different types of functional hyporesponsiveness with distinct mechanisms that need to be discriminated. T cell exhaustion consists of a progressive loss of effector function as a consequence of persistent antigen and inflammation during chronic infection. In the context of self-ignorance, T cells are hyporesponsive due to antigen-inexperience. Central and peripheral tolerance is responsible for the deletion or inactivation of auto-reactive cells. T cell anergy is a result of stimulation without co-stimulatory signals. Finally, senescence corresponds to an irreversible, permanent cell cycle arrest commonly reflected by telomere shortening (Hayflick limit)[203]. Looking more deeply into the process of T cell exhaustion, interestingly, it represents an adaptive state of hyporesponsiveness that, although insufficient to completely clear the pathogen, can provide the host with the ability to control the infection without causing detrimental immunological pathology. Therefore, it does not correspond to a fixed, irreversible, terminal differentiation state, nor an unresponsive T cell state, as has been shown by the development of clinical

responses with the blockade of exhaustion markers or commonly called immune checkpoints. Pre-clinical models[113] and, more recently, clinical trials[204] have shown that these immune checkpoints, including CTLA-4, PD-1, LAG-3, 2B4, and Tim-3 act via nonredundant signaling pathways to establish T cell exhaustion, thus having a synergistic role in this process. Therefore, T cell exhaustion is controlled and 'tuned' by the expression of the immune checkpoint ligands, besides several suppressive factors in the environment, including cytokines such as IL-10 and TGF-β, immune cells including MSDC[205], Tregs[90], and prolonged production of type I IFNs (IFN-α/β) that paradoxically upregulate expression and production of inhibitory molecules. This concept of T cell exhaustion has been translated into the oncology field based on the observation that T cells isolated from human tumors as well as experimental tumor models share many genomic, phenotypic and functional characteristics of exhausted T cells in chronic infections. Therefore, TILs are impaired in the production of effector cytokines, express inhibitory receptors including PD-1, LAG-3, 2B4, Tim-3, and CTLA-4, and display alterations in signaling pathways described for exhausted T cells[92,206,207].

For many years, functionally impaired NK cells have been observed and described in different types of cancer, including breast[97], gastric[208], lung[209] and ovarian[210] cancers. This NK cell dysfunction is characterized by decreased cytotoxic activity, downregulation of expression of activating receptor expression and intracellular signaling molecules, defective proliferation, poor infiltration, decreased cell counts, and defective cytokine production[211]. Nevertheless, several studies have suggested that these functionally impaired NK cells are a consequence of the absence of sufficient activating signals in the TME. Some publications have demonstrated the reversal of this tumor-associated NK cell phenotype after IL-2 stimulation[172,173]. In contrast, our data indicates that even after IL-2 stimulation, NK cells from melanoma patients have a dysfunctional phenotype compared with healthy donors. Therefore, it is not possible to reverse completely the NK cell exhausted phenotype, even through *ex vivo* activation.

As previously shown in a tumor mice model[98], our data suggest that NK cells become progressively exhausted through melanoma stages, making them a potential therapeutic target. Accordingly, NK cell infiltration in tumor tissue is associated with better prognosis[95,175,176], while low activity of peripheral blood NK cells is associated with increased risk of cancer[150]. Different immunotherapeutic NK-cell based strategies have been developed, including cytokines that boost the NK cell response (IL-2, IL-21 or IL-15), adoptive NK cell transfer

and the blockade of inhibitory receptors. Several questions remain regarding the exact mechanism of T cell functional unresponsiveness in the context of cancer. Nevertheless, different hypotheses have been postulated, including: (i) cell-intrinsic self-tolerance programs imprinted in self/tumor antigen-specific T cells[212]; (ii) cell-intrinsic programs induced in tumor-specific T cells that encountered tumor antigen during the early or premalignant, noninflammatory phase of tumor development[213,214]; (iii) cell-intrinsic and extrinsic immunosuppressive factors in the TME including chronic tumor-antigen encounter, expression of inhibitory receptors and availability of their ligands, MSDC, Tregs, cytokines such as IL-10 and TGF-β, and compounds such as nitrate nitrogen (NOS), ROS, nitrogen species, and IDO; and/or (iv) physiological changes within tumors including hypoxia, low nutrient levels, low pH, and/or high interstitial fluid pressure. To our knowledge, the concept of NK cell exhaustion, by analogy with similar findings in $CD8^+$ T cell biology[215], was first described in 2012 by Gill and colleagues, in an adoptive NK cell transfer tumor mice model. The exact mechanism of NK cell exhaustion is still unclear; however, some hypotheses have been postulated. NK cells might become exhausted due to the inhibitory TME, which contains inhibitory cytokines (TGF-β, IL-10 and IDO) and cells (TAMs, Tregs, MSDCs)[142]. Furthermore, sMICA shed from tumor cells also inhibits NK cells' cytotoxicicy[157], and this functional impairment appears to correlate with elevated sMICA sera levels[157]. In this study we have shown that high levels of sMICA in the plasma are associated with shorter recurrence free survival and overall survival. Therefore, MICA may be an independent key independent prognostic factor in melanoma.

Tim-3 has been reported to be expressed on activated $CD4^+$ T cells, $CD8^+$ T cells, monocytes, DCs and NK cells[216], however a high expression of Tim-3 mRNA was observed in human NK cells when compared with other lymphocyte populations[120]. Two studies presented contraditory results regarding Tim-3 function in NK cells from healthy donors; while one showed that Tim-3 inhibits NK cell cytotoxicity[132], the other demonstrated that Tim-3 increases IFN-γ production when stimulated with Gal-9[133]. Contradictory roles have been similarly described for other NK cell receptors such as 2B4[193] and KIR2DL4[194]. The Tim-3 pathway was also described to influence NK cell function in patients with hepatitis B virus infection[134] and atherogenesis[217], in which upregulation of Tim-3 on NK cells correlates with decreased IFN-γ production and cytotoxicity. Moreover, Tim-3 plays a key role in maintaining the process of sepsis, through a negative regulation of the TLR4-mediated

immune responses[128]. In the context of LPS-induced endotoxic shock, Tim-3 acted as a marker of immune exhaustion and Tim-3-positive T cells and NK cells had a lower interferon (IFN)-γ production[218,219]. Our data are consistent with Tim-3 acting as an inhibitory receptor on NK cells from healthy, and most importantly, from melanoma donors. We demonstrate, for the first time, that Tim-3 behaves as an exhaustion marker on NK cells, similarly to its described role in T cells, and it negatively regulates NK cell function in a Gal-9-independent manner.

Interestingly, when we blocked Tim-3 receptor with a soluble antibody we were able to recover, in part, NK cell function from melanoma patients. The Tim-3 blocking antibody binds and internalizes its receptor, decreasing its expression in the membrane of NK cells and the possibility of it binding to natural ligands, including Gal-9, HMGB1, CEACAM1 and phosphatidylserine. Gal-9 is characterized by β-galactoside binding affinity and the presence of an evolutionary conserved sequence, the carbohydrate recognition domain (CRD), that binds to specific carbohydrate moieties on glycoconjugates and is therefore able toto decode and interpret the glycome from the microenvironment[220]. The widespread localization of Gal-9 implies that it exerts a large variety of functional roles, such in, angiogenesis, apoptosis[221] and T cell homeostasis[222]. Gal-9 inhibits T cell function, like proliferation, cytotoxicity and cytokine production, through a Tim-3 interaction[223,224]. Interestingly, galectins are increasingly recognized as prognostic markers for malignancies and galectin-9 is no exception to this[225]. When screening a panel of 84 breast tumors it was observed that, Gal-9 was expressed in half of these tumors and high Gal-9 expression resulted in an increased cumulative disease free survival of these patients[226,227]. In contrast, most current data suggest an inverse relation between Gal-9 expression and cancer progression for the majority of solid tumors. The association of a higher expression of Gal-9 with good prognosis may be explained by the pleiotropic functions of the glycoprotein. It is important to note that most studies did not take into account the extensive Gal-9 splicing. Therefore, it remains to be determined whether the expression of specific splice variants is also different in human tumor tissues[228]. Our study demonstrates that Gal-9 inhibits the immune system by binding to Tim-3 on NK cells, which can contribute to tumor immune evasion.

HMGB1 is a DNA chaperone that maintains nuclear homeostasis as well as an extracellular damage associated molecular pattern molecule (DAMP), that plays a significant role in many diseases, especially inflammatory diseases and cancer[229]. Cancer development is a multistep

process, and HMGB1 seems to contribute to each of these steps of cancer development. HMGB1 has paradoxically been reported to promote both cell survival and cell death by regulating multiple cancer signaling pathways[230]. While extra-cellular HMGB1 acts mainly as a protumor protein due to its cytokine, chemokine, and growth factor activity, intracellular HMGB1 acts as an antitumor protein due to its ability to sustain genome stability and autophagy activity during tumor growth[231,229]. Through the expression of pattern recognition receptors (PRRs), such as TLRs and receptor for advanced glycation endproducts (RAGE), which recognize DAMPs and pathogen-associated molecular patterns (PAMPs) released by stressed cells, DCs play an essential role in initiating innate immune responses against pathogens and tumors. In their recent paper, Chiba and coworkers showed that HMGB1 specifically binds to Tim-3 on DCs' surface and that this HMGB1/Tim-3 complex is then internalized into endosomal vesicles. This protein complex impedes HMGB-1-mediated recruitment of nucleic acids within the endosomes, leading to a decrease of the efficacy of immune responses to nucleic acids. Indeed, Tim-3 competes with nucleic acids to bind to the A box of HMGB1, impeding the HMGB1/DNA association, and thus blocking the activation of innate immune system. Thus, when HMGB1 binds to Tim-3 in tumor-associated DCs, the innate immune responses toward the tumor are blocked resulting in increased tumor expansion[116].

CEACAM1 is a member of the CEA-family of Ig-like transmembrane proteins that is expressed in a wide range of normal tissues and tumors[232]. Early studies have shown that CEACAM1 behaves as a tumor suppressor gene, since its expression is often lost in sporadic colorectal and prostate cancers in humans and, consistent with this, tumor size and number are increased in *Ceacam1*$^{-/-}$ mice exposed to azoxymethane administration[233]. Recent studies in a wide variety of human tumors, including melanoma, lung and colon cancer, have observed that high levels of CEACAM1 expression on tumor cells directly correlate with poor prognosis and tumor metastasis[234,235,236]. In a recent study, Huang and colleagues have shown that CEACAM1 serves as a heterophilic ligand for Tim-3 that is required for its ability to mediate T-cell inhibition, and that this interaction has a crucial role in regulating autoimmunity and anti-tumor immunity. Remarkably, co-blockade of CEACAM1 and Tim-3 leads to enhancement of anti-tumor immune responses with improved elimination of tumors in mouse colorectal cancer models[117].

PtdSer is a negatively charged phospholipid located in the inner leaflet that is exposed on the

surface of cancerous and other pathological cells[237], as well as apoptotic cells. In general, apoptotic cells exposing PtdSer in the outer leaflet of the plasma membrane are specifically recognized by macrophages[238] and DCs[239]. Tim-3 expressing cells can respond to apoptotic cells through TIM-3/PtdSer binding, but the consequence of Tim-3 engagement of PtdSer depends on the polymorphic variants of and type of cell expressing Tim-3. Therefore, tumor cells may circumvent apoptosis and prevent recognition by macrophages through the Tim-3-PtdSer pathway[240].

According to our data, other possible mechanisms of the reversal of NK cell exhaustion are: the blockade of the intrinsic inhibitory pathway of Tim-3 (independently of any ligand); an enhanced ADCC, due to higher CD16 expression; and a better response to IL-2 stimulation, as a consequence of higher IL-2R expression. Tim-3 belongs to the family of immune checkpoints and, like CTLA-4 and PD-1, is a potential therapeutic target. Several *in vitro* and *in vivo* studies have shown that Tim-3 blockade alone or in combination with PD-1 blockade translates into tumor growth control[93,123]. Our data demonstrate that Tim-3 blockade is able to revert, in part, the NK cell exhausted phenotype. This suggests that Tim-3 plays an important role in the process of exhaustion, along with other inhibitory receptors, supporting the combination of different immune checkpoint inhibitors.

NK cells from advanced melanoma patients show an overexpression of Tim-3, displaying low expression of other exhaustion markers, such as PD-1 and CTLA-4. We studied the effect of ipilimumab on NK cells from 10 advanced melanoma patients. Interestingly, we have shown that ipilimumab induces an increase of IL-2R expression in NK cells, and consequently a better response to IL-2 stimulation and enhanced cytotoxicity. More importantly, this effect of ipilimumab on NK cells is associated with a good clinical response. The mechanism behind this effect is not clear; however some hypotheses can be suggested. Even though the expression of CTLA-4 is considerably low in unstimulated peripheral NK cells (1-3% of CTLA-4 positive cells), Stojanovic A *et al* recently demonstrated that both CD28/CTLA-4–expressing NK cells and B7-1/2–expressing myeloid cells are present in the tissue of different tumors, making these NK cells potential therapeutic targets[241]. Another hypothesis is that blockade of CTLA-4 on Tregs that constitutively express this receptor in higher levels, inhibits their suppressive effect on other cells, including $CD8^+$ and NK cells[242]. Finally, it has been demonstrated that patients treated with anti-CTLA-4 antibody blockade generated high-titer anti-MICA antibodies, that are associated with a reduction of circulating sMICA and an

enhancement of NK cell and $CD8^+$ T lymphocyte cytotoxicity, and consequently with a higher therapeutic efficacy[201].

Collectively, our findings show that NK cells in advanced melanoma patients display an exhausted phenotype that develops gradually beginning at stage I and advancing to stage IV. Furthermore, Tim-3 and KIR3DL1 are overexpressed while cytotoxicity and IFN-γ production are impaired in MD NK cells and their levels correlate with the thickness of primary lesion and presence of metastasis. More importantly, Tim-3 behaves as an inhibitory receptor in NK cells from advanced melanoma patients and through its blockade it is possible to reverse this NK cell exhausted phenotype.

CHAPTER 7:

FINAL REMARKS AND FUTURE DIRECTIONS

When I decided to study melanoma, there were only dacarbazine, temozolamide and a few more chemotherapeutic agentes were then available to treat advanced melanoma patients, with really poor clinical responses. At that time, immunotherapy was still a dream followed by some clinicians and researchers, with several past disappointing results.

I joined the Bhardwaj laboratory in 2010 and Ipilimumab was approved by FDA for advanced melanoma the following year. In 2014, anti-PD-1 was approved not only for advanced melanoma, but also for lung cancer, and we all expect it to be approved for other tumor types. There is no doubt that immunotherapy is gaining its space and respect in the oncology field.

The main targets of immunotherapy during these last years have been the DCs, regarding the vaccines, and T cells, through the use of immune checkpoint inhibitors. The aim of our project was to study the role of NK cells, the most relevant anti-tumor cells along with T cells, in the context of melanoma. Our goal was to characterize their phenotype and function through the different melanoma stages and investigate how to manipulate these cells in order to make them fight tumor cells more efficiently. Tim-3 is an immune checkpoint like CTLA-4 and PD-1 on T cells, but with a controversial role in NK cells. Our goal was to check the role of Tim-3 in NK cells from advanced melanoma patients.

This study demonstrated for the first time that NK cells acquire an exhaustion phenotype as melanoma progresses. However the mechanism underlying this process and pathways involved remain unclear. More translational studies are needed to clarify this phenomenon, in order to improve the available immunotherapeutic strategies and define new ones.

Moreover, we have demonstrated that the expression of sMICA in the plasma is an independent prognostic factor and preditor of recurrence in melanoma patients. This finding will more accurately help define the group of patients that would benefit from adjuvant therapy. However, these results will need to be validated in other centres also.

We have found that Tim-3 clearly has an inhibitory function on NK cells from advanced melanoma patients, and its blockade reverses, partially, NK cell exhaustion. These findings may have major implications as Tim-3 becomes a potential therapeutic target. We clarify part of the mechanism of action of anti-Tim-3 mAb; however, a more detailed effect on NK cells and on other cells should be clearly defined.

Finally, anti-CTLA-4 (ipilimumab) induces an upregulation of IL-2R in NK cells, making them more responsive to IL-2 stimulation. Some mechanisms of action of ipilimumab have been postulated, and each of them should be tested in a translational project.

In summary, the results from our research led to the following original conclusions, which can be translated into the clinic:

1. NK cells from melanoma patients acquire an exhausted phenotype with the advancement through stages, making them an appealing therapeutic target. This also suggests that it may be possible to intervene early in the course of tumor progression.

2. sMICA is a potential marker of prognosis and predictor of recurrence in melanoma. sMICA expression in the plasma from melanoma patients offers the great advantage that it can be easily quantified by ELISA.

3. Tim-3 is an exhaustion marker expressed in NK cells from advanced melanoma patients and its blockade reverses their exhausted phenotype. Our *in vitro* studies, together with some preclinical studies suggest that anti-Tim-3 is a good candidate to be included in clinical trials for advanced melanoma patients.

4. Ipilimumab improves NK cell function (increasing IL-2R expression and making these cells more responsive to IL-2 stimulation) in those patients who respond to the treatment. These data support the development of clinical trials with the combination of ipilimumab and IL-2.

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