

UNIVERSITE SORBONNE PARIS CITE

UNIVERSITE PARIS DIDEROT

Ecole Doctorale « Hématologie – Oncogénèse – Biothérapies » (HOB)

UMRS 1160.Alloimmunité – Autoimmunité – Transplantation

DOCTORAT

Hématologie et Oncologie

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Etude des mécanismes immuno-modulateurs de l'interaction anticorps anti-CTLA-4 / molécule CTLA-4 et de ses conséquences dans le renforcement des réponses immunes anti-mélanome

Anti-CTLA-4 antibody / CTLA-4 molecule immuno-modulator mechanisms and its consequences on the reinforcement of anti-melanoma immune responses

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Soutenue le 13 septembre 2016

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RESUME

Le mélanome est un cancer de la peau dont l'incidence est en continuelle augmentation dans le monde entier. Des progrès récents dans la compréhension des mécanismes cellulaires complexes régulant l'immunité du cancer ont conduit à l'élaboration de nouvelles stratégies visant des checkpoints spécifiques de la régulation des réponses immunes. Ipilimumab est un anticorps thérapeutique dirigé contre la molécule CTLA-4. Il a permis, chez les patients atteints de mélanome métastatique, d'augmenter la survie globale et a fait l'objet d'une approbation de la FDA en 2011. Cependant cette thérapie ne semble bénéficier qu'à un nombre restreint de patients et souvent de manière retardée. L'identification de marqueurs immunologiques précoces associés à la réponse clinique et à la survie s'avère donc être nécessaire afin d'apporter des éléments d'orientation. Dans ce contexte, nous avons suivi de manière longitudinale, prospective et retrospective une cohorte de 77 patients atteints de mélanome métastatique. Nous nous sommes intéressés dans un premier temps aux molécules sériques reflétant soit l'importance de l'envahissement tumoral (LDH, S100B et MIA) soit l'implication de mécanismes d'échappement immunitaire (MICA soluble et anticorps anti-MICA). Nous montrons une association entre des faibles concentrations sériques de LDH et S100B, soutenues après première et deuxième dose d'ipilimumab, et la réponse au traitement et la survie. De plus, des niveaux élevés de MICA solubles avant le traitement sont associés à une fréquence plus faible de complications à type d'auto-immunité. Nous avons de plus suivi les populations lymphocytaires avec une attention particulière portée sur les compartiments naïf et mémoires T, les facteurs de transcription impliqués dans la différenciation des lymphocytes T mémoires, les cytokines produites et les récepteurs de chimiokines de lymphocytes T. Nos résultats montrent que le taux de lymphocytes avant l'introduction du

traitement est un facteur prédictif de meilleure survie et de réponse positive à la semaine 16, indépendamment du taux de LDH et de la corticothérapie. De plus, un effet global de l'ipilimumab sur l'expansion des cellules T mémoires classiques a été observé, associé à la réponse au traitement. En revanche, les fréquences des cellules souches T mémoires (T_{SCM}) récemment décrites diminuent malgré une augmentation de la prolifération, suggérant un processus de différenciation. L'ipilimumab induit aussi l'expansion de lymphocytes T exprimant CXCR3, CCR4 et CCR6, permettant une migration vers la peau et les tissus inflammés, avec augmentation de leur capacité à produire des cytokines effectrices. Une augmentation précoce des cellules T CD8⁺ exprimant le facteur de transcription Eomes s'est révélée être associée à un contrôle de la maladie. Enfin, et sur la base des résultats précédents, nous avons étudié la capacité des lymphocytes T mémoires de patients à proliférer après stimulation *in vitro*. Contrairement aux sujets sains, les patients présentent un défaut dans la capacité de prolifération des T_{SCM} qui pourrait être liée à un défaut dans la régulation d'Eomes et Ki-67. Nous proposons ainsi un modèle permettant de suivre de manière précoce les étapes conduisant à la différenciation des T_{SCM} en sous populations mémoires, associées à une réponse clinique sous ipilimumab, et impliquant le facteur de transcription Eomes.

Mots clefs : anti-CTLA-4, ipilimumab, mélanome, cellules souches T mémoires, Eomes

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ABSTRACT

Melanoma is a skin cancer with incidence increasing at dramatic rates worldwide. Ipilimumab, an anti-CTLA-4 therapy developed in the view of counter-balancing the inhibitory role of CTLA-4 in T lymphocytes, was the first immune checkpoint inhibitor demonstrating to extend overall survival in patients with metastatic melanoma, with FDA approval in 2011. However, biomarkers allowing the identification of the subset of patients that will more likely benefit from this immunotherapy or that may allow a good monitoring of patient clinical management during treatment are lacking. The principal objective of this work was to identify potential and early predictive biomarkers of ipilimumab response and/or survival in a cohort of 77 metastatic melanoma patients. Firstly, serum levels of melanoma markers such as LDH, S100B and soluble MICA (and its counter-part anti-MICA antibody), tumour markers associated with tumour development and/or immune escape, were assessed. A correlation between lower baseline levels of LDH and S100B, sustained after the first and second doses of ipilimumab, and treatment response and survival was observed, suggesting their potential utility in treatment monitoring. In addition, higher baseline levels of soluble MICA were found to be associated with a less frequency of immune-related adverse events, which might provide important information for the management of frequent ipilimumab-related adverse events. Secondly, immune markers with a special focus on transcription factors, cytokine secretion and chemokine receptors of T lymphocytes and memory T subsets were assessed. An association between baseline absolute lymphocyte counts and extended overall survival as well as better treatment response was found. In addition, a global effect of ipilimumab on the expansion of conventional memory T cells was observed, which was associated with treatment response. By contrast, frequencies of the recently described

stem-cell memory T cells were shown to decrease despite increased proliferation, suggesting a process of differentiation. Additionally, ipilimumab induced the expansion of CXCR3, CCR4 and CCR6-expressing T lymphocytes and effector cytokines secretion capacity. Early increased levels of Eomes-expressing CD8⁺ T cells were found to be associated with disease control. Lastly, and based on the previous results, we investigated the ability of patients' memory T cells to proliferate under *in vitro* stimulation. We found that, in contrast to healthy subjects, patients possess a defect in the ability of stem-cell memory T cells expansion *in vitro*, that might be related to a defect in Eomes and Ki-67 regulation.

Keywords: anti-CTLA-4, ipilimumab, melanoma, memory stem T cells, Eomes

*To my grandmother Bertinha, who always believed in the power of Education,
to my grandfather Alfredo, who always believed in the power of sense of humour.*

And who strongly believed in me.

ACKNOWLEDGMENTS

It is an honour for me to defend my Ph.D. thesis in the presence of Pr Nathalie Labarrière, Pr Olivier Adotevi, Pr Céleste Lebbé, Pr Eric Tartour and the president Pr Antoine Toubert. I thank them for having taken the time to read, comment and discuss my work. I specially thank Pr Céleste Lebbé for having allowed the collaboration with the Dermatology Service of Saint-Louis Hospital to happen, essential in this work. I also thank Dr Antoine Toubert for having welcomed me in his research unit INSERM U1160 (previously U940). For his support and precious guidance in my researching, as well as for his patience and good mood while directing the team.

I thank *Université Paris-Diderot* for my admission as a Ph.D. student that allowed me, and many students, to engage in a scientific career.

To Dr Hélène Moins-Teisserenc I will not ever be able to thank enough. For having welcomed, encouraged, guided, helped and motivated me during this long journey. For her endless availability, for helping me developing and understanding a complex subject of great interest. For her sense of humour that renders the laboratory a joyful place to work. For her friendship.

I thank Dr Raphael Porcher and Dr Jérôme Lambert for the complex statistical analysis they have performed in this study. It is an honour for me to have their rigor in this work.

I acknowledge Dr Anne Caignard, for having allowed me to pursue my last year under the PAIR melanoma program. For all her guidance and contribution to my work.

I thank all the clinicians of the Dermatology Service of Saint-Louis Hospital that recruited and evaluated the patients included in this cohort, as well as all the technicians and workers involved in the sample collection and preparation.

All the laboratory workers and students, for making me believe that there is not a better environment to work in, for making a pleasure for me to come to work everyday.

Marie-Christine Banus and Farida Gharbi, for their tireless support.

François Marzais and Céline Manier, for our good-mood everyday-lunches.

Laurence Homyrda, Marie Roelens and Alexandra Frazão for their support in my experiments.

Emmanuel for his help in the late stages of my statistical analysis and for his coffee machine.

Fadila, for her willingness in helping me anytime, and for the pleasant times we shared the office.

Nicolas and Juliette, for their timeless support.

Victor, for his availability and kindness.

Meriem Bennabi, for sharing with me the vicissitudes of a thesis, making me feel less lonely.

Meriem Messaoudene, for sharing with me the pleasures of life around a good drink.

Eleonora, Julia, Rita, Louis, Maxime, for having let us create a true friendship.

Joana Guedes and Rita Cruz, for making me believe solid friendships meet no borders. For letting me hope some day we will share the same city again.

Jaime, for being Jaime.

José Oliveira, for making my life in Paris become sweet since the very first day we met.

Clément, for his love. For making me laugh.

My sister, for her support in this last rushing phase of the thesis.

My father, for making me inherit the rational thinking.

My mother, for making me inherit the sensibility.

Both of them, for loving me.

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Abbreviations

AJCC	American Joint Committee on Cancer
ALC	Absolute lymphocyte count
APC	Antigen-presenting cell
ATP	Adenosine triphosphate
<i>BCG</i>	<i>Vaccin bilié de Calmete et Guérin</i>
CAR	Chimeric antigen receptor
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DNAM	Damage-associated molecular pattern
DC	Disease control
ERK	Extracellular signal-regulated kinases
ESMO	European Society for Medical Oncology
Eomes	Eomesodermin
FDA	Food and Drug Administration
FMO	Fluorescence Minus One
GM-CSF	Granulocyte macrophage colony-stimulating factor
HCMV	Human cytomegalovirus
HD	Healthy donor
HLA	Human leucocyte antigen
ICOS	Inducible T cell costimulator
ICOSL	ICOS ligand
IDO	Idoleamine 2,3-dioxygenase
IFN- γ	Interferon- γ
IL	Interleukin
KIR	Killer cell immunoglobulin-like receptors
KO	Knockout
LCMV	Lymphocytic choriomeningitis virus
MAIT	Mucosal-associated invariant T cells
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex

MICA	MHC class I chain-related protein A
MICB	MHC class I chain-related protein B
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinuclotide - hydrogen
NCR	Natural cytotoxicity receptors
NFAT	Nuclear factor of activated T
NK	Natural Killer cell
NKT	Natural Killer cell
ORR	Obective response rate
OS	Overall survival
PBMC	Peripheral blood mononuclear cells
PD	Progressive disease
RPMI	Roswell Park Memorial Institute medium
SLO	Secondary lymphoid organs
TAA	Tumour-associated antigen
T _{CM}	Central memory T cell
TCR	T-cell receptor
T _{EM}	Effector memory T cell
T _{EMRA}	Terminal effector memory T cell
T _{FH}	Follicular helper CD4 T cell
TGF- β	Transforming growth factor- β
T _H	Helper T cell
TIL	Tumour-infiltrating lymphocytes
T _N	Naïve T cell
TNF	Tumour necrosis factor
T _{RM}	Resident memory T cell
T _{SCM}	Stem-cell memory T cell
T _{TM}	Transitional memory T cell
ULBP	Human cytomegalovirus UL16-binding proteins
UV	Ultraviolet

INTRODUCTION

1. Melanoma

1.1. Epidemiology

Melanoma, also called malignant melanoma, is one of the most rapidly spreading cancers in terms of worldwide incidence. It is the 15th most frequent cancer in the world, but it occupies the 7th position in Europe, with more than 100,000 people diagnosed of cutaneous melanoma in Europe in 2012 and over 22,000 melanoma-related deaths (<http://eco.iarc.fr/eucan>). More than in any other cancer type, incidence has dramatically risen in most developed countries over the last 50 years.

Although accounting for only 4 % of all skin cancers, melanoma is responsible for 80 % of skin cancer-related deaths (Miller & Mihm 2006). Cutaneous melanoma is the most common type of melanoma (91 % of melanomas), with rarer forms of melanoma (5.3 %) localizing in the uveal tract, at mucosal sites or with unknown origin (Alegre et al. 2015).

In contrast to other types of tumours, melanoma's incidence is not strongly dependent on age. It is one of the most frequent causes of cancer mortality in ages between 20 and 35 years old (Houghton & Polsky 2002). Lighter skinned patients (i.e. Caucasians) are 5 to 20 fold more affected than darker skinned ones (i.e. African, East Asians, Hispanics). Therefore, melanoma incidence is strongly related to ethnic origin and geographic location, and Europe northern countries are among those with the highest melanoma incidence (Houghton & Polsky 2002; Erdei & Torres 2010).

Although melanoma has a good prognostic survival, with a 5-year survival rate of 92 %, metastatic melanoma has a worse prognosis, with only 14 % of patients surviving for more than 5 years (Miller & Mihm 2006).

1.2. Causes

Melanoma can be originated in any part of the body containing melanocytes and occurs when they begin to grow abnormally. The most important risk factors are family history of melanoma, the presence of multiple or atypical melanocytic *nevi* (moles), and a previous melanoma medical history. Other risk factors include family-inherited traits such as fair skin, freckling and light hair, exposure to ultra-violet (UV) radiation and immunosuppression (Houghton & Polsky 2002).

The main environmental risk factor of melanoma is UV exposure. It can lead to both DNA damaged cells and impairment of defensive response of the skin. Additionally, it can induce growth factor production and the formation of reactive oxygen species that may perturb melanocytes' DNA. However, people working outdoors are less affected by melanoma than indoor workers, which may be explained by the protective status conferred by tanning, that transfers melanocytes-synthesized melanin to keratinocytes, allowing them to absorb and dissipate UV radiation (Miller & Mihm 2006; Bergman & Gruis 2010).

Family history of melanoma accounts for approximately 10 % of melanoma cases (Tsao et al. 2012). Several inherited gene mutations have been identified, 25 to 40 % of them affecting the cyclin-dependent kinase inhibitor 2A gene (*CDKN2A*), also known as multiple tumour suppressor 1 (*MTS-1*) gene, coding for tumour suppressor proteins p16^{INK4A} and p14^{ARF}, which play important roles in cell cycle regulation. Non-familial gene mutations result in most cases in the inactivation of another tumour-suppressor gene, the phosphatase and tensin homologue (*PTEN*). Combination of both *CDKN2A* and *PTEN* mutations in mice leads to melanoma occurrence (Miller & Mihm 2006; Shain & Bastian 2016).

1.2.1. Activation of the mitogenic-activated protein kinase (MAPK) pathway

The mitogenic-activated protein kinase (MAPK) pathway is one of the most important signalling pathways in cancer. At the molecular level, it involves a family of protein

kinases, which transduces extracellular stimuli (such as growth factors, hormones, cytokines and environmental stresses) into intracellular phosphorylation events and protein interactions that ultimately lead to cell proliferation, survival, differentiation, mobility and angiogenesis (Akinleye et al. 2013). Abnormal activation of the MAPK pathway, also known as extracellular-related kinase (ERK) pathway, is frequent in melanoma and stimulates melanoma cell growth. *N-RAS* mutations account for 15 % of the MAPK pathway deregulation, whereas mutations in *B-RAF*, a serine/threonine kinase that transduces regulatory signals from RAS through MEK through MAPK, occur in approximately 50 % of melanomas. The *B-RAF* gene mutation V600E represents 80 % of *B-RAF* mutations, causing an increase in melanoma cell proliferation, differentiation and resistance to apoptosis (Davies et al. 2002).

1.3. Development of metastatic melanoma

According to Clark's model of melanoma progression, five steps lead healthy melanocytes to develop into metastatic melanoma: (1) normal melanocytes proliferate and develop into a benign nevus with a regular dot-like pattern; (2) the nevus acquires lentiginous melanocytic hyperplasia, i.e., the atypical melanocytes begin to aberrantly proliferate, becoming asymmetric with irregular borders and colour; (3) these primary melanoma cells develop radial growth, i.e., begin to proliferate intraepidermally, and subsequently (4) acquire vertical growth, i.e., acquire the ability to invade the dermis and form an expansible nodule; (5) lastly, the tumour cells spread to other areas of skin and organs, establishing a metastatic melanoma (Clark et al. 1984; Miller & Mihm 2006).

1.3.1. Staging and prognosis

The American Joint Committee on Cancer (AJCC) melanoma TNM classification is the most widely accepted staging system for cutaneous melanoma. AJCC TNM classification is based on the thickness (defined using the Breslow scale), ulceration and mitotic rate of the primary tumour (T), the presence of metastases in regional lymph nodes (N), and the existence of distant metastasis (M) (see Table 1) (Balch et al. 2009).

In stages I and II, tumour cells are localised within the primary lesion, with no evidence of spreading to the lymph nodes or other sites. According to this classification, **stage I** melanoma includes primary tumours with less than 1 mm thickness or with a thickness

between 1.01 and 2 mm without ulceration or with a mitotic rate lower than $1/\text{mm}^2$. **Stage II** comprises melanomas with a thickness between 1.01 and 2 mm with ulceration and/or with mitotic rate equal or higher than $1/\text{mm}^2$, as well as tumours with a thickness ranging from 2.01 to 4 mm, or higher than 4 mm in case of absence of ulceration with a mitotic rate lower than $1/\text{mm}^2$. Absence of metastasis represents the major characteristic of both stages I and II. **Stage III** melanoma comprises any tumour thickness and any metastatic lymph nodes, but is characterized by the absence of distant metastasis, whereas **stage IV** includes distant metastasis. Stage IV M1a involves distant skin, subcutaneous or nodal metastases, while M1b involves lung metastasis and M1c all other visceral metastasis or any distant metastasis with concomitant elevated LDH serum levels (Balch et al. 2009).

Patients diagnosed with stage I and II melanoma have an excellent prognosis albeit a non-negligible risk of relapse (Harries et al. 2016), with 10-year survival rates ranging from 93 % (in stage IA) to 39 % (in stage IIC). Prognosis is poorer in stage III melanoma, with 5-year survival rates ranging from 78 % in stage IIIA to 40 % in IIIC. Stage IV melanoma has the poorest prognosis, with one-year survival rates of 62 % for M1a, 53 % for M1b and 33 % for M1c melanomas (Balch et al. 2009).

Table 1 – Melanoma TNM (Primary Tumour, Regional Lymph Nodes, Distant Metastasis) classification according to the American Joint Committee on Cancer (7th edition) (Balch et al. 2009).

Primary Tumour (T classification)		
T	Thickness (mm)	Ulceration status/Mitoses
T1	≤ 1.0	a: without ulceration and mitosis < 1/mm ² b: with ulceration and/or mitoses ≥ 1/mm ²
T2	1.01 – 2.0	a: without ulceration b: with ulceration
T3	2.01 – 4.0	a: without ulceration b: with ulceration
T4	> 4.0	a: without ulceration b: with ulceration

Regional Lymph Nodes (N classification)		
N	No. of metastatic nodes	Nodal metastatic mass
N1	1	a: micrometastasis b: macrometastasis
N2	2 – 3	a: micrometastasis b: macrometastasis c: in transit* ¹ or satellite metastasis without metastatic nodes
N3	≥ 4, or matted nodes, or in transit* ¹ or satellite* ² metastasis with metastatic nodes	

Distant Metastasis (M classification)		
M	Metastatic site	Serum LDH* ³
M1a	Distant skin, subcutaneous or nodal	Normal
M1b	Lung	Normal
M1c	All other visceral metastases Any distant metastasis	Normal Elevated

Anatomic Stage/Prognostic Groups				
State		T	N	M
0	0	<i>In situ</i>	N0	M0
I	IA	T1a		
	IB	T1b		
		T2a		
II	IIA	T2b		
		T3a		
	IIB	T3b		
		T4a		
	IIC	T4b		
	III	IIIA		
IIIB		T1-4b	N2a	
			N1a	
		T1-4a	N2a	
			N1b	
			N2b	
			N2c	
			IIIC	T1-4b
		N2b		
		N2c		
Any T		N3		
IV	IV	Any T	Any N	M1

*¹ In transit metastasis: any skin or subcutaneous metastasis that are more than 2 cm from the primary lesion but are not beyond the regional nodal basin. *² Satellite metastasis: skin or subcutaneous lesions within 2 cm of the primary tumour that are considered intralymphatic extensions of the primary mass. *³ LDH: lactate dehydrogenase.

*¹ In transit metastasis: any skin or subcutaneous metastasis that are more than 2 cm from the primary lesion but are not beyond the regional nodal basin. *² Satellite metastasis: skin or subcutaneous lesions within 2 cm of the primary tumour that are considered intralymphatic extensions of the primary mass. *³ LDH: lactate dehydrogenase.

1.4. Biological markers

Biological markers constitute a field of intensive research in the hope of identifying powerful tools for diagnosis, staging, prognosis and monitoring of the disease development. Ideally, a tumour marker should be easily assessable and have high sensitivity and specificity. Circulating biomarkers are of potential utility, as melanoma cells release numerous compounds either by active secretion or following cell death, including proteins, nucleic acids, metabolites and microvesicles (Alegre et al. 2015). Plus, the

concentration of these biomarkers potentially changes during the course of the disease, making them ideal candidates for tumour monitoring.

Serum proteins are useful tools for melanoma monitoring rather than for diagnosis, mostly due to their limited sensitivity at early stages (Alegre et al. 2015).

1.4.1. LDH

Lactate dehydrogenase (LDH) is an ubiquitous enzyme with subunits of two possible types, either the M (muscle type) or the H (heart-type), whose levels increase with tissue injury, necrosis, hypoxia, haemolysis and myocardial infarction (Miao et al. 2013).

In order to provide the necessary energy for rapidly proliferating tumour cells, most cancer cells switch from oxidative phosphorylation process to an increased rate of glycolysis even under normal oxygen concentrations. Under anaerobic conditions, the lactate dehydrogenase (LDH) enzyme catalyses the reversible transformation of pyruvate to lactate, rendering tumour cells less dependent on oxygen supply (Miao et al. 2013). LDH activity is therefore elevated in hypoxia, a recurrent environment in tumour development. As LDH is not a secreted enzyme, elevated serum levels of LDH are thought to be a result of LDH spillage when melanoma cells outgrow their blood supply (Palmer et al. 2011).

LDH elevated levels in melanoma patients' sera were first described in 1954 (Hill & Levi 1954), having been later associated to the occurrence of metastasis in malignant melanoma, with a high sensitivity for liver metastasis (Einhorn et al. 1974; Garg 1979; Finck et al. 1983). On the basis of LDH association with tumour burden and turnover, as well as with poorer survival in metastatic melanoma (Franzke et al. 1998; Sirott et al. 1993), independently of site and number of metastasis, LDH was soon reported as an independent prognostic factor in patients with stage IV melanoma (Deichmann et al. 1999b). Later studies confirmed the prognostic value of LDH in metastatic melanoma (Bedikian et al. 2008), and LDH became the only tumour marker accepted as a standard prognostic factor of survival by the AJCC in the 2009 revised version (Balch et al. 2009), allowing the discrimination of patients with high levels of this enzyme as stage IVc. However, and even if elevated serum levels of this protein occasionally reveal metastatic relapse, the sensitivity and specificity of LDH is limited (Kluger et al. 2011).

1.4.2. S100B

S100 is a calcium-binding acidic protein family of 20 small molecules, thought to be calcium-sensor proteins modulating biological activity via calcium-binding. They form homo- or heterodimers in the cytoplasm and nucleus and participate in several cellular functions including modulation of cell proliferation, differentiation, motility and apoptosis (Palmer et al. 2011). Within this family, S100-beta subunit (S100B) is a 21 kDa protein predominantly expressed by astrocytes, oligodendrocytes and Schwann cells and, outside the central nervous system, in chondrocytes and melanocytes (Alegre et al. 2015).

S100B was first described in human metastatic melanoma-derived cell lines (Gaynor et al. 1980). It down-regulates the tumour suppressor p53 in melanoma, which is implicated in the down-regulation of anti-apoptotic genes such as Bcl-2 and Bcl-X_L (Lin et al. 2010). However, being also excreted by melanoma cells apparently after cell death, the potential of S100B as a circulating biomarker justifies in-depth research. Due to its differential expression in melanocytes *versus* in melanoma cells, S100B is currently used in immunohistochemical detection of melanoma micrometastasis (Balch et al. 2009).

S100B serum levels increase with melanoma stage progression, with higher levels at stage IIIC and IV (Bonfrer et al. 1998). However, it does not seem to be useful to monitor therapy in melanoma stages I to III (Alegre et al. 2015). Several studies demonstrated a good sensitivity and relevance of S100B serum levels as predictive markers for tumour outcome and survival in advanced metastatic melanoma patients (Axel Hauschild et al. 1999; Egberts et al. 2008; Weide et al. 2013). S100B appears to be a reliable diagnostic marker for newly occurred melanoma metastasis (Krahn et al. 2001), but not in sentinel node status prediction (Egberts et al. 2010). However, its superiority to LDH as an independent prognostic marker is still a subject of inconsistent results (Díaz-Lagares et al. 2011; Weide et al. 2013; Deichmann et al. 1999a).

1.4.3. MIA

Melanoma-inhibitory activity (MIA) is an 11 kDa protein that was first identified in supernatants of melanoma cell lines as an autocrine-secreted tumour cell growth-inhibitor (Bogdahn et al. 1989). MIA has an expression pattern highly restricted to melanocytic tumours, although higher serum levels of MIA have been observed in other malignancies such as gastrointestinal cancer. MIA was initially observed to cause alterations in cell

morphology and to induce detachment of melanoma cell lines from tissue culture plastic, leading to precipitated conclusions of its role as a tumour suppressor factor (Bosserhoff 2005), which misleads its name. Subsequent work developed by Bosserhoff and colleagues demonstrated that MIA secretion by melanoma cell lines to the extracellular compartment allows malignant melanocytes to detach from the extracellular matrix components and correlates with increased ability to metastasize (Bosserhoff et al. 2003). MIA was soon found to be associated with disease stage, with higher serum levels of MIA progressively correlating with increased stages of melanoma (Bosserhoff et al. 1997a; Stahlecker et al. 2000; Meral et al. 2001), underlining its potential in disease monitoring.

1.5. Available treatment for melanoma

1.5.1. Primary melanoma and nodal involvement

Stages I to III melanomas are commonly treated by surgical excision, followed by sentinel lymph node dissection, radical lymph node dissection and isolated limb perfusion in case of nodal involvement and in-transit metastases (Harries et al. 2016; Moreno Nogueira et al. 2013; Agarwala & O'Day 2011).

Interferon- α 2b

Surgical excision and sentinel lymph node dissection is often followed by adjuvant therapy with high doses of interferon- α 2b (IFN α 2b) for selected patients with stages II and III melanoma. IFN α 2b was approved by FDA in 1996 as an adjuvant therapy for resected high-risk melanoma. Although having demonstrated an ORR of 22 % in a phase II clinical trial involving 96 advanced melanoma patients (Creagan et al. 1986), responses were limited to patients with low-volume disease, limiting the use of this immunotherapy in stage IV melanoma. Later, pegylated interferon was tested in a randomized phase III trial in patients with completely resected stage III melanoma, demonstrating improved recurrence-free survival and resulting FDA-approval in 2011 as an adjuvant therapy for patients with melanoma involving lymph nodes (Coit et al. 2016).

1.5.2. Advanced metastatic melanoma

Cytotoxic chemotherapy

Advanced metastatic melanoma has conventionally been treated with chemotherapy including cytotoxic agents such as dacarbazine, approved by United States Food and Drug Administration (FDA) in 1975. Dacarbazine reported objective response rates (ORR) of 15 %, with 2 % of patients surviving for 6 years (Bhatia et al. 2009). Other cytotoxic agents include temozolomide, with overall survival (OS) and ORR that are not statistically different from dacarbazine, and fotemustine. Fotemustine potentially stimulates activity in patients with brain metastases and appears to induce a trend towards improved OS and ORR when compared to dacarbazine (Bhatia et al. 2009; Harries et al. 2016). Although probably inducing a palliative benefit in a subset of patients with metastatic melanoma, cytotoxic chemotherapy remains disappointing, since it does not lead to durable responses and improved median survival. Combined chemotherapy response rates are higher than agents used alone, but at the cost of increased toxicity and with no significant improved survival (Bhatia et al. 2009).

High-dose of interleukin-2

Interleukin-2 (IL-2) cytokine is known to stimulate T and NK lymphocytes proliferation and function, inducing interferon- γ (IFN- γ) and tumour necrosis factor (TNF) production (Bhatia et al. 2009). High-dose of IL-2 therapy was approved by FDA in 1998 for the treatment of metastatic melanoma on the basis of durable complete responses observed in a small subset of patients in eight phase I and II trials. These trials involved 270 patients with metastatic melanoma showed ORR of 16 % including 10 % of partial responses and 6 % of complete responses without evidence of progression for more than 30 months (Atkins et al. 1999), and provided the first evidence that an immune-based treatment could provide durable control of the disease. However, toxicities resulting from this immunotherapy require a selection of patients who are more likely to tolerate it.

Recent advances in immunotherapies for melanoma will be discussed in Chapter 5.

Biochemotherapy

Biochemotherapy combines chemotherapy drugs with the biological agents IL-2 and/or IFN α 2b. Shown to improve median recurrence-free survival when compared to high-dose of IFN α 2b in a phase III randomized trial with fully resected stage III melanoma patients, biochemotherapy is an appropriate option for patients with completely resected high-risk stage III melanoma (Coit et al. 2016).

Targeted therapies

Targeting mutated *BRAF* has led to an exceptional expansion of melanoma treatment options. Two antagonists of mutated *BRAF* were developed: vemurafenib and dabrafenib.

In a phase III clinical trial including 675 treatment-naïve metastatic melanoma patients bearing the BRAF^{V600E} mutation, vemurafenib demonstrated a striking response rate of 48 % in contrast to 5 % in the dacarbazine group (Chapman et al. 2011). It received FDA approval in August 2011 for late-stage or unresectable melanoma patients with the BRAF^{V600E} mutation.

Dabrafenib received FDA approval in May 2013 for unresectable metastatic melanoma patients bearing the BRAF^{V600E} or the BRAF^{V600K} mutations based on a phase III study where it significantly improved progression-free survival (PFS) as compared to dacarbazine (Hauschild et al. 2012). Although experiencing initial profound tumour regression, most patients responding to BRAF inhibitors have evidence of tumour progression within 6 to 8 months after the beginning of therapy. BRAF-mutated patients commonly develop resistance to treatment through activation of alternative pathways or reactivation of MAPK signalling through alternative means (Sullivan & Flaherty 2013).

As activated BRAF phosphorylates MEK, another member of the MAPK signalling pathway, MEK inhibitors were developed including trametinib and cobimetinib, and combined therapies emerged (Ribero et al. 2016). Trametinib demonstrated to improve overall survival compared with chemotherapy in a phase III study (Flaherty et al. 2012), leading to FDA approval alone in 2013 and having been approved in 2014 in combination with dabrafenib. Cobimetinib was approved by FDA in November 2015 for the treatment of unresectable or metastatic melanoma patients with the BRAF^{V600E} or the BRAF^{V600K} mutation in combination with vemurafenib, after demonstrating superiority to vemurafenib alone (Larkin et al. 2014).

2. Melanoma: an immunogenic cancer

In the 1960's, the demonstration that mice could be immunized against syngeneic tumour transplants led to the establishment of the concept of 'tumour-specific antigens' that could be recognised by the immune system (Dunn et al. 2002). This was corroborated by works by Boon and colleagues in the 70's describing *in vitro*-generated mutagenic tumours that failed to form progressive tumours in syngeneic mice (Boon & Kellermann 1977), and later demonstrating that spontaneous mouse tumours do express tumour antigens, although poorly immunogenic ones. The observation that the adoptive transfer of T cells originated from mice that rejected tumour variants protected irradiated mice against the same variant definitely shed light into the role of T lymphocytes in specific-tumour rejection (Coulie et al. 2014).

2.1. Tumour-infiltrating lymphocytes

In the 1980's, studies from tumour-infiltrating lymphocytes (TILs) derived from patients with cancer of various histological origins demonstrated that cell-mediated immune reactions against tumours exist in humans (Rosenberg et al. 1994). In the late 1980's, it was demonstrated that antigens recognized by T cells are small peptides bound to major histocompatibility complex (MHC) molecules and that peptides originating from mutated or aberrant expressed proteins mark cells for cytotoxic T lymphocytes (CTL) recognition (Vigneron 2015; Coulie et al. 2014).

Rosenberg and colleagues pioneered the use of TILs to treat metastatic cancer in murine models to which immune cells with antitumour reactivity were adoptively transferred (Rosenberg et al. 1986). This was followed by a study showing an ORR of 55 % in metastatic melanoma patients treated with *in vitro*-expanded lymphocytes extracted from resected melanomas (Rosenberg et al. 1988). A subsequent study including 86 patients with metastatic melanoma treated with autologous TILs plus high-dose of IL-2 resulted in 34 % of ORR (Rosenberg et al. 1994), reinforcing the potential of lymphocytes in cancer treatment. Moreover, development of vitiligo was associated with clinical response (Rosenberg & White 1996). Vitiligo is a disorder characterized by hypopigmented skin lesions with a predominantly autoimmune aetiology. The onset of autoimmune melanocyte destruction that accompanied tumour regression is considered as a hallmark of efficient

effector cells that also target melanocyte differentiation antigen (Garg et al. 2015). The relative ease in which it is possible to expand TILs from melanoma led to the identification of melanoma-associated antigens.

2.2. Melanoma antigens

Using reverse genetics, an approach made possible by recombinant DNA technology, cDNA libraries derived from patient's melanoma were used to screen the specificity of autologous anti-melanoma CTL. The first human gene coding for a tumour-specific antigen recognised by T lymphocytes was identified in 1991 and named melanoma antigen family A, 1 (MAGE A1) (Bruggen et al. 1991). Using the same approach, several other cancer antigens were subsequently identified in different tumours. Cancers with a high mutation rate such as lung carcinoma and melanoma are more susceptible to bear mutated antigens and are therefore more immunogenic (Vigneron 2015).

Melanoma identified antigens include high tumour specificity antigens, and low tumour specificity antigens.

Antigens with high tumour specificity are derived from point mutations or from cancer-germline genes, or even from viral proteins.

Point mutations arising in ubiquitously expressed genes account for about one half of currently defined tumour-specific antigens in melanoma. They result in melanoma antigens either by generating new epitopes presented by MHC molecules, or by allowing the peptides to bind to MHC molecules.

Melanoma point mutations include mutations in the already mentioned inherited *CDKN2A* gene, as well as mutations in the neuroblastoma RAS (*N-RAS*) gene and in the *B-RAF* gene. *B-RAF* gene mutations are found in approximately 50 % of melanomas. Point-mutated antigens may also result from sporadic mutations that are present on very few tumours (Boon et al. 2006).

Cancer-germline genes are expressed in various types of cancer but not in normal tissues except in germline and trophoblastic cells. Their tumour-specific expression pattern results from the demethylation of their promoter sequence, which occurs in germ cells and in some advanced cancers (Vigneron 2015). In melanoma, cancer-germline genes include the *MAGE* gene family, composed of 25 genes clustered in three subfamilies (*MAGE-A*, *-B* and

-C), as well as other families such as the *BAGE*, the *GAGE*, *LAGE/NY-ESO-1* and *SSX* families (Boon et al. 2006). Originally identified in oesophageal cancer, *NY-ESO-1* is expressed in one third of melanoma, lung, liver, gastric, ovarian and bladder cancers. The function of cancer germ-line genes is not clear. However, *MAGE-1* and *GAGE* have been found to be anti-apoptotic, conferring resistance to TNF- α and Fas, respectively (Gnjatic et al. 2006).

Antigens with low tumour specificity include differentiation antigens, commonly expressed by the tumour and by the corresponding healthy tissue, and antigens derived from genes that are overexpressed in tumours and expressed at low levels in healthy tissues.

Differentiation antigens in melanoma are encoded by *gp100/pmel17*, *Melan-MART-1*, *gp75/TRP1* or *TRP2*, with their respective proteins being often involved in both melanin biosynthesis and melanosome biogenesis. Spontaneous T cell responses directed against these proteins-derived peptides are frequent in melanoma patients and in healthy donors, which can lead to vitiligo (Vigneron 2015; Boon et al. 2006).

Overexpressed antigens in melanoma are encoded by genes such as *PRAME* (Vigneron 2015; Boon et al. 2006). Since overexpressed genes are shared by numerous cancers or different histological origins, they represent interesting targets for immunotherapy development, with however a non-negligible associated-risk owed to the fact that these genes are still expressed, even though at low levels, by healthy tissues.

3. Cancer immunosurveillance and immune-editing

Spontaneous regression of tumours have been described as early as 1742 (Thomas & Badini 2011). Melanoma regression is six times more frequent than other malignant neoplasms (High et al. 2005). The concept of ‘immunosurveillance’ was originally embodied in 1957 by Brunet and Thomas, who speculated that lymphocytes acted as sentinels in the continuous recognition and elimination of nascent transformed cells (Dunn et al. 2002).

Further support of the existence of immunosurveillance was provided in the beginning of the 1990’s, showing that mice lacking NK, T and B cells developed sarcomas more frequently and rapidly, and suggesting a crucial role for lymphocytes and IFN- γ in this process. Additional studies shed light into the importance of TCR-expressing lymphocytes

in immunosurveillance and corroborated the idea that the immune system is able to recognise and eliminate primary tumours and that lymphocytes and cytokines are major actors in this process. This is illustrated at the clinical level by the increased incidence of *de novo* malignant tumours after organ transplantation, that is, in immune-compromised individuals. Similarly, there is the accumulating evidence of a positive correlation between the presence of TILs in a tumour and increased patient survival (Dunn et al. 2002).

3.1. Cancer immunoediting

Immunocompetent individuals can still develop cancer. This fact, associated to studies in the 1980's demonstrating the generation of tumour variants with reduced immunogenicity after the repassage of transplantable tumours through immunocompetent hosts, led to the formalisation of the cancer immune-editing hypothesis. This is based on the principle that the immune system can actually prevent tumour formation and, at the same time, promote tumour variants with reduced immunogenicity (Dunn et al. 2002). According to this model, the immune system exerts a selective pressure towards arising tumours, where less immunogenic tumours are more susceptible to survive and thus to escape immunologic detection and elimination. Supporting this idea were the observations that tumours derived from immunocompetent hosts were less immunogenic than tumours arising in the absence of an intact immune system (Dunn et al. 2002), suggesting that tumours are imprinted by the immunologic environment in which they form.

The three 'Es'

The cancer immunoediting model proposed by Dunn and colleagues in 2002 is composed of three processes, named the three 'Es' of cancer immunoediting: elimination, equilibrium, and escape. The elimination process is comparable to the immunosurveillance concept where, as a consequence of the induction of inflammatory signals following minor tissue disruptions resulting from tumour growth, lymphocytes from the innate immune system (NK, NKT and $\gamma\delta$ -T cells) are recruited. These subsets recognize transformed cells and produce IFN- γ . Secreted cytokines induce limited tumour cell death and promote chemokine production preventing tumour vascularisation and further promoting NK, dendritic cells and macrophage recruitment. The action of these cells results in tumour cell death and subsequent dendritic cell presentation of tumour-cell

debris to T lymphocytes in the lymph nodes. Antigen presentation induces tumour-specific type 1 T helper cells (T_H1) cells that facilitate tumour-specific $CD8^+$ T cells development, followed by homing of specific T lymphocytes into the tumour site along with a chemokine gradient, where they destroy the remaining antigen-expressing cancer cells. If the immune system succeeds in complete elimination of the tumour, the process is complete and does not progress to the subsequent phases. During the equilibrium phase, tumour cell variants that survived immune attack as well as host immune cells enter a dynamic equilibrium. Rapidly mutant tumour cells are progressively generated through a Darwinian selective pressure by the immune system, and begin to expand in an uncontrolled manner which leads to escape (Dunn et al. 2002)(Figure 1).

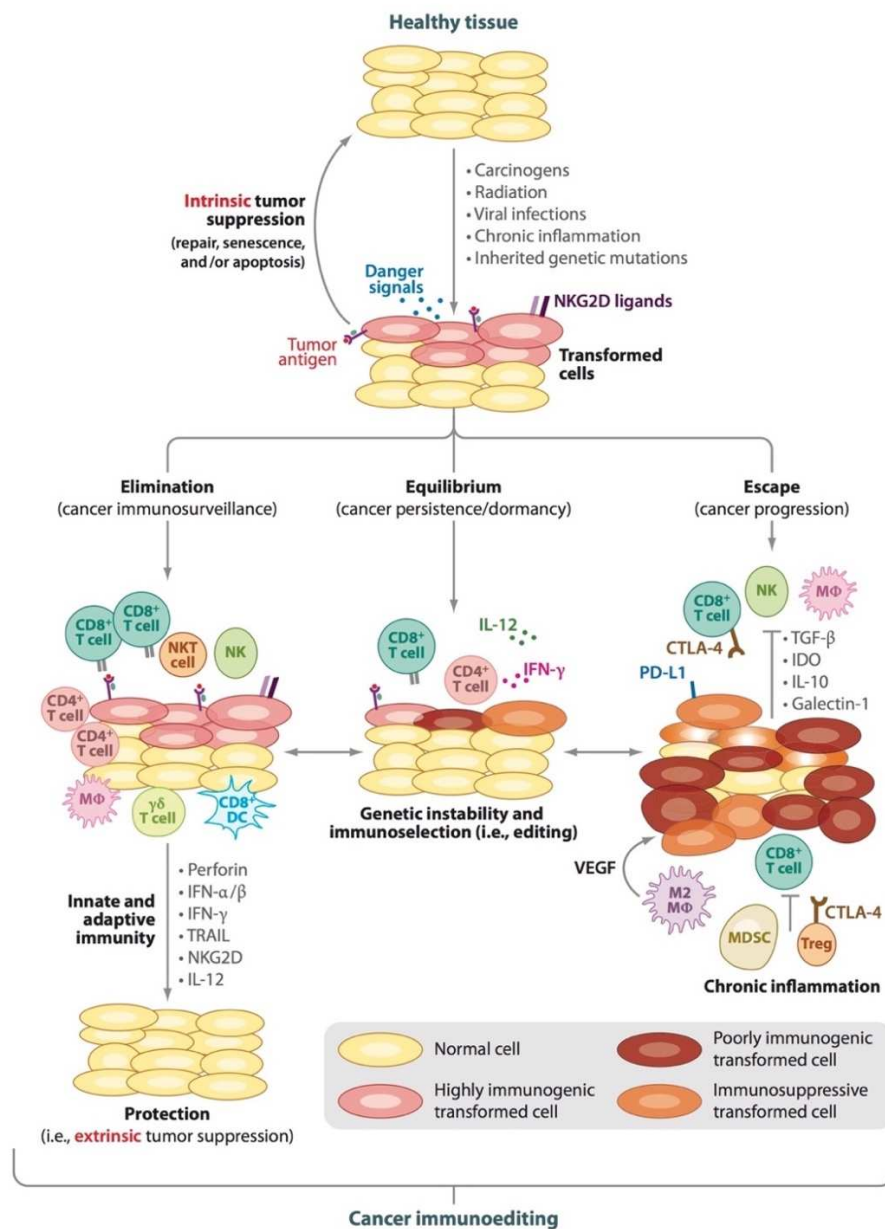


Figure 1 – The three Es of cancer immunoediting: elimination, equilibrium and escape.

Source: Vesely et al., *Annu. Rev. Immunol.* 2011. 29:238

4. Immune actors against cancer

Spontaneous regression of human melanoma has been associated with infiltrating CD4⁺ T cells, with the presence large numbers of Langerhans cells, macrophages and other MHC class- II-expressing cells (Halliday et al. 1995). Further attempt to characterize the TILs within the tumour environment led to the identification of lymphocytes with a CD3⁺, CD4⁺, CD8⁺ and TCR- $\alpha\beta$ phenotype (Mackensen et al. 1993). Moreover, transcriptional analysis of melanoma metastasis revealed an unique immunological signature characterized by the expression of genes associated with NK and CD8⁺ T cell activation (Wang et al. 2008).

4.1. Innate immunity

Innate immunity constitutes the first immunological, non-specific (i.e., antigen-independent) line of defence against a pathogen, which is triggered within minutes or hours after the aggression (Warrington et al. 2011).

The major effector cells of the innate immune system include NK cells, dendritic cells, macrophages, polymorphonuclear leukocytes (including neutrophils, eosinophils and basophils) and mast cells (Liu & Zeng 2012).

Innate lymphocyte lineages were originally believed to be composed by a single lineage – the NK cells –, and are now collectively referred to as innate lymphoid cells (ILCs). ILCs can be divided in helper-like ILCs (including ILC1s, ILC2s and ILC3s), that express IL-7R α and require GATA-3 for differentiation, and killer ILCs, represented by NK cells, that are GATA-3 independent and do not express IL-7R α (Diefenbach et al. 2014). Besides NK cells, the role of other ILCs in cancer has been little studied, but recent studies suggest an implication of these cells in cancer. In colon, NCR ILC3s contribute, via IL-22 and IL-17 production, to the inflammation responsible for sustaining colorectal cancer. The frequency of ILC2 has also been shown to be elevated in gastric cancer patients. By contrast, NRC⁺ILC3 were shown to contribute to melanoma cells' elimination in B16 mice (Vallentin et al. 2015). However, the role of ILCs in human still remains elusive.

4.1.1. Natural killer cells

NK cells are critical players during tumour growth control in immunocompetent hosts. These cells were originally defined by their capacity to kill tumour cells without prior sensitization (Herberman et al. 1975). They are crucial components of innate immunity by virtue of their rapid cytokine secretion capacity. In addition, their role in modulating adaptive immune responses has been increasingly documented (Fehniger et al. 2003). NK cells originate from the common lymphoid progenitor in the bone marrow and reside in spleen, peripheral blood, as well as in lymph nodes, uterus, thymus, gut and liver, and can migrate to secondary lymphoid organs (SLO) to contribute to the first line of defence against pathogens (Moretta et al. 2014). When activated, NK cells release cytokines that amplify the inflammatory responses and contribute to growth and activation of granulocytes and monocytes. In SLO, they provide an early source of IFN- γ that might directly interact with naïve T lymphocytes and with dendritic cells to indirectly promote T_H1 responses. Besides IFN- γ , NK cells produce other cytokines such as the proinflammatory TNF- α , the immunosuppressive IL-10, and growth factors such as the granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-3, as well as several chemokines (Vivier et al. 2011).

Each NK cell expresses several inhibitory and activator receptors. The balance between the resulting signals following their engagement determines the biological response of an NK cell.

In normal conditions, the engagement of inhibitory receptors by their cognate ligands – mostly classical and non-classical MHC class I molecules – keeps NK cells inhibited. This phenomenon is referred to as the ‘missing-self’ hypothesis (Ljunggren & Karre 1990).

Inhibitory receptors that recognise polymorphic epitopes of MHC molecules include the killer-cell immunoglobulin-like receptor (KIR) family and the CD94/NKG2A heterodimer surface molecule. Other NK-cell inhibitory receptors that have non-MHC-molecule ligands were identified, which might explain why self-tolerance is maintained in individuals with abnormally low levels of MHC class I. These include receptors such as CD244, CD66a and CD161b (Kumar & McNerney 2005).

The discovery of activating receptors on NK cells capable of recognizing various self-encoded ligands that are expressed when a cell is under stress started to change the paradigm. This led to the description of a phenomenon in which activating receptors may

trigger the activation of NK cells even in the presence of inhibitory signals. This system is referred to as ‘induced-self’ or ‘stressed-self’ recognition (Eagle & Trowsdale 2007). Tolerance or activation of NK cells effector function is hence a result of a delicate balance between positive and negative signals.

NK cells activating receptors include NKG2D and a small class of specialized receptors termed the natural cytotoxicity receptors (NCRs) comprising NKp46, NKp30 and NKp44. Other activator receptors are CD16 (or FcγRIII) – whose key-role rely on its capacity to provide antibody-dependent-cytotoxicity against IgG-coated target cells –, and NKp80, LFA-1 and DNAM-I (Moretta et al. 2014).

Recently, it was shown in mice that Ly49H⁺ NK cells undergo clonal-like expansion during mouse CMV infection capable of generating long-lived memory NK cells. These cells preferentially reside in spleen and liver and are able to expand upon re-stimulation (Sun et al. 2009). In humans, several groups identified a subset of NKG2C⁺ NK cells capable of robustly expand after human cytomegalovirus (HCMV) infection. These cells reactivate after rechallenge and persist over one-year in CMV-seropositive patients (Björkström et al. 2011). It has therefore become evident that NK cells can acquire an immunological memory response after pathogen and non-infectious exposure, which was traditionally associated to the adaptive immune system (O’Sullivan et al. 2015).

NKG2D

NKG2D is the most studied NK activator receptor. Originally identified in 1991, NKG2D is a type II transmembrane protein with a C-type lectin-like extracellular domain encoded by a highly conserved gene (*KLRK1*). NKG2D is expressed on the surface of NK cells, γδ-T and CD8⁺ T cells, as well as on a small subset of CD4⁺ T cells and invariant NKT (iNKT) cells (Eagle & Trowsdale 2007).

NKG2D associates with the adaptor DNAX-activation protein 10 (DAP10) to form a stable structure. Upon interaction with one of its ligands, NKG2D transduces, via DAP10, the activation of a specific signalling cascade, leading to degranulation and cytokine production (Zwirner et al. 2007). The modulation of NKG2D expression is regulated by cytokines allowing transcriptional and posttranscriptional processing of NKG2D and DAP10. In humans, IL-2, IL-7, IL-12 and IL-15 lead to an upregulation of NKG2D on the

cell surface, whereas transforming growth factor- β (TGF- β), IFNB1 and IL-21 exert a downregulation (Lanier 2015).

NKG2D recognizes an extensive repertoire of ligands (NKG2DLs) that are encoded by at least eight genes in the host's genome (Eagle & Trowsdale 2007).

NKG2D ligands (NKG2DLs)

NKG2DLs show a restricted expression in normal tissues. They are generally absent in normal adult cells but upregulated in response to cellular stress, like heat-shock, tumourogenesis, genotoxic stress and stalled DNA replication. Therefore, they represent crucial immunosurveillance tools. In NKG2D-knockout(KO) mice, arising aggressive tumours express high levels of NKG2DLs, highlighting the evidence for surveillance of primary tumours by NKG2D (Guerra et al. 2008).

In humans, NKG2D ligands are grouped into two families: the MHC-class I chain-related protein A (MICA) and B (MICB), that are closely related as they share 84 % amino acid identity, and the HCMV UL16-binding proteins 1 to 6 (ULBP1-6) family (also known as RAET1: retinoic acid early transcript) (Lanier 2015). Engagement of NKG2DLs results in NK cell activation and costimulation of $\gamma\delta$ -T cells and antigen-specific $\alpha\beta$ -CD8⁺ T cells, leading to cytotoxic activity and cytokine production (Paschen et al. 2009).

MICA and MICB are upregulated or expressed *de novo* in stress conditions and in various malignancies including tumours of epithelial origin, carcinomas (breast, lung, colon, kidney, ovary and prostate), melanomas and some leukemias (Ge et al. 2011; Nausch & Cerwenka 2008). MICA is also constitutively expressed at low levels on intestinal epithelium of the gut and thymus, as well as on endothelial cells, fibroblasts and monocytes (Chen & Gyllenstein 2014). Interestingly, MICA is associated with increased frequencies of tumour-infiltrating $\gamma\delta$ T cells (Salih et al. 2002). Like MIC molecules, ULBP proteins are also expressed by tumours of different origins, such as leukemia, gliomas and melanoma (Nausch & Cerwenka 2008).

A strategy of tumour immune evasion involves the proteolytic shedding of NKG2DLs from tumour's cell surface, through the action of metalloproteases (Groh et al. 2002; Waldhauer & Steinle 2006; Salih et al. 2002). In addition, soluble MIC-A (sMICA) and -B mediate the downregulation of NKG2D by rapid endocytosis and partial lysosomal degradation of NKG2D, resulting in the impairment of CTL and NK cell cytotoxicity

(Roda-Navarro & Reyburn 2009). As a consequence, high levels of soluble NKG2DLs are frequently found in a various types of cancer, including lung, colorectal, breast, ovarian, prostate and melanoma (Holdenrieder et al. 2006; Paschen et al. 2009; Raulet et al. 2013; Wu 2014).

Natural cytotoxicity receptors (NCRs)

Natural cytotoxicity receptors (NCRs) play a major role in NK cell-mediated cytotoxicity of transformed cell (Pahl & Cerwenka 2015). Three human NCRs have been identified: NKp30, NKp44 and NKp46.

NKp30 is expressed on both activated and resting NK cells. It is a major activating receptor involved in tumour cell lysis. *In vitro* studies demonstrated that it also has a unique role in targeting non-transformed cells such as immature dendritic cells. NKp30 is upregulated by IL-2 and down-regulated by TGF- β . The first identified NKp30 ligands were of viral origin. The HLA-B-associated transcript 3 (BAT3), a nuclear protein ectopically found at the plasma membrane upon stress, was also shown to bind and trigger NKp30 (Kaifu et al. 2011; Lam et al. 2013). More recently, B7-H6 has been identified as a ligand for NKp30. B7-H6's expression appears to be limited to tumour cells. It does not bind any other NCR, has not been detected in most normal adult tissues, and has been observed in several tumour cell lines including lymphoma, leukaemia, carcinoma and melanoma as well as on primary blood cells (Matta et al. 2013). A soluble form of B7-H6 was detected in melanoma patients, and shedding of B7-H6 was shown to be dependent on metalloproteases, whose inhibition led to enhanced NKp30-dependent NK degranulation (Schlecker et al. 2014).

NKp44 is only expressed on circulating activated NK cells. It has been shown to be responsible, along with NKp46, for the recognition of viral hemagglutinins from influenza virus. It also plays a key role in the killing of several tumour cell lines, promoting the release of cytotoxic granules, IFN- γ and TNF- α . However, NKp44 can also be engaged by tumour cells, resulting in NK cell death through upregulation of Fas ligand expressed on the NK cells (Horton & Mathew 2015). NKp44 ligands include viral hemagglutinin and hemagglutinin-neuraminidase or orthomyxoviridae and the envelope protein of flaviviridae. Recently, other ligands were identified: PCNA and a splice variant of MLL5 (Brusilovsky et al. 2015).

NKp46 is expressed in all human NK cells. Its engagement promotes cytokine secretion and cytotoxic activity by NK cells. Its potential ligands comprise hemagglutinin of influenza vaccina and ectromelia virus, also shared with NKp44, as well as cell surface heparin sulphate proteoglycans expressed by some tumour cells (Lam et al. 2013).

4.2. Adaptive immunity

Adaptive immunity is triggered when innate immunity is ineffective in eliminating infectious agents or malignant cells. In contrast to innate immunity, adaptive immunity is antigen-dependent and antigen-specific. Its primary functions are to recognize specific ‘non-self’ antigens in the presence of ‘self’ antigens, triggering immunologic effector pathways that eliminate infected or transformed cells. Additionally, adaptive immunity has the capacity for immunological memory generation, allowing a rapid and specific immune response following antigen re-exposure (Warrington et al. 2011).

Adaptive immunity is composed by different subsets of cells including B cells and T cells, (mainly CD4⁺ helper T cells and CD8⁺ cytotoxic T cells).

Besides growing evidence of B cells playing a role in antitumour immune responses (for review and references therein (Guy et al. 2016; Fremd et al. 2013; Linnebacher & Maletzki 2012)), this immune subset of adaptive response was not explored in this work and will not be further developed here.

T lymphocytes play a crucial role in anti-tumour immunity, as they recognise tumour-associated antigens presented via MHC class I or II on APCs. They derive from pluripotent hematopoietic stem cells and migrate after shifting into T lineage to the thymus. They are then submitted to thymic maturation, allowing the positive selection of T cells that are able to recognize self-MHC molecules and the negative selection of clones that are able to recognize self antigens. This process results in the release of naïve T cells in peripheral blood and SLO (Broere et al. 2011; Purnama et al. 2013).

4.2.1. Naïve T cells (T_N)

Naïve T lymphocytes (T_N) continuously recirculate between SLOs, the lymph and the blood stream without proliferating, repeating the cycle until eventual activation by antigenic peptides (Broere et al. 2011; Purnama et al. 2013). Antigenic presentation promotes clonal T cell activation and differentiation into memory subsets.

Mature T_N released from the thymus harbour a given epitope specificity. The pool of naïve T cells remains nearly constant during adult life. T_N homeostasis and survival is promoted by IL-7-induced signals that sustain high levels of the anti-apoptotic protein Bcl-2, and by TCR-contact with self-ligands operating at an intensity just below than the required to enter cell cycle. This allows T_N cells to survive for long time without proliferation (Fomby & Cherlin 2011; Boyman et al. 2007).

T_N cells display a naïve phenotype characterized by the expression of low levels of CD44 and high levels of the lymph node homing receptors CD62L (L-selectin), CCR7, the IL-7 receptor subunit- α (CD127) and CD27 (a member of the TNF receptor superfamily). CD62L allows T_N to roll on endothelial venules, which promotes CCR7 engagement to its ligands. CCR7 ligands comprise the lymphoid chemokine ligand 19 (CCL19) and 21 (CCL21), displayed by fibroblastic reticular cells, which construct and support the T cell zones in SLO. CCR7 interaction with its ligands promotes T-cell continuous scanning of dendritic cells (Sallusto et al. 1999; Takada & Jameson 2009). The interactions between antigen-specific T cells and dendritic cells provide the necessary signals for specific T cell activation.

4.2.2. Specific antigen recognition

Specific T cells recognize their cognate antigen as a peptide presented in the context of a particular self-human leucocyte antigen (HLA) allele. $\alpha\beta$ -TCR T lymphocytes can be divided into two major groups on the basis of their co-receptor markers: $CD4^+$ and $CD8^+$ T cells. $CD4^+$ T cells interact specifically with the combination of one peptide and one class II HLA molecule (HLA-DP, -DQ or -DR), expressed on APCs such as dendritic cells, macrophages and B cells. Likewise, $CD8^+$ T cells recognise peptidic fragments presented by HLA class I molecules (HLA-A, -B and -C), that are expressed by all nucleated cells. This phenomenon is referred to as restricted antigen recognition.

Monocyte-derived dendritic cells, depending on the local cytokine environment and other stimulatory signals, initiate and control the quality, magnitude and orientation of the immune response by driving the differentiation of naïve $CD4^+$ T cells into T_H1 or T_H2 cells (for a review and references therein (Ueno et al. 2007)). Dendritic cells express a large collection of membrane and intracellular receptors, referred to as pattern recognition receptors, which behave as immune sensors (Medzhitov & Janeway 1997).

In the absence of inflammatory process, dendritic cells constitutively patrol the circulation, peripheral tissues, lymph and SLO in an immature state. Dendritic cells activation occurs when the immune sensors are triggered by microbial components or in response to inflammatory cytokines or even in certain contexts by self-molecules, such as dying cells, cancer cells, releasing molecules collectively named cell damage-associated molecular pattern molecules (DAMP). Dendritic cells enter into a maturation program comprising the upregulation of the costimulatory molecules CD80, CD86 and CD40, the translocation of MHC class II molecules to the cell surface and the secretion of cytokines that reshape other immune effectors (Caux et al. 1994; Ueno et al. 2007). The maturation signals also induce in dendritic cells the surface expression of the lymph node homing chemokine receptor CCR7.

In SLO, engagement of the specific TCR triggers an intracellular cascade of signalling events that result in naïve T cell activation. This first step of antigen recognition (also called signal 1) is not sufficient to trigger an immune response. The second signal (signal two) is provided by a limited number of co-stimulatory molecules (such as CD28 and CD40L) expressed on T cells that synergize with TCR-CD3 signalling to initiate T cell priming. Their engagement is required to provide full T lymphocyte activation that results in clonal expansion and differentiation into functional effector cells of distinct subsets (Shrikant et al. 2010). Once activated, T cells alter their expression of various homing receptors such as integrins, selectins and chemokine receptors, allowing their migration to peripheral tissues and inflamed sites where they recognize their target antigen (Mueller et al. 2013).

4.2.3. Effector T cells

Depending on the nature of cytokines produced in the surrounding milieu by APCs, NK and T cells, as well as on the valency of TCR ligation and costimulation, CD4⁺ T cells differentiate into different lineages of effector T helper cells, while CD8⁺ T cells form effector cytotoxic T lymphocytes following antigen-encounter (Angelica & Fong 2008; Kato & Nariuchi 2000).

CD4⁺ T cells or T helper (T_H) cells

Naïve CD4⁺ T cells can differentiate into different subsets of effector T_H cells with specific functions. Not endowed with cytotoxic or phagocytic capacities, these cells participate in the immune response by releasing cytokines that induce both B cell-dependent antibody production and cytotoxic immune response, being also potent activators of dendritic cells (Hivroz et al. 2012; Annunziato et al. 2015). Moreover, T_H cells are also required for effective CD8 T cell responses mostly at low levels of inflammation. In this context, CD4⁺ T cells may directly activate CD8⁺ T cells via CD40 or IL-2 stimulation, or activate dendritic cells that subsequently present the antigen to CD8⁺ T cells (MacLeod et al. 2010).

T_H1 cells drive type 1 responses, essential to fight viruses and other intracellular pathogens and to eliminate cancerous cells. The development and regulation of T_H1 response is crucially exerted by IFN- γ , which is produced by NK cells and macrophages, and by IL-12, a pro-inflammatory cytokine expressed by dendritic cells. IL-12 promotes the expression of T-bet, inducing cells to commit to T_H1 program. T_H1 cells produce IFN- γ , which activates the bactericidal activities of macrophages, as well as lymphotoxin- α , TNF and IL-2. They express chemokine receptors such as CXCR3, CCR2 and CCR5, promoting their recruitment to inflammatory sites. T-bet is considered as the hallmark transcription factor of these cells (Annunziato et al. 2015; Hirahara & Nakayama 2016; Kato & Nariuchi 2000).

IL-4 secretion commits helper T cells to the T_H2 program. T_H2 cells secrete IL-4, IL-5, IL-6 and IL-13, TNF- α and some produce IL-9, as well as modest amounts of IL-2. IL-4 and IL-13 are essential in humoral response by promoting the switching process of B cells for antibody production, whereas IL-5 plays a role in eosinophils activation. Under T_H2 conditions, chemokine receptors such as CCR3 and CCR4 are preferentially expressed (J. Zhu et al. 2010; Janikashvili et al. 2015; Yadav et al. 2013; Farber et al. 2014).

Other T_H have been described both *in vivo* and *in vitro*: type 17 T helper cells (T_H17), follicular T helper (T_{FH}) and type 22 and 9 helper cells (T_H22 and T_H9). T_H17 cells provide protection against bacterial and fungal infections. They arise in response to TGF- β and IL-6, and produce IL-17A, IL-17F, TNF and GM-CSF, key cytokines in neutrophils activation, as well as IL-21 and IL-22, with key roles in inflammation. T_{FH} reside in follicles and initiate germinal B cell responses to generate antibodies. T_H22 produce IL-22, that

promotes epithelial cell homeostasis and antimicrobial defence at mucosal surfaces, being also important in tissue repair. T_H9 produce IL-9 and are involved in airway inflammation (J. Zhu et al. 2010; Janikashvili et al. 2015; Yadav et al. 2013).

CD8⁺ T or cytotoxic T cells

Features of CD8⁺ T cells such as the ability to recognise peptides presented by almost all nucleated cells and capacity to directly kill antigen-expressing cells makes them essential in the control of intracellular pathogens infection and malignant transformation (Warrington et al. 2011).

Similar to T_H cells, a dichotomy is observed within CD8⁺ T cells allowing their classification into two subsets: cytotoxic T cells 1 (T_C1 cells) and 2 (T_C2) (Annunziato et al. 2015).

T_C1 cells play a key role in response to intracellular pathogens and tumours and their expansion is driven by IL-2, IL-12 and IFN- γ cytokines. Similar to T_H1 cells, T_C1 lymphocytes secrete IFN- γ and TNF- α cytokines within the immunological synapse. In addition, T_C1 exhibit a high cytolytic potential, being capable of releasing cytotoxic molecules such as granzyme B and perforin. Perforin creates pores in the target cell, allowing the delivery of granzyme B which induces programmed cell death, thus eliminating cells that have become cancerous or infected with viruses. Their cytolytic potential and IFN- γ production are dependent on T-bet activation and can be regulated by Eomesodermin (Eomes) transcription factor. These cells express the chemokine receptors CCR5 and CXCR3 (Mittrücker et al. 2014; Annunziato et al. 2015).

T_C2 lymphocytes participate in helminths and venoms response. These pathogens induce the release of IL-4 cytokine, which induces naïve T cells development into T_C2 lymphocytes producing IL-4, IL-5 and IL-13, providing help in Ab production by B cells (Annunziato et al. 2015).

T_C17 lymphocytes were identified in 2008 after the observation that T-bet and Eomes-KO mice failed to differentiate into functional cytotoxic cells capable of intracellular pathogen destruction following virus infection (Intlekofer et al. 2008b). Instead, they differentiate into an IL-17A, IL-21 and IL-22 cytokine-producing T population, named T_C17. In physiological conditions, T_C17 differentiation is triggered in response to extracellular bacteria and fungi (Annunziato et al. 2015).

Stepwise differentiation from naïve to memory T cells

A T cell response to an acute infection can be characterized by three distinguishable phases: clonal expansion, contraction of the T cell population and memory formation. At the peak of the primary response, the contraction phase begins and lasts 1-2 weeks, resulting in apoptosis following activation-induced cell death. During this phase, the majority (90-95 %) of effector T cells dies, leaving a residual population of long-lived memory cells (Mueller & Mackay 2015; Angelica & Fong 2008).

Effector-to-memory cell transition is influenced by the expression of homing molecules, cytokine receptors, and the TCR signal strength (increased TCR signal strength results in decreased memory potential) (Mueller & Mackay 2015). In increased inflammation conditions within a rich cytokinic milieu, the ratio of terminally differentiated or shorter-lived CTLs to memory precursor cells augments. Similarly, deficiency (or shortage) in IL-12 or IFN- γ preserves memory precursor cells (Kaeche & Cui 2012).

Memory T cells can already be distinguished from developing-effector T cells during T cell activation in primary infections: they up-regulate CD44 and IL-7 receptor subunit- α (CD127) as well as B cell lymphoma-2 (Bcl-2), and, unlike effector T cells, they do not lose CD127 and CD27, they do not up regulate KLRG1 and they express low levels of CD25 (Mittrücker et al. 2014; Kaeche & Cui 2012). However, memory cells can also arise from cells that have undergone effector differentiation during the primary response. These latter are able to survive the contraction phase, although they may be in disadvantage for survival (MacLeod et al. 2010).

Several models have been proposed to explain how memory and effector cells arise from a pool of naïve T cells (Kaeche & Cui 2012) (Figure 2):

- (a) **Separate-precursor model:** supports that multipotent T_N cells are ‘pre-programmed’ to originate either effector or memory T cells based on information received during thymic development. Currently, little evidence exists to support this model.
- (b) **Decreasing-potential model:** supports that greater effector proliferation and terminal effector functions result from repetitive stimulation of T cells with antigens and pro-inflammatory cytokines. These cells possess lower memory formation potential, in contrast with cells with lower antigen exposure, that acquire memory T cell properties. This model suggests the existence of a spectrum of effector T cells at various differentiation states according to the cumulative history of encountered signals during

infection, with the potential for memory potential formation decreasing along with repetitive antigen exposure.

(c) **Signal strength:** the formation of heterogeneous T cell populations is dependent on the overall strength of antigen exposure (signal 1), co-stimulation (signal 2) and cytokine exposure (signal 3) during T-cell priming. Combination of strong signals results in greater clonal expansion and selects T cells competent to form memory T cells. However, when delivered in excess, these signals can result in terminal effector T cell differentiation. This model differs from the decreasing-potential model in that 'cell-fate' results from signals delivered early during T-cell priming, rather than from successive rounds of stimulation.

(d) **Asymmetric cell fate model:** supports that a single precursor T cell divides asymmetrically as the activated T cells clonally expand, giving rise to both effector and memory T cells. This asymmetric division can occur as early as the first cell division. Evidence indicates that the daughter cell that inherits the immunological synapse receives stronger TCR and co-stimulatory signals. When integrated with the 'decreasing-potential model', this suggests that asymmetric division following repetitive antigen-exposure promotes terminal differentiation, simultaneously preserving a less-differentiated population with greater memory cell potential and stem cell-like qualities.

These models are not mutual exclusive and they all result in the formation of effector cells with a spectrum of differentiated states, with one end of the spectrum comprising effector T cells with memory potential and longevity, and the other including terminal effector T cells.

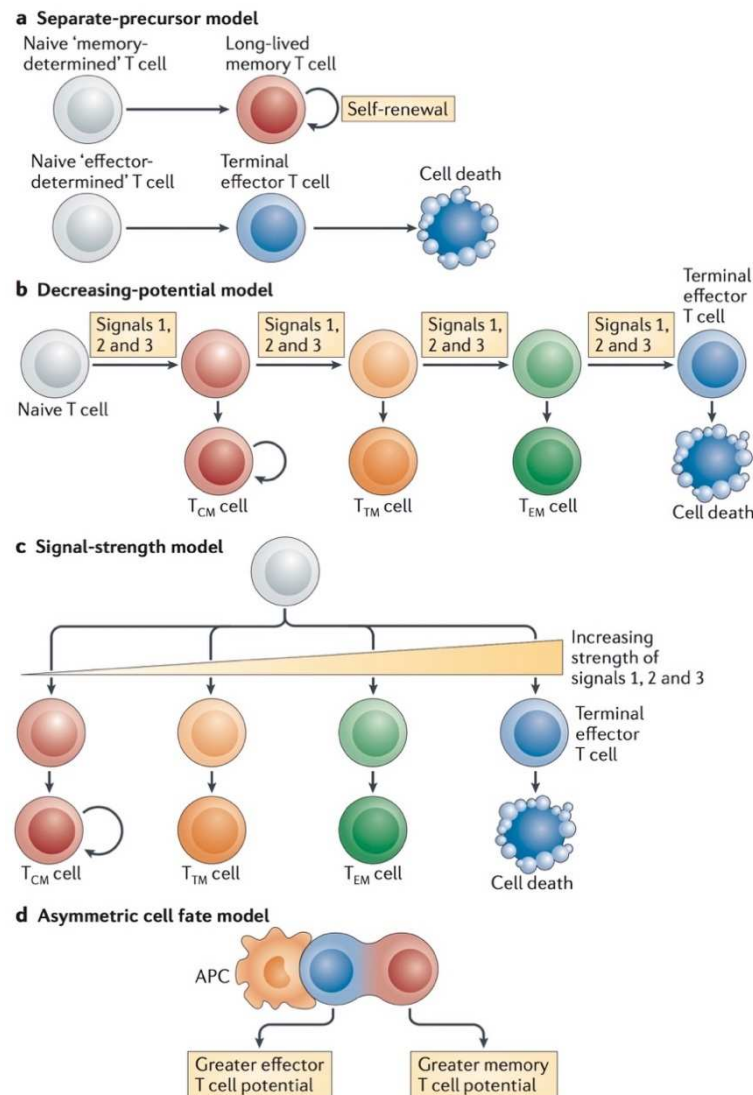


Figure 2 – Models for effector and memory T cell heterogeneity generation.
 Source: Kaech *et al.*, Nature Reviews Immunol. 2012. 12(11):753

4.2.4. Memory T cells

Memory T cells have a lower threshold for activation through the TCR and are thought to on costimulation than T_N cells. The memory compartment comprises a variety of memory be distinguished by their phenotypic and functional properties, as well as by their genetic cells have distinct homing characteristics and effector functions (Sallusto et al. 1999). subset characterization using a combination of cell surface markers such as CD45RA, was reported with the definition of central memory (T_{CM}), transitional memory (T_{TM}), and terminally effector memory (T_{EMRA}) lymphocytes, leading to a more accurate definition (Table 2, Xinglei Shen, et al. 2005; Sallusto et al. 2004). A summary of different memory T cells can be found on

Central Memory (T_{CM})

The heterogeneity of memory T cells was first identified in 1999 after Sallusto and colleagues' findings that the expression of the CCR7 chemokine homing receptor to lymph nodes allows the classification of CD45RA⁺ memory T cells into two subsets, with distinct functional properties. The CD45RA⁺CCR7⁺ subset displays lymph-node homing potential and is defined as central memory T cells. T_{CM} are devoid of both inflammatory and cytotoxic functions, and efficiently differentiate into the subset of CD45RA⁺CCR7⁺ effector memory T cells (Sallusto et al. 1999).

T_{CM} express high levels of CD62L and possess long telomeres. Like naive T cells, T_{CM} readily traffic to lymph nodes, where they reside for some hours to scan antigens, after which they migrate to the circulation via the lymphatics (Mueller et al. 2013). Like T_N, T_{CM} also upregulate (and to a higher extent) CD40L, suggesting a potent role in dendritic cells activation, which is CD40L-dependent (Sallusto et al. 1999). Besides, they have a great proliferation capacity and produce larger amounts of IL-2 but low levels of IL-4, IL-5 and IFN- γ . *In vitro* stimulation of naïve T cells with cognate antigen followed by high doses of IL-2 drives terminal differentiation to tissue-homing effector cells, whereas IL-15 or low doses of IL-2 promote the retention of CCR7 expression. This results in low cytotoxic function, together with IFN- γ -secreting capacities. After adoptive transfer, these latter cells home avidly to lymph organs where they mediate rapid recall responses, consistent with the central memory phenotype (Sallusto & Lanzavecchia 2001). T_{CM} also express high levels of chemokine receptors such as CXCR4 and CCR4, which are thought to allow them to respond to a high spectrum of chemokines and to more efficiently interact with dendritic cells. Additionally, they express CXCR3, CCR5, CCR10 and CLA, although they are only moderately efficient to migrate to inflamed tissues and to the skin (Sallusto et al. 1999; Kaeck 2014).

Effector Memory (T_{EM}) T cells

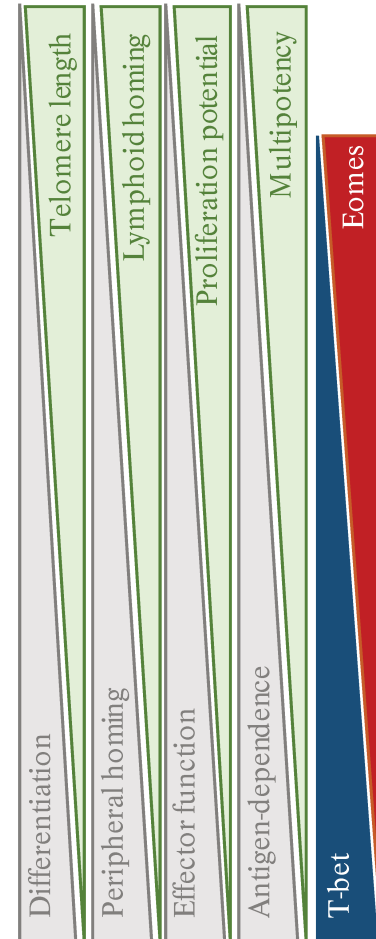
T_{EM} lack the expression of CCR7, which allows them to migrate to inflamed tissues to perform immediate effector functions. In comparison to T_{CM} , T_{EM} express lower levels of CD62L and possess shorter telomeres (Sallusto et al. 1999; Kaech 2014).

T_{EM} preferentially reside in peripheral compartments. In accordance, they express high levels of $\beta 1$ - and $\beta 2$ -integrins, which are required for homing to inflamed tissues. They also express tissue's specific homing receptors such as CD103, CLA and CCR10, these later two allowing them to relocate to the skin. They can express the chemokine receptors CXCR3 and CCR5, which endow them with a greater capacity to migrate to inflamed tissues. In peripheral tissues, T_{EM} cells are able to perform effector functions including inflammatory responses and cytotoxicity. T_{EM} produce high amounts of IL-4, IL-5 and IFN- γ , depending on CD4 and CD8 subsets, but modest amounts of IL-2 (Sallusto et al. 1999; Kaech 2014). Additionally, $CD8^+ T_{EM}$ contain perforin granules for immediate cytotoxicity (Mahnke et al. 2013).

Based on telomere length, CCR7 expression and cytokine production following anti-CD3 stimulation *in vitro* in short-term cultures, a stepwise differentiation from T_N to T_{CM} and then to T_{EM} cells is suggested (Sallusto et al. 1999).

Table 2 - Naive and memory T cell subsets.

	Location	Phenotype		Chemokine receptors	Cytokines		Summary
T_N	Thymus Spleen Lymph nodes Blood	CD45RA ⁺ CD62L ⁺ CCR7 ⁺ CD27 ⁺	CD28 ⁺ CD127 ⁺ Bcl-2 CD44 CD95 ⁻	CCR4 +/- CXCR3 + in CD8 CXCR4	IL-2		Continuously recirculate between secondary lymphoid organs, lymph and blood without proliferation, until eventual activation by antigenic peptides.
T_{SCM}	Spleen Blood	CD45RA ⁺ CD62L ⁺ CCR7 ⁺ CD27 ⁺ IL-2RB ⁺	CD28 ⁺ CD127 ⁺ Bcl-2 CD122 ⁺ CD95 ⁺	CCR4 CXCR3 +/- in CD4 CXCR3 ++ in CD8 CXCR4 +	IFN- γ TNF IL-2		Rare memory population. Superior survival potency, ability for asymmetric division, high proliferation potential and can differentiate into other memory subsets.
T_{CM}	Thymus Spleen Lymph nodes Blood	CD45RO ⁺ CD62L ⁺ CCR7 ⁺ CD27 ⁺	CD28 ⁺ KLRG ⁻ CD95 ⁺	CCR4 + CCR5 CCR6 +	CCR10 CXCR3 + CXCR4 ++ CLA	IL-2 ++ TNF + IFN- γ Perforin +/-	Traffic into lymph nodes. Have a low activation threshold and, upon restimulation in SLO proliferate and differentiate into T _{EM} .
T_{TM}	Bone marrow Spleen Lymph nodes Blood	CD45RO ⁺ CCR7 ⁻ CD27 ⁺	CD28 ⁺ CD95 ⁺	CCR4 ++ CCR5 + CCR6 ++	CXCR3 + CXCR4 ++	IFN- γ ++ TNF + IL-2 Gzm A + GzmB + Perforin +	Effector capacities between those of T _{CM} and T _{EM} .
T_{EM}	Bone marrow Spleen Lymph nodes Blood	CD45RO ⁺ CD62L ⁻ CCR7 ⁻ CD27 ⁻ CD28 ⁻	KLRG ⁺ CD95 ⁺ CD103 CLA	CCR4 ++ CCR5 ++ CCR6 ++	CCR10 + CXCR3 + CXCR4 + CLA +	IL-4 IL-5 IFN- γ + TNF ++ Modest IL-2 Gzm A ++ GzmB ++ Perforin ++	Preferentially reside in peripheral compartments, with great capacity to migrate to inflamed tissues, where they perform effector functions including inflammatory response and cytotoxicity.
Effector T	Spleen Lymph nodes	Short-lived: KLRG ^{hi} CD127 ^{low} IL-2RA ^{hi} Long-lived: KLRG ^{low} CD127 ^{hi} IL-2RA ^{low}		T _C 1: CCR5, CXCR3 T _C 2: CCR3, CCR4 T _H 1: CCR5, CCR2, CXCR3 T _H 2: CCR3, CCR4		T _C 1: IFN- γ ++ Gzm B ++ Perforin ++ TNF- α ++ T _H 1: IFN- γ ++ IL-2 TNF	Traffic to peripheral tissues where they perform effector functions and differentiate in short-lived terminally effector or long-lived memory precursor cells.
T_{EMRA}	Spleen Lymph nodes Blood	CD45RA ⁺ CD62L ⁻ CCR7 ⁻ CD27 ^{low} CD127 ^{low}	CD28 ⁻ KLRG ⁺ CD57 ⁺ Bcl-2 ^{low}	CCR4 +/- CCR5 + CXCR3 CXCR4 +	Gzm A +++ GzmB +++ Perforin +++ IFN- γ ++		Low proliferative potential, indicating terminal differentiation. Contain the largest amounts of perforin with potent effector functions.



Transitional Memory T cells (T_{TM})

While T_{CM} cells mostly co-express CD27 and CD28 along with CCR7 and CD62L, the inverse is not true. This led to the identification of transitional memory T cells. T_{TM} constitute an intermediate subset of memory cells that are more differentiated than T_{CM} but not as differentiated as T_{EM} cells, having already lost CCR7 and CD62L expression, but not CD27 (Mahnke et al. 2013).

Based on CCR7 and CD27 markers, three populations may thus be defined: the $CCR7^+CD27^+$ subset, corresponding to T_{CM} , the T_{EM} subset lacking both markers, and a third $CCR7^+CD27^+$ intermediate subset, the T_{TM} .

In vitro stimulation using a variety of culture conditions and stimulation reveals a stepwise differentiation from $CD8^+ T_{CM}$ to T_{TM} to T_{EM} , with progressive telomere shortening, lower proliferative capacities and increased susceptibility to apoptosis in T_{EM} . T_{EM} and T_{TM} secrete higher levels of IFN- γ and decreased levels of IL-2 than T_{CM} , consistent with the acquisition of effector cytokine secretion with the loss of CCR7 expression (Fritsch, X Shen, et al. 2005). When activated, these cells are able to produce certain amounts of granzyme B and perforin, although in less quantities than T_{EM} (Mahnke et al. 2013).

Terminally Differentiated Effector Memory T cells (T_{EMRA})

Memory cells typically display a loss of multipotency while differentiation proceeds, losing proliferative capacity and telomerase activity. Simultaneously, cumulative exposure to antigens with age induces the accumulation of memory T cells.

Terminal effector memory T cells are a subset of memory cells in humans (with no counterpart in mouse) expressing CD45RA as do naïve T cells, but lacking CCR7 and CD62L, and generally not expressing CD27 nor CD28. However, some $CD8^+ T_{EMRA}$ cells express CD27 and display phenotypical and functional features that are intermediate between naïve and effector T cells. The expansion potential and telomere length are the lowest in T_{EMRA} , constituting the most differentiated of all memory subsets. These cells are observed in both $CD4^+$ and $CD8^+$ T compartments, but predominant in the $CD8^+$ T cell subset. T_{EMRA} cells express CXCR3 and CCR5, although at lower levels than T_{EM} . They also express markers of senescence such as KLRG-1 and CD57, and have strong effector

activity, since they carry the largest amount of perforin. (Sallusto et al. 2004; Mahnke et al. 2013; Saule et al. 2006).

The fact that CD45RA can be reexpressed on memory T cells following cytokine-driven but not antigenic-driven proliferation suggests that T_{EMRA} cells are primarily generated through homeostatic rather than antigen-dependent pathways, that is, without the need of TCR cross-linking with antigen. T_{EMRA} are hence a subset of T_{EM} cells with strong effector capacities that provide strong killing and effector activity via bystander activation by cytokine receptors (Sallusto et al. 2004; Reinke et al. 2013).

Although a stepwise differentiation from T_{CM} to T_{EM} to T_{EMRA} has been suggested (Mahnke et al. 2013), the lineage relationship between CD8⁺ T cell subsets is not clear. Others suggested that T_{EMRA} are generated from a T_{CM} subset upon homeostatic proliferation in the absence of antigen (Geginat et al. 2003). However, a clear dichotomy exists between T_{CM} and the two effector memory subsets (T_{EM} and T_{EMRA}). While T_{CM} have a greater expansion potential and self-renewal capacity, both T_{EM} and T_{EMRA} strongly express genes involved in effector function (Willinger et al. 2005). It was proposed that, in response to antigenic stimulation T_{CM} differentiate into T_{EM}, whereas in response to homeostatic cytokines they differentiate into T_{EMRA} (Sallusto et al. 2004; Willinger et al. 2005).

Stem Memory T cells (T_{SCM})

Since naïve T cells generate all different subtypes of effector and memory T cells, it has been suggested that the repopulation of memory cells subsets was a result of ‘stem cell-like’ characteristics maintained within distinct subsets of memory cells that would be capable of repopulating terminal differentiated effector lymphocytes in response to antigen. T_{CM} were thought to constitute this stem cell-like subset, in contrast to T_{EM} which, after a limited number of cell divisions, undergo terminal differentiation (Stemberger et al. 2009).

Recently, the *in vitro* induction of Wnt-β-signalling by using Wnt3A or inhibitors of glycogen synthase kinase-3β during mouse T cell-priming was shown to induce the inhibition of CD8⁺ T cell differentiation into effector T cells. Instead, it promoted the generation of a population with the CD44^{low}CD62^{high} phenotype similar to that of T_{CM} cells, but endowed with distinct functional properties. These included proliferative and

antitumour capacities exceeding those of T_{CM} and T_{EM} cells. This $CD8^+$ stem cell-like population identified in mice was designated $CD8^+$ memory stem T cells (T_{SCM}) (Gattinoni et al. 2009).

Additionally, T_{SCM} cells were shown to confer superior immunity than T_{CM} and T_{EM} cells after adoptive transfer into B16 mice in combination with gp100 and IL-2, improving survival (Gattinoni et al. 2009).

Two years after the identification of T_{SCM} cells in mice, the same team described the natural occurrence of this population in humans in $CD4^+$ and $CD8^+$ T subsets. T_{SCM} represent about 2-3 % of all circulating $CD4^+$ and $CD8^+$ T lymphocytes in healthy donors, consisting of a subset of $CD45RA^+CD45RO^-CCR7^+CD62L^+CD27^+CD28^+IL-7Ra^+$ naïve-like cells that also express the Fas receptor (CD95) and CD122. Like in mice, T_{SCM} human cells express Bcl-2, and CXCR3. Additionally, and compared to T_N cells, human T_{SCM} express higher levels of LFA-1, CXCR4, and less amounts of CD38 and CD31, and have a low TREC content, reflecting several rounds of division. They are endowed with higher proliferative potential than T_{CM} cells and enhanced survival capacity. Similar to conventional memory T cells, these cells possess rapid recall ability with the capacity to secrete IFN- γ , IL-2 and TNF- α cytokines upon antigen encounter. They constitute the least differentiated memory subset, undergo robust proliferation in the presence of IL-7 and IL-15 and are long-lived memory T cells with multiple viral- and self-tumour specificities. They possess asymmetric division, with the ability to both self-renew and to differentiate into more specialized cells *in vitro*. T_{SCM} cells in healthy donors represent a substantial proportion of the total antigen-specific $CD8^+$ T cell memory subsets. Plus, transferred T_{SCM} cells into a xenograft tumour mice model showed high proliferative and survival ability of these cells, resulting in tumour regression and cure. This suggests a higher potential of T_{SCM} cells compared to conventional T_{CM} and T_{EM} memory subsets in adoptive immunotherapy (Gattinoni et al. 2011).

Resident Memory T cells (T_{RM})

For several years, it remained unresolved whether T_{EM} cells could permanently reside in non-lymphoid tissues or if they continuously recirculated through tissues. Recently, it was observed that some memory T cells permanently reside in peripheral tissues after clearance of the infection without recirculating through the blood. These cells have been

designated tissue-resident memory T cells (T_{RM}). T_{RM} derive from precursors that enter tissues at the effector phase of immune responses, remaining in that compartment. They are present in many, if not all, organs and tissues and are best described for memory $CD8^+$ T cells, although non-recirculating subsets of $CD4^+$ T_{RM} have also been described in lungs, genital tract and skin. Similarly to T_{EM} cells, T_{RM} express high levels of CD44 and low levels of CD62L (Schenkel & Masopust 2014). T_{RM} cells can be distinguished from circulating T_{EM} and T_{CM} cells by the coexpression of the C-type lectin CD69 and the αE integrin CD103, though T_{RM} cells lacking CD103 are also observed in intestines, SLO and liver (Mueller & Mackay 2015).

T_{RM} cells reside at mucosal borders and seem to operate as sentinel cells at the portals of antigen entry, and are thought to play a pivotal role in protection of mucosal surfaces and epithelia from invading pathogens (Kaech 2014; Mahnke et al. 2013).

Interaction of CD103 on tumour-specific TILs with its ligand, E-cadherin, is required for the positioning of cytotoxic granules near the interface and their delivery into the target. Additionally, CD103 induces costimulation in activated CTL by triggering signals that cooperate with TCR-mediated signalling. CD103 is expressed at high levels by mucosal $CD8^+$ T lymphocytes and by a large proportion of $CD8^+$ T cells infiltrating epithelial tumours, such as pancreatic and colorectal. Recently, it was shown that $CD8^+103^+$ TILs in non-small lung carcinoma are T_{RM} , emphasizing their role in promoting intratumoural responses (Djenidi et al. 2015). However, the presence of T_{RM} in epithelial tumours and their role in antitumour responses have not been systematically addressed so far.

4.2.5. Recall response of memory T cells

Once a secondary infection has been established at a peripheral site, memory T cells present in the tissue, usually T_{EM} and T_{RM} , provide an immediate response. Pro-inflammatory cytokines and chemokines are produced *in situ*, leading to the recruitment of circulating memory T cells of various antigen-specificities into the site of the infection. The antigen-specific memory cells generate a secondary effector T-cell response, with the differentiation of T_{CM} into secondary effector cells. At the same time, antigen-bearing-dendritic cells migrate to the lymph nodes, where they activate lymph memory T cells. This generates secondary antigen-specific T cells that traffic through the bloodstream to the

infected tissue. Together, these three stages result in a rapid pathogen clearance during a secondary infection (Woodland & Kohlmeier 2009; Kaech & Cui 2012).

4.3. Regulators of T cell fate

4.3.1. Regulatory T cells (T_{reg})

T_{reg} are a highly suppressive subset of $CD4^+$ T cells characterized by the expression of the master regulatory transcription factor Foxp3. The importance of T_{reg} was evidenced when adoptive transfer of CD25-depleted T cells induced multi-organ autoimmunity in recipient animals. Later, mutations in the gene of Foxp3 in both mice and humans were shown to result in fatal autoimmunity (Bluestone & Abbas 2003; Yadav et al. 2013). Foxp3 is a crucial transcription factor in directing the regulatory program of T_{reg} , although some T_{reg} cell signature molecules can be expressed, at least to a certain extent, without Foxp3 expression (Ohkura et al. 2013). T_{reg} represent an essential counterbalance to adaptive immune responses by maintaining self-tolerance and immune homeostasis, whereas in malignancies they promote tumour progression by suppressing effective anti-tumour immunity. Of note, T_{reg} control T cell responses to cancer antigens that are derived from self-constituents, but are less suppressive to T cells recognizing foreign antigens (Chousterman & Swirski 2015).

T_{reg} can arise either in the thymus (natural occurring T_{reg} , nT_{reg}) or in the periphery (pT_{reg}) in case of antigen-exposure of naïve $CD4^+$ T cells. nT_{reg} constitutively express Foxp3 and are a polyclonal subset of T cells that result from self-reactive T cells that are not deleted in the thymus, being associated with high TCR/MHC-peptide affinity. These cells migrate to peripheral tissues where they suppress various effector lymphocytes, especially helper T cells (Ohkura et al. 2013). pT_{reg} are induced under subimmunogenic conditions and result from antigen-exposure of naïve $CD4^+$ T cells in the periphery (Janikashvili et al. 2015; Ohkura et al. 2013). These two subsets can be distinguished from *in vitro*-induced Foxp3 T_{reg} (iT_{reg}). These later result from TGF- β -induction of Foxp3 in conventional T cells, but so far do not recapitulate the functional and phenotypic characteristics of pT_{reg} , as they are less stable in the expression of T_{reg} cell function-associated molecules, such as Foxp3 (Yadav et al. 2013; Ohkura et al. 2013).

T_{reg} express the IL-2-receptor α -chain (CD25), as well as the TNF-superfamily member GITR. Expression of IL-7R (CD127) is low in T_{reg} , allowing them to be distinguished from activated effector T cells. Additionally, T_{reg} constitutively express the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), a T cell coinhibitory receptor (Walker 2013). Foxp3 expression is not specific of T_{reg} cells, as it can also be transiently expressed by nonsuppressive $CD4^+$ T cells. In this context, it was demonstrated that there is a human T_{reg} -specific demethylation of a conserved region in the first intron of the *FOXP3* gene (*FOXP3i1*) (Baron et al. 2007; Lucas et al. 2012), which does not occur in activated conventional $CD4^+$ T cells or in Foxp3-expressing TGF- β -treated cells despite expression of Foxp3. However, in contrast to blood, demethylated *FOXP3i1* is not specific to T_{regs} in melanoma tumour microenvironment, since some melanoma cell lines also carry this specific demethylation (Lucas et al. 2012), but it remains thus far the most specific marker of human T_{regs} .

T_{reg} cells can be divided into different phenotypic and functional subsets. Naïve or resting T_{reg} possess circulatory characteristics that are similar to naïve conventional $CD4^+$ T cells, with weak suppressive activity and possessing the $CD45RA^+Foxp3^{low}CD25^{low}$ phenotype. These can differentiate into effector T_{reg} (eT_{reg}) cells upon antigen encounter, possessing the $CD45RA^+Foxp3^{hi}CD25^{hi}$ phenotype. eT_{reg} are terminally differentiated and highly suppressive (Liston & Gray 2014; Chousterman & Swirski 2015; Nishikawa & Sakaguchi 2014).

T_{reg} carry out their regulatory function through diverse mechanisms such as secretion of inhibitory cytokines IL-10, TGF- β and IL-35, suppression of the function of APCs via CTLA-4, consumption of IL-2 and ATP degradation. CTLA-4 appears to have a key-role in T_{reg} inhibitory functions since CTLA-4-deficient T_{reg} show impaired suppression functions *in vitro* (Sojka et al. 2009). Human CTLA-4 haploinsufficiency causes Foxp3 $^+$ T_{reg} dysregulation, effector T cells hyperactivation and lymphocytic infiltration of target organs (Zhang et al. 2013). Additionally, anti-CTLA-4 treatment blocks T_{reg} functions both *in vitro* (Takahashi et al. 2000) and *in vivo* (Read et al. 2000). T_{regs} were also shown to induce CD80 and CD86 suppression on APCs (Oderup et al. 2006), probably in a CTLA-4 dependent manner (Qureshi et al. 2011).

In several cancers including melanoma, non-small cell lung, gastric and ovarian cancers, T_{regs} efficiently infiltrate the tumour microenvironment through chemo-attraction. They

expand locally and differentiate into a T_{reg} cell subpopulation (mainly eT_{reg}) capable of recognizing self-antigens including tumour antigens. Proliferated/differentiated T_{reg} cells efficiently suppress the activation of tumour antigen-specific effector cells. High tumour infiltration of T_{regs} is associated with poor survival (Chousterman & Swirski 2015; Nishikawa & Sakaguchi 2014).

4.3.2. T-bet and Eomes

In effector cells

Functional development of distinct T cell populations is regulated by transcription factors including T-bet and Eomesodermin (Eomes) (Pearce et al. 2003).

T-bet is a T_H1 -specific T box transcription factor encoded by *Tbx21* gene initially described as a master regulator of T_H1 cells commitment. It is now well established that innate cells also express T-bet (Annunziato et al. 2015).

T-bet is responsible for non-redundant control of lineage commitment of NK cells and in T_H lymphocytes (along with STAT4) by regulating the expression of their hallmark cytokine, IFN- γ . T-bet-KO mice show impaired IFN- γ production by $CD4^+$ T and NK cells with increased IL-4 and IL-5 production, and are susceptible to intracellular pathogens (Mullen et al. 2002). Additionally, T-bet-KO mice fail to control melanoma metastasis due to defective NK cells (Werneck et al. 2008).

In NK cells, T-bet was shown to be responsible for the development of immature NK cells expressing the TNF-related apoptosis-inducing ligand (TRAIL) (Lazarevic et al. 2013). T-bet also contributes to the expression of IFN- γ , perforin and granzyme B in NK cells (Y. Zhu et al. 2010).

T-bet activates approximately half of T_H1 -specific genes related to cytokine expression (IFN- γ , LT- α and TNF), chemokine ligands (CCL3 and CCL4) and receptors (CXCR3 and CCR5), that are required for effector function and migration and for recruitment of other immune cells into the site of inflammation (Lazarevic et al. 2013).

In addition, T-bet is sufficient to induce IFN- γ when ectopically expressed (Szabo et al. 2002), and overexpression of T-bet in fully differentiated T_H2 or T_H17 cells endows them with the ability to produce IFN- γ , reminiscent of T_H1 cells (Lazarevic et al. 2013).

T-bet also contributes to the expression of IFN- γ , perforin and granzyme B by cytotoxic CD8⁺ T (Y. Zhu et al. 2010). However, the observation that T-bet-KO mice exhibited normal IFN- γ production by CD8⁺ T cells implies the existence of a T-bet-independent pathway for CD8⁺ T cells and probably NK cells effector function and homeostasis (Intlekofer et al. 2005; Y. Zhu et al. 2010).

Eomesodermin (Eomes) is a T-box factor highly homologous to T-bet. Its expression by effector CD8⁺ T cells triggers IFN- γ , perforin and granzyme B production. It is highly expressed on activated CD8⁺ T cells as well as on resting and activating NK cells, but weakly expressed in CD4⁺ T cells. Eomes was suggested to regulate the T-bet independent control of cytotoxic effector function (Pearce et al. 2003).

Lymphocytic choriomeningitis virus (LCMV)-specific CD8⁺ T cells from T-bet and Eomes double-KO mice fail to differentiate into functional killers and lead to IL-17-secreting lineages. This causes an excessive neutrophil recruitment, revealing a role of T-bet and Eomes in suppressing T_C17 alternative fate (Intlekofer et al. 2008a). T-bet and Eomes have also been shown to prevent T_C2 responses (Y. Zhu et al. 2010).

In NK cells, Eomes allows the complete maturation of NK cells by silencing TRAIL expression (Lazarevic et al. 2013).

In CD8⁺ T cells, T-bet is rapidly induced following TCR and IL-12R signalling, and is required for the early production of IFN- γ and granzyme B. T-bet expression is amplified by IFN- γ and IL-12 production, which repress Eomes transcription. Eomes is induced later in a RUNX3-dependent fashion and its expression is amplified by IL-2 secretion (Kaech & Cui 2012). Eomes can replace T-bet to promote IFN- γ and granzyme B production and, along with STAT5, regulates perforin expression in these cells (Lazarevic et al. 2013). As so, T-bet expression is maximal during effector phase whereas Eomes expression progressively increases in the transition of effector to memory phases of CD8⁺ T cell response (Shrikant et al. 2010).

In summary, T-bet is expressed in T_H1 cells, regulating IFN- γ expression. In NK cells, T-bet is responsible for an immature state, while Eomes allows their complete maturation, and both contribute to IFN- γ and granzyme secretion. In CD8⁺ T cells, T-bet is rapidly induced and responsible for early effector functions, whereas Eomes is later induced in the transition of effector to memory phases, and both transcription factors induce IFN- γ and

granzyme B production. Therefore, T-bet and Eomes play major roles in effector functions of NK and T cells.

Eomes and T-bet were also shown to be critical regulators of T cell-mediated immune responses against cancer. They are crucial for tumour infiltration by CD8⁺ T cells, which is associated with better clinical outcomes in certain types of cancer. Indeed, the lack of both Eomes and T-bet results in a reduced expression of CXCR3, disfavours migration of T_H1 cells and effector CD8⁺ T cells to inflamed tissues and tumours (Y. Zhu et al. 2010).

In memory T cells

T-bet and Eomes in memory CD8⁺ T cells

The transcriptional programmes controlling terminal effector cell differentiation and memory cell potential were proposed to be based on a gradient expression of transcription factors where T-bet and Eomes display reciprocal expression patterns (Figure 3).

Strong IL-12R signalling promotes the terminal differentiation of short-lived effector cells (SLECs) by enhancing STAT4-mediated mTOR kinase activity, which in turn increases T-bet expression and represses Eomes. As T-bet expression increases during an infection, effector CD8⁺ T cells acquire more terminally differentiated phenotypes with less proliferative capacities and longevity. Therefore, T-bet-, IL-12- or mTOR-deficiencies results in the absence of SLECs and promotes long-lived memory precursor effector cells (MPECs) (Lazarevic et al. 2013). Conversely, Eomes seems to counter-regulate T-bet-induced terminal differentiation of effector T cells. Eomes favours the formation of long-lived memory cells, possibly in response to Wnt signalling and to transcription factor 1 (TCF1). Thus, CD8⁺ MPECs surviving the contraction phase generate a reservoir of memory cells progressively expressing Eomes and downregulating T-bet levels. Hence, the T-bet-to-Eomes ratio is higher at the effector phase and lower at memory cell stages (Kaeche & Cui 2012).

As so, T_{CM} cells express high levels of Eomes, whereas T_{EM} cells express high levels of T-bet, consistent with a more differentiated phenotype. This is illustrated by a gradient of T-bet/Eomes during post-thymic differentiation of CD8⁺ T cells (Lazarevic et al. 2013; Kaeche & Cui 2012).

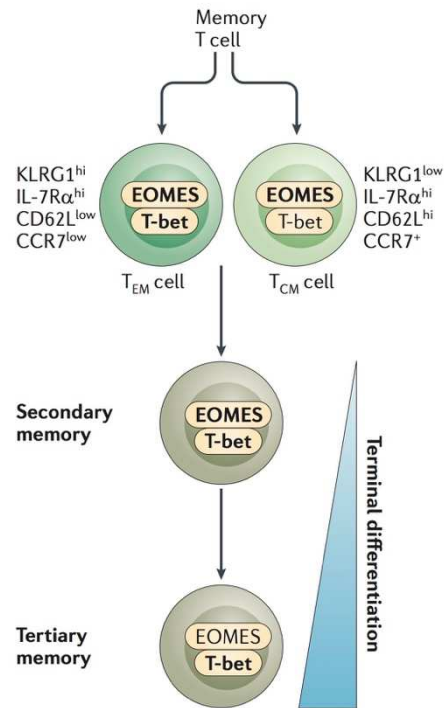


Figure 3 – T-bet and Eomes expression in memory T cells generation and differentiation.

Source: Lazarevic, Nature Reviews Immunol. 2013. 13(11):785

T-bet and Eomes in memory CD4⁺ T cells

The features allowing a proper distinction of a committed memory precursor from an effector CD4⁺ T cell are less well established. Infection with LCMV in mice produces a population of T_H1 cells positive for PSGL1 and LY6C. The LY6C^{hi} subset has features of terminally differentiated effector cells such as increased T-bet, CXCR3, IFN-γ and granzyme B expression. By contrast, the LY6C^{lo} counterpart is less differentiated and express intermediate levels of T-bet, consistent with CD4⁺ T_{CM} cells. This subset has longer lifespan and proliferates better upon antigen recall. Loss of T-bet results in an expansion of PSGL1^{hi}LY6C^{lo}T_H1 memory precursor cells (Lazarevic et al. 2013; Marshall et al. 2011). This suggests a role of T-bet expression in the balance between terminal differentiation and memory cell potential in CD4⁺ memory compartment.

However, another study described the formation of virus-specific memory CD4⁺ T cells even in the absence of T-bet following LCMV infection in mice (Mollo et al. 2013). Therefore, the contribution of T-bet and even Eomes to the development of memory CD4⁺ T cells remains unclear.

T-bet and Eomes in memory T cell homeostasis

Memory T cells seem to depend on different homeostasis factors based on their localisation. At mucosal sites, memory T cells with low levels of CD122 (the β -subunit of IL2R and IL-15R) may rely on cross-reactive TCR-mediated interactions for survival, as a result of high antigen density at these sites.

By contrast, in blood, lymphoid tissues, spleen and bone marrow, CD8⁺ memory T cells are preferentially maintained by cytokines such as IL-7 and IL-15. These are constitutively produced by dendritic cells. In this context, memory CD8⁺ T cells do not require antigens or MHC-I contact for maintenance. They need IL-7 for survival and IL-15 for homeostasis. IL-15-dependent lineages are considerably depleted in Eomes and T-bet double-KO mice (Intlekofer et al. 2008a; Intlekofer et al. 2005). This is due to the fact that CD122 expression is controlled by T-bet and Eomes (Li et al. 2013), via CD122-encoding gene (*Il2rb*) transactivation.

CD4⁺ memory T cells depend on IL-7 for slow turnover and survival, although it is now clear that IL-15 also contributes to their homeostasis. Besides, these subset also requires TCR signalling and/or MHC class-II molecules for functional maintenance and survival (Farber et al. 2014; MacLeod et al. 2010).

4.3.3. The CD154/CD40 pathway

CD40 and CD154 (also known as CD40L) molecules belong to the TNF superfamily. While CD40 is constitutively expressed on APC, CD154 is expressed on T lymphocytes following activation, mainly CD4⁺ T cells and also endothelial cells. CD154/CD40 interaction induce the upregulation of B7 molecules on APC and triggers a bidirectional signal allowing both T and B cell activation, playing an important role in both cell-mediated and humoral immunity. Therefore, this pathway represents an attractive target in immunomodulatory therapy, having prompted the development of anti-CD154 monoclonal antibodies whose efficacy is currently being tested in the context of autoimmunity and transplantation (Yamada et al. 2002; Pinelli & Ford 2015).

4.3.4. OX40 (CD134)

OX40 (CD134) is a costimulatory molecule belonging to the TNF receptor superfamily. OX40 is expressed on both CD4⁺ and CD8⁺ activated T cells, subserving costimulatory

functions. OX40 is also constitutively expressed on T_{regs}. Strong crosslinking of TCR/CD3 can induce OX40 expression, but under physiological conditions other costimulatory molecules such as CD28 promote its upregulation, as well as cytokines such as IL-2 or IL-4. Its only known ligand, OX40L (CD252), is mainly present on APCs although other subsets such as NK cells can also express it. OX40/OX40L interaction triggers intracytoplasmic pathways associated to T-cell signalling such as the nuclear factor (NF)- κ B and the nuclear factor of activated T cells (NFAT). This results in enhanced expression of cyclin A, Bcl-2 antiapoptotic molecules, cytokine receptors and cytokine secretion (Aspeshlagh et al. 2016). The strong activity of OX40-OX40L interactions in driving T cells activation prompted the development of agonistic monoclonal antibodies against OX40 with potential immunostimulatory activities, shown to induce the formation of long-lived memory CD4⁺ T cells in mice (Redmond et al. 2014).

4.3.5. CD137 (4-1BB)

CD137 is a costimulatory molecule belonging to the TNF receptor superfamily. It is expressed on activated T cells, being also detectable on T_{reg}, dendritic cells and NK cells. Its cognate ligand (CD137L) is mainly expressed on APCs. CD137/137L interaction provides T cell co-stimulation in a CD28-independent manner. CD137 promotes CD4⁺ helper T-cell proliferation and cytokine production and enhances cytotoxic T cell responses. The development of anti-CD137 agonistic monoclonal antibodies as cancer immunotherapies rapidly followed these observations, and have led to eradication of large tumours in mice, including sarcomas and mastocytomas, increasing T cell proliferation, differentiation into memory T cells and apoptosis-resistance. Additionally, anti-CD137 Ab was shown to elicit antitumour immune responses in CD137^{-/-} mice in a melanoma model (Yonezawa et al. 2015). A randomized phase II clinical trial studied an anti-CD137 human mAb (BMS 663513) as a second-line monotherapy in metastatic melanoma. However, the study was discontinued due to a high incidence of grade 4 hepatitis (Simeone & Ascierto 2012). Nonetheless, another phase I study assessing the safety, tolerability and pharmacokinetics of the same antibody is ongoing (NCT01471210).

4.3.6. Inducible co-stimulator (ICOS)

ICOS is a homodimeric protein member of the extended CD28/B7/CTLA-4 Ig superfamily. It is synthesised *de novo* after T cell activation and retained in many memory

T cells, enhancing T cell proliferation, lymphokine secretion and providing help for antibody secretion by B lymphocytes. ICOS delivers a CD28-independent signal inducing the production of IL-10, IL-21, IL-17 and IL-4 (but not IL-2), having an important role in T_{FH} cells formation and in the regulation of both T_H1 and T_H2 differentiation (Hutloff et al. 1999; Leung & Suh 2014; Group et al. 2001). Being essential for T-cell activation, ICOS appears to have also a regulatory role since, along with impaired T cell activation, ICOS-KO mice show enhanced susceptibility to experimental autoimmune encephalomyelitis (Dong et al. 2001). Indeed, ICOS was also detected in T_{reg}, influencing its function and homeostasis (Leung & Suh 2014; Ceeraz et al. 2013).

ICOS genes locate in human chromosome 2 along with CD28 and CTLA-4 genes (Soskic et al. 2014). ICOS ligand (ICOSL, also known as CD275) is an homolog of B7 expressed on APC cells (Ceeraz et al. 2013). In humans, ICOSL can also bind CD28 and CTLA-4. It is also expressed by tumours, notably by melanoma cells, where it was shown to engage ICOS on activated T_{reg}, promoting tumour escape (Martin-Orozco et al. 2010).

4.3.7. Programmed cell death protein 1 (PD-1)

PD-1 inhibitory receptor is a member of the Ig superfamily related to CD28 and CTLA-4. It is expressed by CD4⁺ and CD8⁺ thymocytes, mature T and B cells and occasionally found in macrophages (Wong et al. 2007). PD-1 possesses an important role in regulation of the effector phase of T cell responses, with two natural ligands: programmed death ligand 1 (PD-L1, also known as B7-H1 or CD274) and PD-L2 (also known as B7-HC or CD273) (Leibson 2004). PD-L1 is frequently expressed by cancer cells, such as melanoma cells, and in tumour-infiltrating macrophages, whereas PD-L2 is primarily expressed by dendritic cells and macrophages, although low levels are occasionally found in activated T cells (Hamid et al. 2013). PD-1 regulates T cells during long-term antigen exposure, as in chronic viral infections and cancer. Its interaction with PD-L1-expressing tumour cells and PD-L2-expressing APCs in the tumour environment results in the inhibition of T cell proliferation, cytokine release and cytotoxicity (Ribas 2012). PD-1/PD-L1 interactions occur in the periphery, i.e., at the tumour site (Robert et al. 2013).

4.3.8. The CD28/CTLA-4 pathway

CD28

CD28 is a 44 kDa type I transmembrane protein expressed on the surface of the majority of naïve CD4⁺ and CD8⁺ T cells. It is the major co-stimulatory receptor required for T cell priming, whose engagement is also known as the ‘second signal’ that leads to full activation of T cells and the generation of T-cell memory. Its two ligands, CD80 (B7-1) and CD86 (B7-2), are predominantly found on APCs. CD28 engagement induces T cell IL-2 production, enhancing proliferation and survival by upregulation of bcl-2 and bcl-xL (Vacchio et al. 2005). Delivery of signal 1 (TCR/MHC+peptide interaction) with insufficient CD28 co-stimulation induces an anergy state that can be seen as a self-tolerance mechanism. CD28-deficient mice lack effector T cell functions (Green et al. 1994).

CD28 plays an important role in T-helper differentiation and seems to be highly involved in the generation of T_H2 responses notably in the context of weak TCR signalling (Soskic et al. 2014). CD28 is also thought to be important in T_{reg} homeostasis. The requirement of CD28 signal for the memory subset activation is less established as memory T cells have lower activation thresholds, and CD28 expression is downregulated from the surface of highly differentiated mature T cells (Soskic et al. 2014).

Cytotoxic-T lymphocyte antigen 4 (CTLA-4)

CTLA-4 is a negative costimulator of T lymphocytes. Not expressed by naïve T cells, it is induced 2 to 3 days following T cell activation. Antibody-mediated crosslinking of CTLA-4 results in decreased IL-2 transcription, inhibition of T cell proliferation, and cell cycle arrest at G1 to the S phase of the cell cycle (Krummel & Allison 1996). In contrast to PD-1/PDL1, whose interactions occur in the periphery, interaction of CTLA-4 with its ligands takes place mostly in the lymphoid organs (i.e., centrally (Robert et al. 2013)).

CTLA-4 discovery

CTLA-4 was first identified in late 1980’s after screening of mouse cytotoxic-T-cell-derived cDNA libraries. It is a 223-amino-acid transmembrane protein of the immunoglobulin gene superfamily with a single variable domain, and it is mainly expressed on activated T

lymphocytes (Brunet et al. 1987). CTLA-4 shares a high degree of sequence similarity and is mapped to the same chromosomal band as CD28. To test its affinity with B7 molecules, Linsley's team constructed a soluble CTLA-4 that was shown to bind specifically B7 with 20-fold higher affinity than CD28 (Linsley et al. 1991). In the same study, soluble CTLA-4 was shown to inhibit T cell proliferation *in vitro*. Later, anti-human CTLA-4 antibodies were developed and were shown to enhance T cell proliferation following anti-CD3 and anti-CD28 *in vitro* stimulation (Linsley et al. 1992). In addition, cross-linking TCR and CTLA-4 inhibits T cell proliferation even when CD28 is engaged (Krummel & Allison 1995). Finally, generation of CTLA-4-KO mice showed that these mice die within 4 weeks after birth due to rapid and fatal lymphoproliferation with multi-organ tissue destruction, demonstrating a critical role of CTLA-4 in regulating T cell activation and in maintaining immunologic homeostasis (Tivol et al. 1995).

CTLA-4 is a key element in immune tolerance and the main negative regulator of T-cell mediated antitumour immune responses (Takahashi et al. 2000; Read et al. 2000).

CTLA-4 trafficking

In contrast to CD28, which is expressed on the cell surface, CTLA-4 localization is largely intracellular. After synthesis in rough endoplasmic reticulum and maturation in the Golgi, CTLA-4 is transported to the plasma membrane. Then, AP-2 protein binds to the cytoplasmic domain of CTLA-4, resulting in clathrin-mediated endocytosis. As a result, CTLA-4 either recycles back to the cell surface or undergoes lysosomal degradation (Soskic et al. 2014; Walker & Sansom 2011). In naïve T cells, surface CTLA-4 expression is inhibited by CTLA-4 binding to AP50, a subunit of AP-2. Following TCR engagement, CTLA-4 expression increases both transcriptionally and post-translationally, and CTLA-4 is uncoupled from AP50, resulting in increased surface expression (Comin-Anduix et al. 2010; Soskic et al. 2014). However, CTLA-4 is not stabilized at the T cell surface during activation, but is continuously internalized, resulting in both recycling and lysosomal degradation (Qureshi et al. 2012). The absence of ligand binding results in a steady-state where 90 % of CTLA-4 is intracellular (Qureshi et al. 2012).

CTLA-4-expressing cells

CTLA-4 is expressed by both CD4⁺ and CD8⁺ T cells 2 to 3 days following T cell activation, and is constitutively expressed by T_{reg}.

Conventional (as opposed to T_{reg}) and memory T cells have been shown to possess a significant intracellular reservoir of CTLA-4, which appears to be much smaller in naïve T cells. The increase in CTLA-4 expression from naïve to memory T cells may relate to the need for CTLA-4, as T cells may only need CTLA-4 after activation in order to regulate the response (Jago et al. 2004).

CTLA-4 mode of action

CTLA-4 prevents or terminates T cell activation, constituting a non-redundant pathway for self-tolerance. Its main role is to regulate CD28-dependent T cell activation. Several mechanisms have been proposed for the underlying molecular basis of CTLA-4 inhibitory functions that are not mutually exclusive, including intrinsic and extrinsic models of CTLA-4 function.

Intrinsic models of CTLA-4 function

(a) **CTLA-4 outcompetes with CD28 for ligand binding.** Competition would occur due to the higher affinity of CTLA-4 for B7 receptors (50 to 100 fold greater than CD28), depriving T cell of CD28-mediated costimulation. In support of this model, a truncated CTLA-4 gene lacking its cytoplasmic domain retarded the disease onset in CTLA-4-deficient mice (Masteller et al. 2000). This suggests that the CTLA-4 molecules that cannot transduce signals retain some immunoregulatory function, by outcompeting with CD28 for ligand binding.

(b) **CTLA-4 delivers a T-cell intrinsic inhibitory signal,** that is, CD80/86 receptors engage the CTLA-4 pathway that, *per se*, induces a negative signal that regulates TCR signalling. This concept is based on the observation that CTLA-4 cross-linking results in T cell inhibition of proliferation and IL-2 production. Indeed, CTLA-4 was shown to recruit phosphatase, and to induce decreased phosphorylation in several key proteins downstream the TCR. Additionally, it was shown to inhibit the activation of the ERK, required for IL-2 production. Other studies suggested that CTLA-4 inhibits lipid raft formation on naïve T cells, which contain signalling proteins and whose formation is

induced following TCR ligation. However, despite the existence of several studies reporting the intrinsic activity of CTLA-4, these studies are sometimes inconsistent, and a cohesive signalling pathway for CTLA-4 inhibitory function has not yet been reported (Rudd 2008; Walker & Sansom 2011).

(c) **CTLA-4 signalling is ligand-independent.** This model postulates that a splice variant of CTLA-4, consisting of a membrane anchored cytoplasmic molecule without the extracellular domain, inhibits T cell activating by constitutively transducing negative signals. In support of this, the overexpression of this CTLA-4 splice variant in T cells results in inhibition of T cell responses and delayed disease in CTLA-4-KO mice (Chikuma et al. 2005). Thus, CTLA-4 seems to confer inhibitory function in the absence of ligand ligation. However, the fact activated T cells proliferate robustly even expressing substantial levels of CTLA-4 constitutes one limitation to this model.

(d) **CTLA-4 expression keeps T cell mobile,** reducing their dwell time on APCs and thus preventing them from priming. However, T cell motility in CTLA-4-deficient mice is not altered. Therefore, although it is clear that the contact time between T cells and APCs is important in the regulation of T cells responses, the role of CTLA-4 in this context remains unclear (Walker & Sansom 2011).

In addition, the role played by CTLA-4 in regulating antigen-specific T cell responses may also depend on the strength of the stimulatory conditions encountered during activation. When T cells are stimulated below a given activation threshold (weak TCR affinity or impaired antigen presentation, currently reported in melanoma), they will not be able to commit to entering cell cycle. In the absence of CTLA-4 signalling (i.e., with the adjunction of anti-CTLA-4 antibodies), T cell activation would be shifted, so that cells receiving weaker amounts of stimulation could now become activated and proliferate. When T-cell stimulation is optimal (stronger TCR affinity for the epitope as in well differentiated memory T cells), CTLA-4 has a greater capacity to translocate and stabilize to the cell surface, leading to an attenuation of the T-cell response (Egen & Allison 2002). Thus, it could be postulated that blocking CTLA-4 function would shift the responding population toward higher affinity TCRs, resulting in a polyclonal response of reduced diversity.

Extrinsic models of CTLA-4 function

The concept of CTLA-4 being capable of extrinsic T cells regulation emerged after the demonstration that chimeric mice containing a mixed bone marrow of CTLA-4^{-/-} and

normal T cells do not develop lymphoproliferative disease and are healthy (Bachmann et al. 1999). Moreover, the observation that CTLA-4-deficient T cells in such mixed chimeras showed no signs of spontaneous activation suggested that CTLA-4 can function cell-extrinsically, being able to regulate the activation status of other T cells. Additionally, after injection of LCMV in this mice, T cells lacking CTLA-4 became activated, expanded and contracted indistinguishably from CTLA-4-expressing T cells (Bachmann et al. 2001). However, the mechanisms underlying this regulation are not clear, and several models have been proposed that could allow for a cell-extrinsinc function of CTLA-4 (Figure 4).

(e) **CTLA-4 mediates indoleamine 2,3-dioxygenase (IDO) activation.** IDO catalyses tryptophan degradation, resulting in suppressive activity on T cells. It has been reported that CTLA-4-Ig (Grohmann et al. 2002) and CTLA-expressing T_{regs} (Fallarino et al. 2003) induce IDO activation in CD80- and CD86-expressing APCs. However, these findings have not always been reproducible. Indeed, abatacept, a CTLA-4-Ig fusion protein used in clinical settings, does not activate IDO although inducing immune suppression. Additionally, IDO-deficient mice do not develop the lymphoproliferative disease reported in CTLA-deficient mice. Therefore, the link between IDO and CTLA-4 remains unclear (Walker & Sansom 2011).

(f) **CTLA-4 stimulates T_{reg} production of TGF- β .** As cross-linking of CTLA-4 in murine CD4⁺ T cells was shown to induce TGF- β production, which inhibits APC or T cell function (Chen et al. 1998), CTLA-4 in T_{regs} was thought to exert regulatory role on T cells. However, CTLA-4-deficient T cells in mixed bone marrow chimeric mice have the same *Tgfb* mRNA level as wild-type mice, and regulation by CTLA-4-expressing T cells does not seem to rely on TGF- β or IL-10 (Walker & Sansom 2011).

(g) **CTLA-4 can capture its ligands by trans-endocytosis.** It has been shown that T_{regs} can reduce the expression of CD80 and CD86 on dendritic cells *in vitro*, and that down-modulation could be inhibited by anti-CTLA-4 antibody (Oderup et al. 2006). Latter, it was described that CTLA-4 expressed on T_{regs} and on conventional T cells can capture CD80 and CD86 by a process of trans-endocytosis (Qureshi et al. 2011). The transferred ligands are then degraded inside the CTLA-4-expressing cell in lysosomes, consistent with the known lysosomal trafficking of CTLA-4. Capture of ligands by CTLA-4-expressing cells was observed both *in vitro* and *in vivo*, resulting in impaired CD28 costimulation (Qureshi et al. 2011).

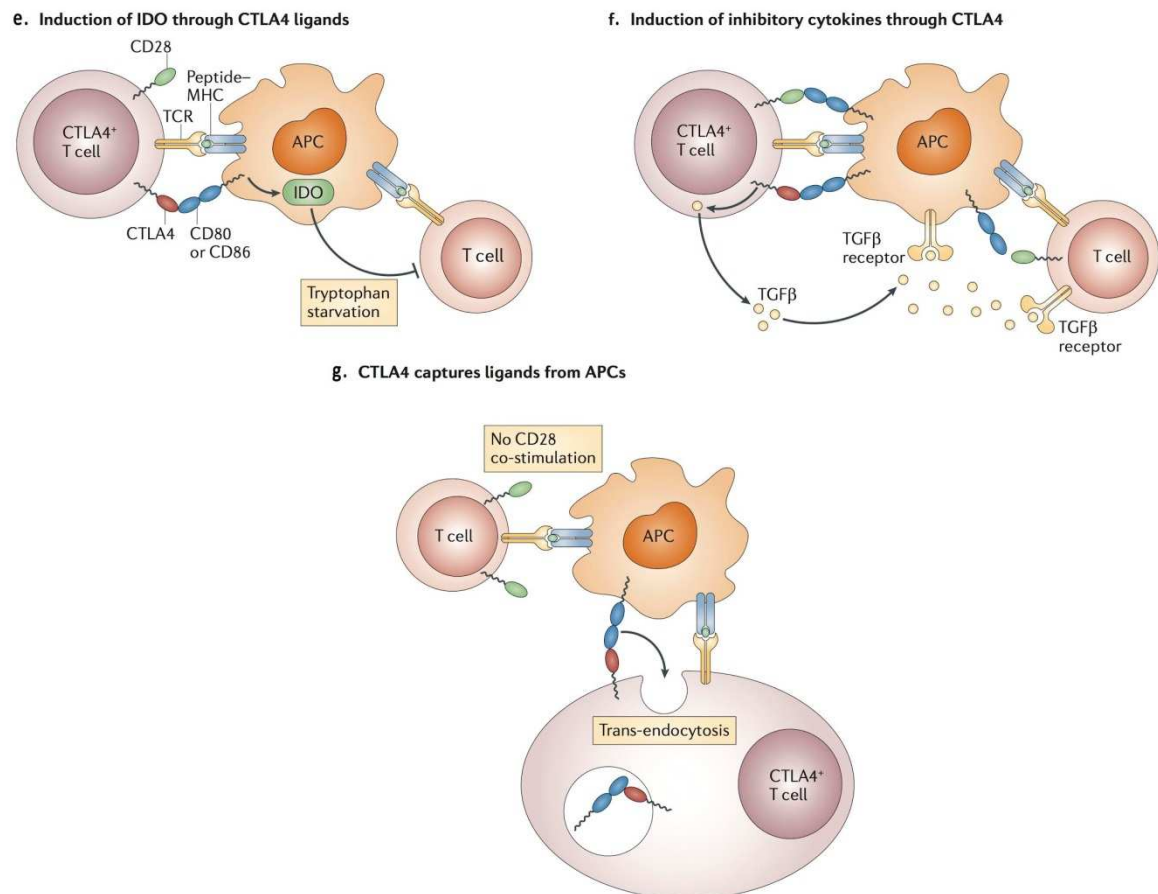


Figure 4 – Extrinsic models of CTLA-4 function.
Adapted from Walker & Samson, *Nature Reviews Immunol.* 2011. 11(12):859

CTLA-4's mode of action remains controversial. The recent model of transendocytosis is consistent with the ligand competition model and would explain the extrinsic role of CTLA-4 suggested by chimeric mice, but intrinsic mechanisms of CTLA-4 cannot yet be excluded. However, it is unlikely that CTLA-4 triggers so many disconnected pathways and the next years will surely elucidate the definite pathway employed by CTLA-4 during inhibitory responses.

5. Immunotherapy in melanoma

The potential of immunotherapy was first documented by William Coley in 1890, reporting the case of a patient with a sarcoma who achieved tumour shrinking with complete remission following a *Streptococcus pyogenes* wound-infection. He then decided to administer a bacteria-containing vaccine to treat cancer patients with dramatic responses, which became known as “Coley’s toxins” infection (Hoption Cann et al. 2003).

In the 1960's, the observation that mycobacteria such as tubercle bacilli of the *BCG* strain inhibited grafted tumours brought back to stage the interest in cancer immunotherapies (Komenaka et al. 2004). One decade later, a review of the clinical features of spontaneous regression of melanoma observed in 73 patients shed light into the immunologic role behind tumour remission (Nathanson 1976). In the 1980's, IL-2-activated lymphocytes were shown to target tumour cells *in vitro*, and exogenous cytokines were investigated in large-scale clinical trials for metastatic melanoma. IFN- α was the first exogenous cytokine to demonstrate anti-melanoma activity, and IFN- α 2b became in 1995 the first immunotherapy approved by FDA for adjuvant treatment for stage IIB/III melanoma. IL-2 was the second exogenous cytokine demonstrating anti-melanoma activity, having been approved by FDA in 1998 for metastatic melanoma. The identification of shared melanoma-associated antigens and cancer-germline antigens expressed by melanoma arose the interest in peptide and protein vaccines, designed to stimulate specific antitumour immune responses (Kirkwood et al. 2008).

One of the main obstacles of immunotherapies is tumour-induced immune suppression. When the role of immune costimulatory and regulatory molecules became elucidated, checkpoint immunotherapies were developed in the hope of overcoming immune tolerance. This chapter will focus on the recent advances in immunotherapy in the context of melanoma.

5.1. Peptide vaccines

The identification of melanoma antigens set the beginning of the development of cancer vaccines, in the view of breaking immunological tolerance and promoting antitumour immune responses against melanoma antigens. Several clinical trials have employed peptide or peptide-pulsed dendritic cells as vaccines, containing melanoma-associated antigens such as MART-1, gp100 and tyrosinase (Wang 2001). Vaccination with dendritic cells loaded with allogenic tumour-cell lysate has been shown to induce antitumour CD8⁺ T cell responses in metastatic melanoma patients (Bercovici et al. 2008). Vaccination with gp100 peptide resulted in high levels of T lymphocytes capable of recognizing melanoma cells *in vitro* and led to a clinical study combining gp100 peptide vaccine and IL-2 in the hope of synergising immune responses against melanoma cells. In this study, the vaccine plus IL-2 group showed an overall clinical response of 16 % in contrast to 6 % in the IL-2-

only group (Schwartzentruber et al. 2011). Although results from numerous clinical trials using melanoma peptides resulted in the presence of CTLs, this immune response did not always correlate with tumour regression, which could be explained by the difficulty in obtaining specific CTLs with high avidity or by tumour escape from immunosurveillance (Garg et al. 2015).

5.2. Adoptive T cell therapy

Autologous T cells have emerged as a powerful immunotherapy option for metastatic melanoma patients. Patient-derived tumour-associated antigens (TAA)-specific T cells are expanded outside the patient's body and re-infused into the blood stream to destroy cancer cells (Mirzaei et al. 2016). These tumour-specific T cells can be generated by several ways, including the expansion of **TILs**, as well as from **T-cells transduced with high-affinity TCRs** against major melanosomal tumour antigens, or by transducing T cells with chimeric antigen receptors (**CAR**) composed of hybrid immunoglobulin light chains with endo-domains of T-cell signalling molecules (Wu et al. 2012).

TILs are isolated from primary or secondary tumours and are then expanded *in vitro* with IL-2. Adoptive cell therapy with TILs in combination with IL-2 have shown early promising results in the context of metastatic melanoma (Rosenberg et al. 1988; Rosenberg et al. 1994), as mentioned in Chapter 2.1. More recently, in a cohort of 93 metastatic melanoma patients treated with autologous TILs administered with IL-2 following different lymphodepleting preparative regimens, ORRs between 49 and 72 % were reported (Rosenberg et al. 2011). Clinical results of various published TILs adoptive cell transfer trials consistently demonstrated ORRs of 40-50 %, with durable complete responses in 10-20 % of metastatic melanoma patients (Itzhaki et al. 2013).

Genetically engineered T cells with high affinity TCRs have been developed in order to broaden the TIL therapy beyond a narrow subset of tumours characterized by extensive T cell infiltrates, as well as to overcome other limitations including the surgery to obtain tumour tissue, and the difficulty to identify TILs specificity, persistence and anti-tumour activity *in vivo* (Ascierto et al. 2015). Current approaches include T cell genetic manipulation to express specific TCRs or CARs that recognize specific tumour antigens.

TCR transgenic T cells are generated from peripheral blood and transduced with a retrovirus encoding for a specific TCR. As a result, a great number of TAA-specific cells

can be generated for adoptive transfer (Wu et al. 2012). In the first clinical trial using TCR-transduced T cells, melanoma patients'-derived PBLs were transduced with the genes encoding the α and β chains of the anti-MART-1 TCR, which were cloned from a TIL clone derived from a metastatic melanoma patient who had achieved complete regression following TILs adoptive cell transfer. The resulting MART-1 TCR-engineered T cells were adoptively transferred into 17 selected HLA-A*0201 metastatic melanoma patients, resulting in durable engraftment and an ORR of 13 % (Morgan et al. 2006). Subsequent clinical trials using a MART-1 or gp-100 higher avidity TCRs resulted in response rates of respectively 30 and 19 % (Itzhaki et al. 2013). However, a non-negligible proportion of patients developed major toxicities related to normal melanocytes destruction. Several studies have thus focused on the cancer testis antigen NY-ESO-1. ORR were reported in four of six patients in a clinical trial where melanoma patients received adoptive cell transfer of autologous T cells genetically engineered to express a TCR against NY-ESO (Robbins et al. 2011). However, the recognition being restricted by a given HLA allele limits this therapy to 30-35 % of melanoma patients. In addition, the transfer of modified T cells with only one selected recognition antigen might lead to tumour escape by antigen loss (Ascierto et al. 2015; Itzhaki et al. 2013). However, and despite low initial clinical ORRs, TAA-specific TCR technology remains a potential option for melanoma patients that are not eligible for TIL adoptive cell therapy.

Chimeric antigen receptors were developed in order to circumvent these limitations. **CARs** are constituted by a single-chain variable fragment derived from a monoclonal antibody specific for a tumour cell-surface molecule, coupled to the transmembrane and cytoplasmic signalling domains of the TCR complex (CD3- ζ) and costimulatory molecules (CD137 or CD28). By including both tumour antigen recognition domains and T-cell signal domains, CARs promote lymphocyte activation upon antigen encounter, and can potentially redirect the effector functions of a T cell towards any protein and non-protein antigens, bypassing the need of MHC-restricted targets and broadening the potential application of adoptive T cell therapy (Mirzaei et al. 2016). CAR T cell treatment has met striking results in the context of hematologic malignancies such as acute lymphocytic leukaemia (Brentjens et al. 2013). Adoptive transfer of CAR-modified T cells for melanoma is in early clinical stages of development, with an ongoing phase I clinical trial (NCT02482532) of CAR T cells targeting the GD2 antigen, a protein with highly restricted expression in melanoma. However, some limitations in this technique still exist, including the requirement of

antigen expression at the cell surface, limited half-life of CAR-modified T cells in the host, and the potential toxicity due to the expression of common antigens on normal cells (Itzhaki et al. 2013).

5.3. Oncolytic viruses

Wild-type and modified oncolytic viruses are designed to destroy cancer cells by directly injecting a virus into the tumour where it both kills the cancer cells and releases GM-CSF that stimulates the immune system. Talimogene laherparepvec (T-VEC) is an oncolytic virus therapy based on a modified herpes simplex virus (HSV) type 1 designed to both replicate in and lyse tumour cells while inducing regional and systemic antitumour immunity. T-VEC saw its approval by FDA in October 2015 for the treatment of melanoma lesions in the skin and lymph nodes after demonstrating, in a phase III clinical trial involving 436 patients with unresectable stage III or IV melanoma, an improvement of overall survival when compared to subcutaneous GM-CSF (Andtbacka et al. 2015).

5.4. Immune checkpoint inhibitors

5.4.1. Anti-CTLA-4 antibodies

When the role of CTLA-4 as an inhibitor receptor of T lymphocytes became clear, it was rapidly hypothesized that blocking CTLA-4/B7 molecules interaction would enhance effector CD4⁺ and CD8⁺ T responses and block T_{reg} inhibitory functions, which allowed the emergence of immunotherapy strategies based on anti-CTLA-4 monoclonal antibodies (mAb). Mouse-derived CTLA-4 blocking antibodies were shown to induce enhanced antitumour immunity and decreased IL-2 production following administration in tumour transplanted mice (Leach et al. 1996).

Two fully human monoclonal antibodies were then developed in the hope of improving patients immune status: the IgG1 κ monoclonal anti-CTLA-4 antibody ipilimumab (MDX-010, Medarex/Bristol-Meyers Squibb) and the IgG2 monoclonal anti-CTLA-4 antibody tremelimumab (CP-675 206, Pfizer).

Although tremelimumab has shown objective response rates of 6.6 % and a median overall survival of 10.0 months in unresectable stage III or IV melanoma patients (Kirkwood et al.

2010), this antibody was abandoned after failing to demonstrate significant survival superiority to chemotherapy in a phase III trial involving 655 treatment-naïve unresectable stage IIIC/IV melanoma patients (Ribas et al. 2013).

Ipilimumab

Ipilimumab, formerly known as MDX-010, was first developed by Dr. James Allison's laboratory at the University of California-Berkeley and later acquired through a patent acquisition by Medarex startup, which became a subsidiary of Bristol-Meyers Squibb in 2009 (Mansh 2011).

A first phase I trial of ipilimumab included 7 metastatic melanoma patients and 2 ovarian carcinoma patients (Hodi et al. 2003). Among them, three metastatic melanoma patients showed extensive tumour necrosis with lymphocyte and granulocyte infiltrates and the 2 ovarian cancer patients were stabilized. No serious toxicities were observed. Another phase I/II study, including 88 patients with metastatic melanoma, compared different schedule and doses of ipilimumab. Grade 3/4 immune-related adverse events (IrAEs) were reported in 14 % of patients, with the highest rate occurring under doses of 10 mg/kg (Weber et al. 2008).

The first phase III trial included 676 unresectable stage III or IV melanoma patients with previous treatment failure. Patients were randomized to receive ipilimumab (3 mg/kg) with or without gp100 vaccine, or to receive gp100 alone (Hodi et al. 2010). Median OS was shown to be improved in the groups receiving ipilimumab (10 months) than in patients receiving gp100 alone (6.4 months). Grade 3/4 IrAEs were observed in 10-15 % of patients treated with ipilimumab.

A second phase III study involved 502 treatment-naïve patients with metastatic melanoma. They were randomly treated with either ipilimumab (10 mg/kg) and dacarbazine or dacarbazine plus placebo. An improved overall survival was observed in the group receiving ipilimumab plus dacarbazine (11.2 vs. 9.1 months), but at the cost of grade 3/4 IrAEs occurring in 56.3 % of patients (Robert et al. 2011).

These phase III trials resulted in the approval of ipilimumab by FDA in 2011 for the treatment of metastatic melanoma at a regimen of 4 doses of 3 mg/kg every three weeks.

Data from recent clinical trials suggest that a subset of melanoma patients following ipilimumab treatment can achieve long-term survival of at least 5 years (McDermott et al. 2014).

Recently, another phase III trial assessed the usefulness of ipilimumab as adjuvant therapy for patients with completely resected stage III melanoma at high risk of recurrence, and included 951 patients who were randomly assigned to receive ipilimumab at 10 mg/kg or placebo (Eggermont et al. 2015). Median recurrence-free survival was 26.1 months in the ipilimumab group, compared to 17.1 in patients receiving placebo. This study led to the approval by FDA of high-dose ipilimumab as adjuvant treatment of melanoma patients with involvement of regional lymph nodes greater than 1 mm in diameter who have undergone complete resection, including total lymphadenectomy (Coit et al. 2016).

Clinical trials are ongoing to test ipilimumab in the treatment of uveal, ocular and mucosal melanoma, lymphoma, neuroblastoma, sarcoma, small cell lung cancer, non-small cell lung carcinoma, hepatocellular carcinoma, renal cell carcinoma, colorectal, prostate, kidney and bladder cancer (<http://www.clinicaltrialsregister.eu/>).

Immune-related adverse events (IrAE)

As reported above, IrAEs are common under ipilimumab therapy and are mainly related to the diminished threshold of T cell activation. A retrospective review of 14 completed phase I-III trials pooling 1498 patients treated with ipilimumab at various doses alone or in combination with other agents reported IrAEs in 64.2 % of patients, with grade 3/4 in 17.8 % of cases (Ibrahim et al. 2011).

The most common IrAEs are rash and pruritis (65 % of patients) with an onset of 2-3 weeks after the beginning of the therapy and are usually managed with topical corticosteroids and antipruritic agents (Quirk et al. 2015). The second most common type of IrAE involves the gastrointestinal tract, being reported in 33 % of patients (mostly diarrhea and/or colitis). Less than 5 % of cases involve the endocrine system (hypophysitis and thyroiditis), liver, eye, kidney, nervous system and pancreas (Ibrahim et al. 2011; Quirk et al. 2015). They can be life threatening, resulting in death in less than 1 % of patients, but they can be managed using specific treatment guidelines allowing the appropriate use of immunosuppressive therapy (Ibrahim et al. 2011).

Although the induction of IrAEs was thought to be associated durable objective response rates (Downey et al. 2007), so far IrAEs are not established as predictors of tumour response under ipilimumab immunotherapy (Kong et al. 2010).

5.4.2. Anti-PD-1 antibodies

As previously described, PD-1 negatively regulates the effector phase of T cell responses, taking place in the periphery. As so, it was early hypothesised that the blockade of this interaction would enhance the preferential activation of cancer-specific T lymphocytes in the periphery, resulting in fewer toxic side effects than those seen with CTLA-4 blockage (Hamid et al. 2013; Ribas 2012) (Figure 5).

Several anti-PD-1 antibodies were developed, including pembrolizumab, nivolumab and pidilizumab.

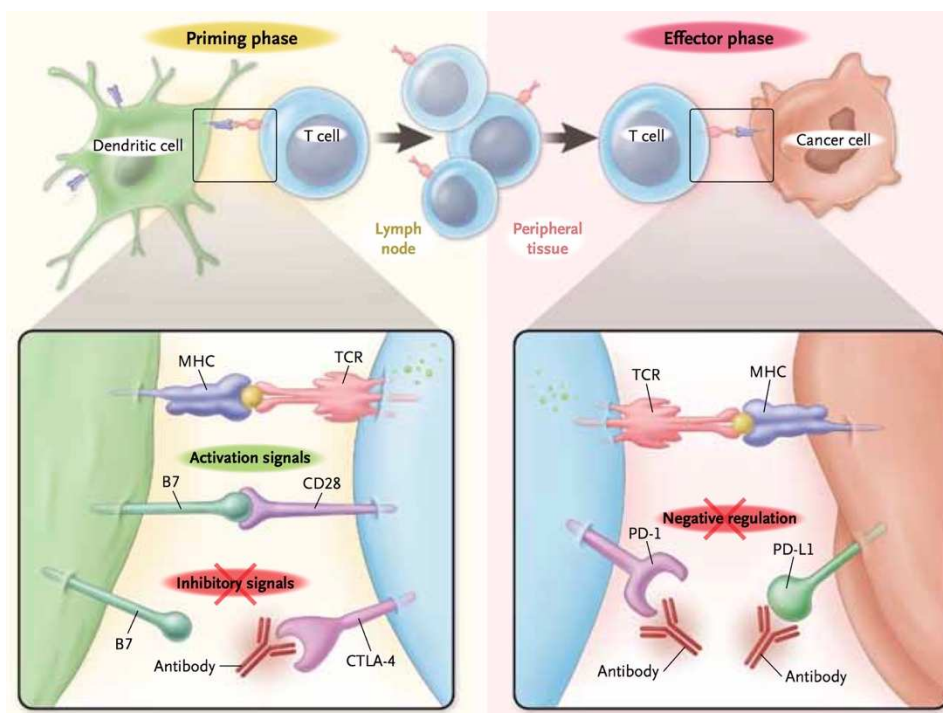


Figure 5 – Blockade of PD-1 or CTLA-4 signalling in tumour immunotherapy.

Source: Ribas *et al.* 2012 N Eng J Med 2012. 366(26):2518

Pembrolizumab

Pembrolizumab is a high affinity humanized IgG4 monoclonal anti-PD-1 antibody, designed to block PD-1 interaction with its PD-L1 and PD-L2 ligands. Pembrolizumab demonstrated ORR of 26 % in a phase I clinical trial involving 173 patients with advanced melanoma whose disease had progressed after at least two ipilimumab doses (Robert et al.

2014). Grade 3 irAEs were reported in 3 % of patients. This study prompted its accelerated approval by FDA in September 2014 for patients with ipilimumab-refractory unresectable or metastatic melanoma.

In a larger phase I clinical trial involving 411 patients with melanoma, ORR was of 34 % and pembrolizumab showed manageable safety profile (Ribas et al. 2014).

Recently, a phase III study (C Robert, Schachter, et al. 2015) demonstrated the superiority of pembrolizumab to ipilimumab, where 834 advanced melanoma patients were randomly assigned to receive pembrolizumab (at two different dosing-schedules) or ipilimumab, resulting in progression-free survival rates of over 47 % for the pembrolizumab group in contrast with 26.5 % in the ipilimumab group. In addition, response rate was over 33 % in the two groups receiving pembrolizumab, and 11 % in the ipilimumab group. Adverse events were also lower in pembrolizumab groups (13.3 and 10.1 vs 19 % in the ipilimumab group).

This prompted the FDA approval of pembrolizumab in December 2015 as a first-line treatment of patients with advanced melanoma.

Finally, an ongoing phase I/II demonstrated that a treatment regimen of pembrolizumab with low-dose ipilimumab was effective and tolerable for patients with advanced melanoma, with 36 % of patients experiencing grade 3/4 drug-related AEs. (Atkins et al. 2015).

Nivolumab

Nivolumab is a fully human IgG4 antibody directed against PD-1 approved by FDA in December 2014 for unresponsive metastatic melanoma patients. This approval followed phase III clinical trial involving 631 unresectable or metastatic melanoma patients that had progressed after ipilimumab or BRAF inhibitor, with ORR of 32 % observed in the group of patients receiving nivolumab alone versus 11 % in the chemotherapy group (Weber et al. 2015). Another study involving 418 previously untreated patients showed superior ORR and overall survival in the group receiving nivolumab alone compared to the group of patients receiving dacarbazine (Robert et al. 2015b).

A recent phase I dose-escalation study (Robert et al. 2015c) involving 142 treatment-naïve metastatic melanoma patients randomly assigned to receive either ipilimumab plus nivolumab or ipilimumab plus placebo resulted in ORR of 61 % in the nivolumab group in

contrast to 11 % in the placebo group. However, grade 3 or 4 AEs were observed in 54 % of patients in the combination therapy in contrast to 24 % receiving ipilimumab alone. Following this study, FDA approved nivolumab in combination with ipilimumab in September 2015 for treatment of patients with wild type BRAF V600.

Another phase III study (Larkin et al. 2015) randomly assigning 945 previously untreated unresectable stage III or IV melanoma patients to nivolumab alone, nivolumab plus ipilimumab or ipilimumab alone showed a progression-free survival of, respectively, 6.9, 11.5 and 2.9 months, further supporting the superiority of nivolumab to ipilimumab, and emphasizing the synergistic effect of immunotherapy combination. However, IrAEs were observed in 54 % of patients receiving the combination therapy in contrast to 24 % of patients receiving ipilimumab alone. On the basis of this study, FDA approved nivolumab as first-line treatment of patients with advanced melanoma in November 2015.

Pidilizumab

Pidilizumab is humanized IgG1 κ recombinant monoclonal anti-PD-1 antibody shown to be capable of inducing T and NK cell mediated tumour regression in experimental human melanoma. A phase II clinical trial involving patients with metastatic melanoma that did not previously respond to ipilimumab or to anti-BRAF therapy demonstrated good tolerability and substantial 12 month-survival comparable to other anti-PD-1 mAbs in heavily pre-treated patients (Atkins et al. 2014). Pidilizumab appears to have a favourable toxicity profile. However, response rates in advanced melanoma appear to be lower than those reported with other anti-PD-1 inhibitors.

5.4.3. Anti-PD-L1 antibodies

Another approach to target the PD-1 inhibitory pathway is through antibodies directed against PD-L1, frequently expressed by melanoma cells. Three anti-PD-L1 antibodies are currently under development: MEDI-4736, an IgG1 antibody with an ongoing phase I clinical trial; MPDL3280A, another IgG1 antibody that recently demonstrated response rates of 21 % in a phase I study including metastatic melanoma patients; and BMS-936559, a humanized IgG4 antibody that demonstrated in a phase I study to extend overall survival in melanoma patients, with just 9 % of patients experiencing grade 3 or 4 treatment-related AEs (Philips & Atkins 2015).

5.4.4. Biomarkers of immune checkpoint inhibition

Immune checkpoint inhibitors provide variable response rates in patients with metastatic melanoma. The identification of biomarkers that may accurately identify the subset of patients that will more likely respond to the different immune checkpoint inhibitors has been the subject of intensive research in the past recent years.

In routine clinical testing, several peripheral blood biomarkers have been examined in the context of ipilimumab and anti-PD-1/PD-L1 inhibition. Absolute lymphocyte counts (ALC) superior to 1000/ μ L after one or two doses of ipilimumab have been shown to correlate with clinical benefit and improved OS in metastatic melanoma patients (Ku et al. 2010; Di Giacomo et al. 2011; J Delyon et al. 2013; Bjoern et al. 2016; A. Martens et al. 2016). By contrast, ALC levels during treatment were not predictive of ORR when melanoma patients were submitted to nivolumab alone or in combination with ipilimumab (Johnson et al. 2015; Friedman & Postow 2016). Early studies showed the prognostic utility of absolute neutrophil counts and the neutrophil-to-lymphocyte ratio in solid tumours (Sirott et al. 1993), with recent studies reporting higher baseline levels associated with worse prognosis in advanced melanoma patients submitted to ipilimumab (Ferrucci et al. 2016; Zaragoza et al. 2016). An early increase in absolute eosinophil counts associated with improved OS in advanced melanoma patients treated with ipilimumab (Delyon et al. 2013) and with anti-PD-1 therapy (Friedman & Postow 2016) has also been described.

LDH has also been the subject of several ipilimumab studies, with lower levels having been associated with better ORR and OS in metastatic melanoma patients (Di Giacomo et al. 2011; Simeone et al. 2014; Valpione et al. 2015; Weide et al. 2016). High relative levels of eosinophil and lymphocyte counts, as well as low levels of LDH were also shown to favour OS in melanoma patients submitted to pembrolizumab (Weide et al. 2016).

However, to date, none of these routinely monitored biomarkers are established as predictive of immune checkpoint treatment response or survival.

Besides routinely measured biomarkers, other biomarkers have also been studied in the context of immune checkpoint inhibitors. Better response to ipilimumab was shown to be associated with the presence of circulating NY-ESO-1 serum antibodies or NY-ESO-1-specific CD4⁺ and CD8⁺ T cells (Yuan et al. 2008; Yuan et al. 2011), as well as with Melan-A-specific CD8⁺ T cells (Klein et al. 2009), but did not always met reproducibility (Goff et al. 2009). Interestingly, Weide *et al.* (Weide et al. 2014) found an inverse

correlation between the presence of melanoma-specific T cells and the levels of myeloid-derived suppressor cells (MDSC), an immune-suppressive cell population, suggesting a causal effect with clinical relevance. Other studies reported a negative influence of higher levels of circulating MDSC, as well as of T_{reg}, in ORR and OS under ipilimumab treatment (Simeone & Ascierto 2012; Kitano et al. 2012; Martens et al. 2016).

The tumour microenvironment has also received great attention in the biomarker setting as it is thought to influence the outcome of patients submitted to immune checkpoint inhibitors. Early increase in TILs under ipilimumab treatment has been associated to better clinical activity (Hamid et al. 2011). Moreover, under pembrolizumab, higher levels of CD8⁺, PD-1 and PD-L1-expressing cells at the invasive tumour margin and inside tumours were observed in responding metastatic melanoma patients, in contrast to non-responders (Tumeh et al. 2014). Several studies focused on PD-L1 expression by tumours, as their engagement by PD-1-expressing T cells promotes tumour evasion. In a phase I trial with nivolumab, an association was found between patients bearing PD-L1-positive tumours and ORR (Topalian et al. 2010). Others described similar findings (Grosso et al. 2013), although metastatic melanoma patients appeared to have clinical benefit under nivolumab regardless of tumour PD-L1 expression.

Unfortunately, none of the described peripheral blood and tumour-based biomarker have been prospectively validated as markers allowing a pre-selection of patients for anti-CTLA-4 and anti-PD-1/PD-L1 immunotherapies.

OBJECTIVES

This PhD thesis was composed of three parts based on three main objectives:

- 1) To identify potential **melanoma circulating biomarkers** associated with ipilimumab treatment response, involving the longitudinal assessment of patients' levels of LDH (a protein excreted by tumours in high hypoxia conditions), S100B (a melanocytic protein involved in the downregulation of p53 and apoptosis in melanoma), MIA (a protein involved in the extravasion and metastatis of melanoma), soluble MICA (frequently found in tumours and known for promoting NKG2D downregulation in NK and CD8⁺ T lymphocytes), and anti-MICA antibodies.
- 2) To identify potential peripheral **immune-related biomarkers** associated with ipilimumab treatment response, with a focus on T lymphocytes, memory T cell compartments including the recently identified stem-cell memory T subset, as well as on chemokine receptors, effector cytokines such as IFN- γ , TNF- α and granzyme B and the transcription factors Eomes and T-bet;
- 3) Based on potential identified immune-related biomarkers, to perform *in vitro* studies with patients'-derived peripheral blood mononuclear cells allowing a better insight of the immune actors and cellular mechanisms involved in melanoma responses to ipilimumab.

RESULTS

Article 1

Ipilimumab reshapes T cell memory subsets in melanoma patients with clinical response

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Purpose: Metastatic melanoma is an aggressive tumour whose prognosis remains poor, despite recent impressive advances in melanoma treatment approaches. Biomarkers of response to innovative melanoma therapies that would allow a better patient management as well as a pre-selection of patients to different arising immune- and targeted therapies are lacking. Current FDA-approved ipilimumab therapy provides extended survival in patients with advanced metastatic melanoma. However, the current monitoring of this anti-CTLA-4 therapy doesn't allow the early detection of the potential for clinical benefit.

Experimental design: Peripheral blood mononuclear cells from a cohort of 77 metastatic melanoma patients treated with ipilimumab after failure of at least one previous treatment were analysed before each treatment dose (at 3 weeks intervals), for a total of 4 doses, in order to identify potential immune-related biomarkers to the therapy.

Results: Absolute lymphocyte counts (ALC) at the baseline were associated with better clinical outcome and expanded overall survival. An early expansion of CD4⁺ and CD8⁺ T lymphocytes including central and effector memory T cells was observed, correlating with better response to treatment. Frequency of stem-cell memory T cells was shown to decrease following the first two doses despite enhanced proliferation, suggesting a process of differentiation. Increased T-bet and Eomes transcription factors were detected in CD8⁺ cells, with higher increases in Eomes levels correlating with disease control. Higher levels of granzyme B and TNF- α expression, as well as of CXCR3, CCR4 and CCR6 were observed, suggesting enhanced effector functions and migration capacities.

Conclusion: Ipilimumab reshapes the memory T cell compartment, involving central, effector, terminal differentiated and stem-cell memory cells. Moreover, CTLA-4 blockade favours the expression of master transcription factors essential in effector T functions and memory homeostasis, as well as of chemokine receptors required for tumour and skin homing.

ORIGINAL RESEARCH

Ipilimumab reshapes T cell memory subsets in melanoma patients with clinical response

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ABSTRACT

Purpose: Therapy targeting CTLA-4 immune checkpoint provides increased survival in patients with advanced melanoma. However, immunotherapy is frequently associated with delayed and heterogeneous clinical responses and it is important to identify prognostic immunological correlates of clinical endpoints. **Experimental design:** 77 patients with stage III/IV melanoma were treated with ipilimumab alone every 3 weeks, during 9 weeks. Blood samples were collected at the baseline and before each dose for in depth immune monitoring. **Results:** The median follow-up was 28 mo with a median survival of 7 mo. Survival and clinical benefit were significantly improved when absolute lymphocyte count at the baseline was above $1 \times 10^9/L$. Notably, ipilimumab had a global effect on memory T cells, with an early increase of central and effector subsets in patients with disease control. By contrast, percentages of stem cell memory T cells (TSCM) gradually decreased despite stable absolute counts and sustained proliferation, suggesting a process of differentiation. Higher proportions of $eomes^+$ and $Ki-67^+$ T cells were observed, with enhanced skin homing potential and induction of cytotoxic markers. **Conclusion:** These results suggest that CTLA-4 blockade is able to reshape the memory subset with the potential involvement of Eomes and memory subsets including TSCM.

Abbreviations: ALC, absolute lymphocyte count; CLA, cutaneous addressin; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; DC, disease control; eomes, eomesodermin; HD, healthy donor; HR, Hazard Ratio; IrAEs, immune-related adverse events; NR, non-responder; OS, overall survival; PBL, peripheral blood lymphocyte; TCM, central memory T cell; TEM, effector memory T cell; TEMRA, terminal effector memory T cell; TIL, tumor-infiltrating lymphocyte; TN, naive T cell; TSCM, stem cell memory T cell; TTM, transitional memory T cell

ARTICLE HISTORY

Received 7 October 2015
Revised 17 December 2015
Accepted 18 December 2015

KEYWORDS

CTLA-4; immunotherapy; ipilimumab; melanoma; memory T cells; TSCM

Introduction

Melanoma is one of the most rapidly spreading cancers in terms of worldwide incidence. Until recently, survival outcomes for stage IV melanoma patients treated with standard therapies such as dacarbazine have been poor, with a median survival less than 8 mo and a 5-y survival rate of approximately 10%.^{1,2} Melanoma is a long-lasting cancer known for its ability to induce specific immunological responses, providing the first evidences of successful immunotherapy by activating the immune system with high doses of IFN- α or IL-2.³ The use of autologous tumor-infiltrating lymphocytes (TIL) in metastatic melanoma by Rosenberg and colleagues⁴ Three decades ago emphasized the potential role of tumor-specific T cells⁵ and paved the way for the development of melanoma specific vaccines. However, patients with detectable tumor Ag-specific T cells can still develop progressive metastatic melanoma. Results

from many clinical trials indicate that the common denominator of therapy benefit in melanoma is the augmentation of host immunity. Recent advances in the understanding of the complex cellular mechanisms regulating cancer immunity have led to the development of new strategies aimed at targeting specific immune-checkpoints.⁶ The early stages of T cell activation are regulated by the interaction of B7-1 and B7-2, whose expression is restricted to professional antigen presenting cells, with their counter receptors CD28 and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4).⁷ The costimulatory signal through CD28 lowers the threshold for full activation, allowing lower affinity T cell clones to become activated. Subsequently, CD28 primes the system for regulation by increasing CTLA-4 on the T cell surface (for a review, see ref.⁸). CTLA-4 is a key element in immune tolerance and a main negative regulator of T-cell-mediated antitumor immune responses. It out competes

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✉ Supplemental data for this article can be accessed on the publisher's website.

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the interaction of CD28-B7 molecules and antagonizes early T cell activation, IL-2 production, cell cycle progression and modulation of TCR signaling.⁹ Therefore, blockade of CTLA-4 aims to prevent the attenuation of T cell activation, potentiating tumor-specific T cells.^{10,11} Ipilimumab, previously known as MDX-010, is a fully humanized IgG1 monoclonal antibody against the extracellular domain of CTLA-4. Since the initial human studies in previously vaccinated melanoma patients,^{12,13} several trials have assessed the efficacy of anti-CTLA-4 therapy alone or in association with targeted therapy, chemotherapy, peptide vaccine or other immunotherapies (for a review, see ref.¹⁴). A survival benefit with ipilimumab was demonstrated in two pivotal phase III trials in a subset of patients with advanced melanoma, leading to its approval by the US Food and Drug Administration in March 2011.^{15,16} Data from subsequent clinical trials also suggested that a proportion of patients treated with ipilimumab can achieve survival of at least 5 y.¹⁷ A recent pooled analysis of 12 studies reported a remarkable effect in terms of durability illustrated by a 3-y survival rate of 22%.¹⁸

However, this therapy appears to benefit only a subset of patients. Consequently, identifying prognostic immunologic correlates of clinical endpoints is a challenging issue. Although durable objective clinical responses are found to be associated with the induction of immune-related adverse events (irAEs),¹⁹ autoimmune disorders do not predict improved antitumor response.²⁰ Immunological monitoring has always constituted a relevant aspect of completed and ongoing clinical trials. Several immunologic endpoints with a strong biologic rationale are proposed to correlate with clinical activity. Increased absolute lymphocyte count (ALC) during the therapy is found to be associated with clinical benefit and overall survival (OS) in uncontrolled studies.^{21,22,23,24} In a clinical trial using tremelimumab, another humanized anti-CTLA-4, increased levels of memory and activated T cells were reported in 3 out of 12 patients achieving disease control (DC).²⁵ Several reports

demonstrate immunological changes under ipilimumab therapy, shedding light on *in vivo* mechanisms of anti-CTLA-4, but their pertinence to predict clinical responses and OS remains to be clarified.²⁶⁻²⁹ In this report, we present results from the longitudinal immunological monitoring of a cohort of 77 ipilimumab-treated patients. The extended characterization of peripheral lymphocyte subsets allowed us to define early markers of survival and/or clinical response, such as ALC at the baseline. We report major changes within the memory T cell subsets, which are associated with response to the treatment, and a potential implication of T memory stem cells (TSCM).

Results

Patient clinical characteristics, response to treatment and immune-related adverse events

The majority of patients included in this study were stage IV (90%) (Table 1). The median follow-up was 28 mo with a median survival of 7 mo in the cohort of patients treated with ipilimumab alone (95% IC 6–10). DC group was defined as patients achieving complete response (CR), or partial response (PR) or stable disease (SD) at week 16, whereas NR group included patients with progressive disease (PR) or death before week 16. DC was reported in 30% of cases. 52 patients received the total course of four cures ipilimumab and presented a better clinical response at week 16, with 35% of these patients achieving DC, compared to 24% in the group of patients receiving less than four doses of ipilimumab ($p = 0.01$). This was expected since the number of doses of anti-CTLA-4, reflecting the continuation of the therapy, depends on a good tolerability of the treatment by the patient, potentiating a better response. The overall survival was however not affected by the dose number of anti-CTLA-4 (data not shown). Patients receiving less

Table 1. Patients characteristics. The whole cohort is described as well as the two groups of patients treated or not with the full course of four cures of ipilimumab. Adverse events were graded according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (version 4.0).

Patients	Whole cohort n = 77	< Four cures of ipilimumab n = 25	Four cures of ipilimumab n = 52	p value
Age, mean (SD) y	59.8 (16.6)	59.8 (16.3)	59.8 (17.0)	1
Women, no. (%)	35 (45)	11 (44)	24 (46)	1
AJCC stage at inclusion, no. (%)				0.81
IIIc	8 (10)	4 (16)	4 (8)	
IV	4 (5)	1 (4)	3 (6)	
IVM1a	2 (3)	0	2 (4)	
IVM1b	7 (9)	2 (8)	5 (10)	
IVM1c	56 (73)	18 (72)	38 (73)	
Response at week 16 (WHO), no. (%)				0.01
CR	1 (1)	0	1 (2)	
PR	4 (5)	0	4 (8)	
SD	19 (25)	6 (24)	13 (25)	
PD	36 (47)	8 (32)	28 (54)	
Death < w16	17 (22)	11 (44)	6 (12)	
Overall survival (95 % CI)				0.10
at 6 mo	62 % (52 to 74)	36% (21 to 61)	75% (64 to 88)	
at 12 mo	25 % (17 to 37)	20% (9 to 44)	28% (18 to 43)	
at 24 mo	11 % (5 to 21)	12% (4 to 35)	10% (4 to 24)	
Adverse events, no. (%)				
Total	36 (47)	15 (60)	21 (40)	0.14
Grade 3	16 (21)	10 (40)	6 (12)	0.007
Grade 4	2 (3)	1 (4)	1 (2)	0.55

than four doses were the ones with a higher frequency of grade 3 irAEs ($p = 0.007$), resulting in treatment discontinuation.

IrAEs occurred 49 d in median after the beginning of treatment with values ranging from 7 to 186 d. The most clinically significant IrAE was enterocolitis (grade III/IV in 14% of cases) followed by rash/pruritus or hepatitis (5%). These IrAEs were in most cases treatable with vigilance and early intervention with corticosteroids. Of note, we did not find any correlation between patients who develop IrAEs and those who achieved clinical benefit (data not shown).

ALC at the baseline is a predictive marker of survival and clinical response

The success of therapies aimed at immune checkpoints relies on the ability of the immune system to mount specific and sustained antitumor responses. Therefore, the immune status at the baseline may be especially relevant. Our results showed that ALC before Ipilimumab initiation was lower in patients when compared to healthy donors (HD) (median = $1.18 \times 10^9/L$ versus median = $1.58 \times 10^9/L$ respectively, $p = 0.00008$). This was mainly due to a defect in both $CD4^+$ ($p = 0.005$) and $CD8^+$ T cells ($p = 0.006$), with a more pronounced defect in effector memory $CD8^+$ T cells ($p < 10^{-6}$). B and NK-cell counts were similar in patients and HD (data not shown). Characteristics of patients T cells at the baseline are represented in Figs. 1A and B and are consistent with an activated phenotype, as evidenced by increased percentages of HLA-DR+ and granzyme B+ $CD8^+$ T cells and a lower expression of CD28 on $CD8^+$ T cells. Although not significant, a shift toward a higher CTLA-4 expression was detected on memory $CD8^+$ T cells of patients when compared to healthy donors (HD) ($p = 0.057$).

In addition, the cutaneous addressin CLA, as well as CCR4 and CXCR3 were expressed at higher levels in patients.

We then delineated a threshold value of ALC = $1 \times 10^9/L$ below which only 2.5% of HD was found. A median survival of 11 mo (95%CI 7–14) was observed in the group with a baseline ALC $\geq 1 \times 10^9/L$, vs. 5 mo (95%CI 3–10) in the group with ALC $< 1 \times 10^9/L$ ($p = 0.003$) (Fig. 1C). The prognostic impact of baseline ALC was still significant when adjusting for baseline LDH (Hazard Ratio HR = 0.49, 95%CI = 0.28–0.86) or for the use of glucocorticoids (HR = 0.5 95%CI 0.29–0.88). Moreover, the proportion of patients achieving disease control was higher in the group with an ALC $\geq 1 \times 10^9/L$ at the baseline (44% vs. 19% if ALC $< 1 \times 10^9/L$, $p = 0.04$).

Ipilimumab induces early immunological changes of T cell subsets

A significant increase in ALC, $CD4^+$ and $CD8^+$ T cells absolute counts was observed 3 weeks after the first dose of ipilimumab ($p = 0.0006$, $p = 0.0002$ and $p = 0.008$, respectively) with values remaining stable from week 3 to week 12 (data not shown). A marked increase in HLA-DR activated T cells was also reported, lasting at least over 12 weeks for $CD8^+$ T cells (Fig. 2A). An early and transient upregulation of CD25 on $CD4^+$ and $CD8^+$ T cells was observed at week 3 (data not shown).

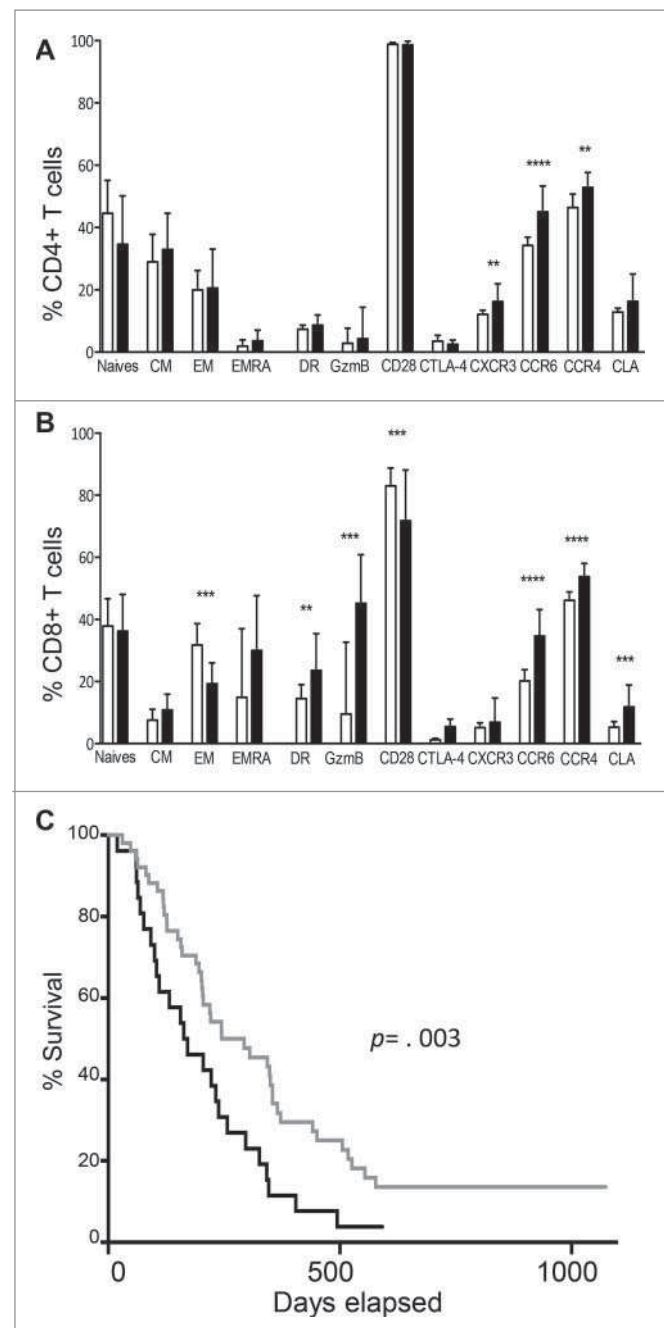


Figure 1. Patients immunological status at the baseline. Percentages of $CD4^+$ (A) and $CD8^+$ (B) T cell subsets (CM for central memory, EM for effector memory, EMRA for terminally effector memory, GzmB for Granzyme B). Results from HD and patients are shown as open bars and black bars respectively. *for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$. (C) Kaplan–Meier survival curves from patients with ALC $\geq 1 \times 10^9/L$ (gray line, $n = 43$) or $< 1 \times 10^9/L$ (black line, $n = 26$) at the baseline.

Naive and memory T cell subsets were identified based on CD45RA and CCR7 expression. Naive cells (TN) were characterized as $CD45RA^+ CCR7^+$, central memory T cells (TCM) had the $CD45RA^+ CCR7^+$ phenotype, effector memory T cells (TEM) were defined by the lack of expression of these markers and terminal effector memory (TEMRA) exhibited $CD45RA^+ CCR7^-$ phenotype (Fig. S1A). An early effect of ipilimumab was detected on $CD4^+$ and $CD8^+$ TCM and TEM subsets with an increase in both percentages and absolute counts

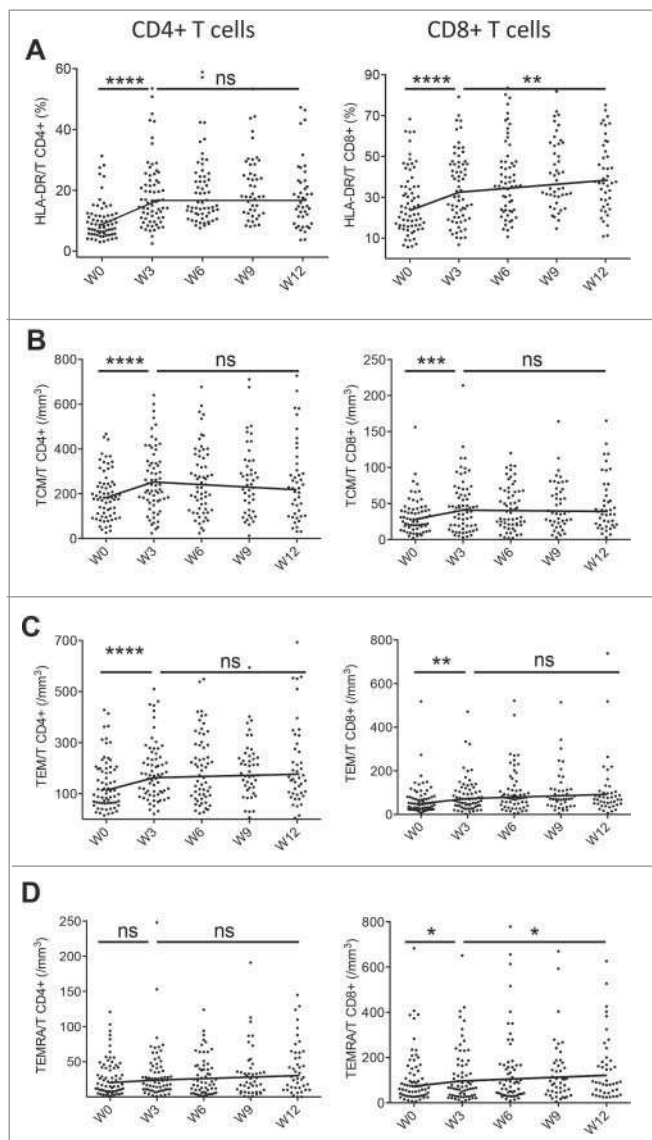


Figure 2. Ipilimumab induces activated and memory T cells. The two slope model is visualized by the black line. CD4⁺ and CD8⁺ T cell subsets are presented in the left and the right panels respectively. (A) Percentage of HLA-DR⁺ T cells, absolute counts per mm³ of (B) CM, (C) EM and (D) EMRA. The same results are observed in terms of percentages of parental subsets (data not shown). * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$.

(Figs. 2B, C), whereas the increase of CD8⁺ TEMRA subset was delayed (Fig. 1D). These results suggest that the major dynamic changes between naïve and memory subsets operate within 3 weeks after the first injection of ipilimumab.

ICOS (CD278) is a receptor belonging to the CD28/CTLA-4 family expressed upon T-cell activation.³⁰ It has been shown that ICOS critically controls the pool size of both effector memory and regulatory T cells.³¹ In our study, comprising the global cohort of 77 patients, no significant changes in the frequency of ICOS⁺ CD4⁺ T and CD8⁺ T cells from baseline to week 6 were observed (Fig. S2).

Eomes and T-bet are the two T-box transcription factors which are involved in the generation and function of effector and memory CD8⁺ T cells. Our results showed that ipilimumab treatment was associated with the expansion of lymphocytes expressing eomes (CD4⁺ and CD8⁺ T cells) and T-bet

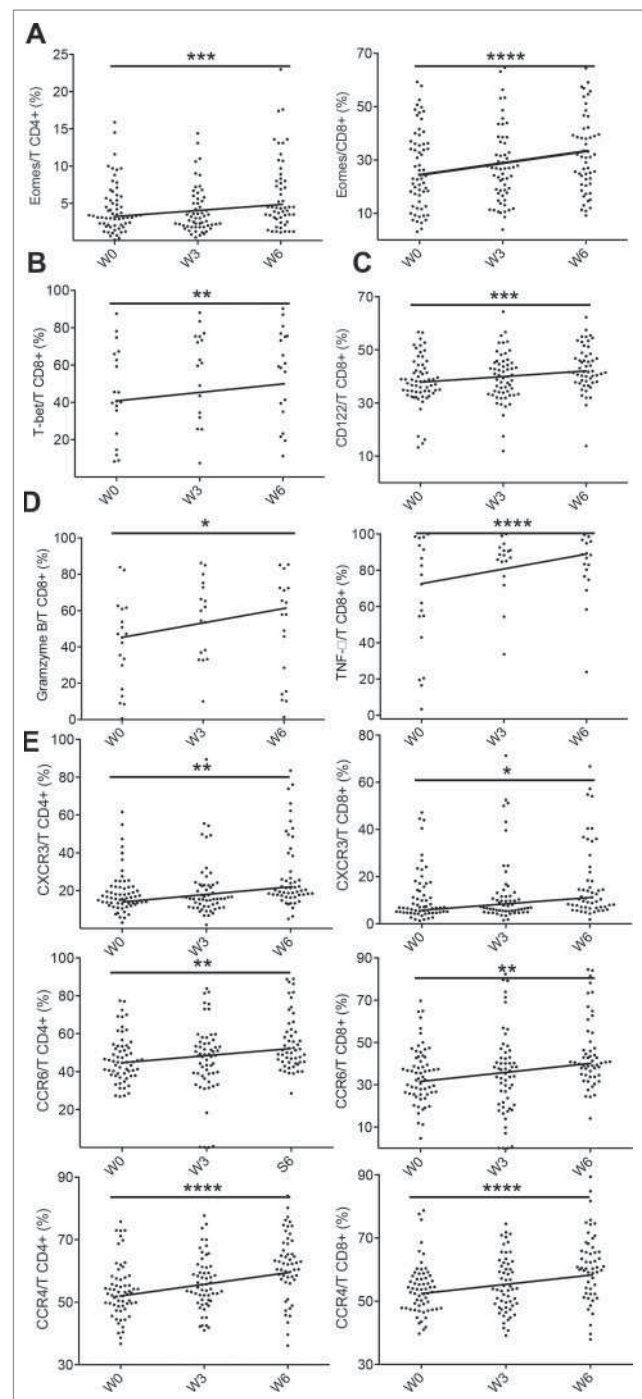


Figure 3. Pharmacodynamic changes during ipilimumab therapy. Expression of eomes on CD4⁺ and CD8⁺ T cells (A), T-bet (B) and CD122 (C) on CD8⁺ T cells. Cytotoxic markers expression (GzmB and TNF-α) on CD8⁺ T cells (D). Evolution of chemokine receptor expression in T cells (E). Note: T-bet and GzmB were evaluated on 20 patients as mentioned in material and method section. * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$.

(CD8⁺ T cells) (Figs. 3A and B). These transcription factors cooperate to maintain memory CD8⁺ T cells homeostasis through the expression of IL-2Rβ (CD122), favoring IL-15 mediated signaling and proliferation of memory T cells.³² The increased expression of CD122 (Fig. 3C) on CD8⁺ T cells was consistent with the enhanced expression of T-bet and eomes. In addition, cytotoxic molecules such as TNF-α and granzyme B were induced on CD8⁺ T cells but not in CD4⁺ T cells during the course of treatment, whereas no changes in IFNγ

expression were observed in T cells (Fig. 3D and data not shown). Finally, CTLA-4 blockade had an effect on chemokine receptors with an expansion of CXCR3, CCR6 and CCR4 positive T cells from week 0 to week 6 (Fig. 3E).

Occurrence of IrAEs is not associated to any of the biomarkers tested in this study

Although targeted immunotherapies such as ipilimumab are well related to the induction of IrAEs, we did not find any association between the occurrence of such events and the activation status of lymphocytes, assessed by CD25, HLA-DR and ICOS expression on T cells, at baseline and during the course of immunomonitoring. Similarly, none of the biomarkers used in our experimental design was found to be associated with the development of IrAEs (data not shown).

Clinical response is associated to the generation of memory T cells with a potential role of T memory stem cells (TSCM)

An increase in ALC between week 3 and week 12 was significantly associated with DC ($p = 0.013$). Importantly, although no association between the evolution of total count of CD4⁺ T cells and clinical response was observed, the early raise of

CD4⁺ TCM and TEM (from week 0 to week 3) was significantly higher for patients achieving DC than for non-responders (NR) (Fig. 4A). This early augmentation in CD4⁺ TEM was followed by an increase between week 3 and week 12 in patients achieving DC, and a decrease in patients with no response (median slope = -2.1 and $+5.9$ in NR and DC respectively, $p = 0.025$).

In addition, patients with DC had a slightly delayed, but sustained increase of effector and terminal effector memory CD8⁺ T cells (Fig. 4B) with a higher proportion of CD8⁺ T cells expressing eomes (median slope = 0.7 and 2.1 in NR and DC respectively, $p = 0.008$) (Fig. 4C). Of note, the two response groups had similar immunological profiles in terms of ICOS⁺ T cell evolution (data not shown).

Further phenotypic characterization of memory T cells was performed for 20 patients (10 DC and 10 NR, randomly chosen), allowing the study of transitional memory T cells TTM (CD45RA-CCR7-CD27⁺)³³ and the recently identified TSCM (defined as CD45RA+CCR7+CD27+CD95⁺) (Fig. S1B).³⁴ Upon ipilimumab therapy, percentages of both CD4⁺ and CD8⁺ TSCM significantly decreased whereas absolute counts remained unchanged (Figs. 5A and B). In order to analyze the dynamics amidst memory T cell subsets, we followed the expression of the Ki67 protein, a cellular marker for proliferation and cell cycling.³⁵ Ipilimumab treatment resulted in a significant increase of CD4⁺ and CD8⁺ T cell proliferation (median slope = $+0.6$, $p < 10^{-15}$ and $+0.8$, $p < 10^{-5}$, respectively), involving TCM, TTM and TEM subsets, and remaining barely detectable in naive T cells (Fig. 5C). Notably, both CD4⁺ and CD8⁺ TSCM had significant proliferative activity and CD4⁺ TSCM exhibited an enhanced proliferative capacity from week 0 to week 6 (median slope = $+0.4$, $p = 0.034$).

Discussion

Anti-CTLA-4 is the first therapeutic agent aimed at overcoming cancer immune tolerance. It relies on the ability of T cells to enhance antigen recognition by shifting their activation threshold.

Prior to ipilimumab therapy, patients are characterized by a lymphopenia, which may reflect either the tumor burden, through tumor-derived factors,³⁶ or previous conventional therapies such as dacarbazine.³⁷ In the present study, we reported that ALC above $1 \times 10^9/L$ before therapy was significantly associated with an extended survival and disease control. Moreover, only patients mounting a sustained expansion of total lymphocytes, as well as CD4⁺ and CD8⁺ T cells during the course of immunotherapy were associated with a higher rate of disease control at week 16. Our data reinforce previous results from clinical trials involving either ipilimumab at a higher dose or another CTLA-4 abrogating antibody, tremelimumab.^{21,22,23,38} Prior to treatment, patients' circulating T cells displayed a phenotype compatible with a chronic activation by the tumor, with a lower expression of the costimulatory molecule CD28 and increased percentage of HLA-DR and granzyme B positive CD8⁺ T cells, when compared to HD. In addition, although not significant, a trend toward higher percentages of TCD8⁺ T cells expressing CTLA-4 by was also observed, suggesting a post-activation status of T cells. An early expansion of CD4⁺ and CD8⁺ T cells was reported at the induction of

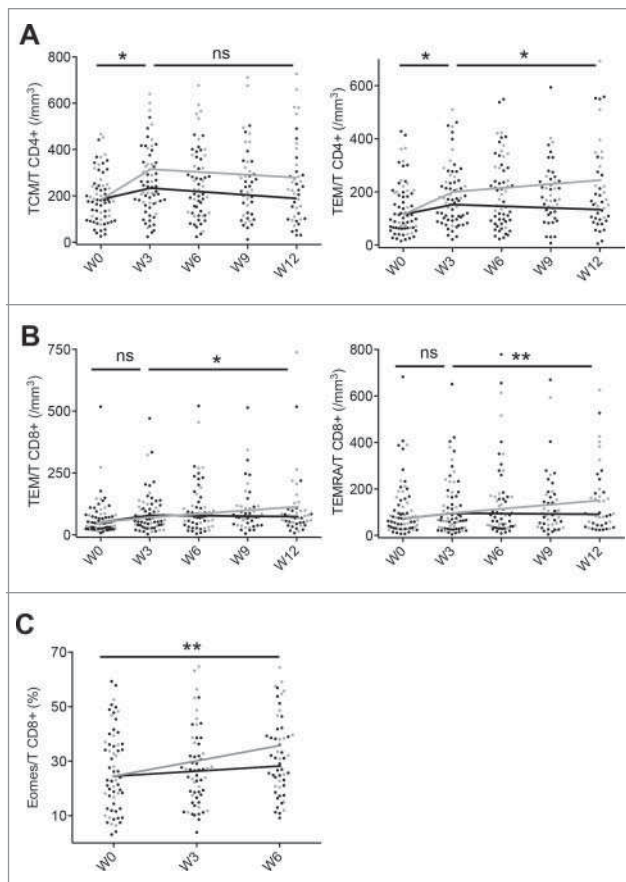


Figure 4. CTLA-4 blockade and markers associated with clinical response. Patients were subdivided in two groups, DC (gray line and dots) and NR (black line and dots). Clinical benefit is associated with a more marked increase of memory subsets (A and B) and of eomes expressing CD8⁺ T cells (C). *for $p < 0.05$, ** for $p < 0.01$

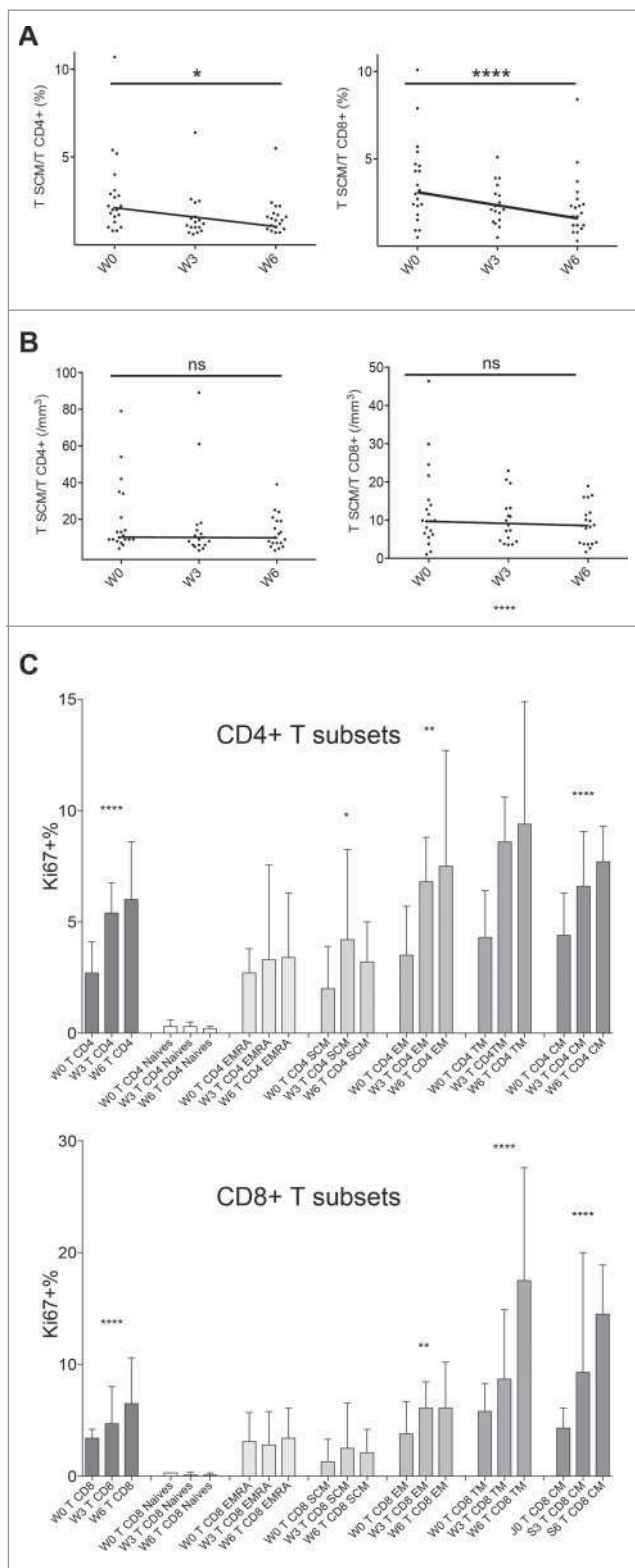


Figure 5. Ipilimumab induces a dynamic interplay between TSCM and classical memory T cells. Among memory subsets, only TSCM decrease in percentages (A) with constant absolute counts (B). The proliferative capacity of naïve, memory and TSCM subsets was assessed by Ki67 expression (C).

immunotherapy, with CD8⁺ T cells gradually expressing higher levels of granzyme B and TNF- α .

A correlation between CTLA-4 blockade and ICOS induction has been reported in several studies.³⁹ An increase of

ICOS^{hi} CD4⁺ T cells producing IFN γ was first observed in six bladder cancer patients, in the absence of any association with clinical outcome.⁴⁰ In a subsequent study, Carthon *et al.* showed that increases in ICOS^{hi} CD4⁺ T cells were more pronounced with 10 mg/kg ipilimumab treatment than with 3 mg/kg. In addition, these authors correlated the increased and sustained frequency of ICOS^{hi} CD4⁺ T cell to improved overall survival in an independent cohort of 14 melanoma patients treated with 10 mg/kg ipilimumab.⁴¹ More recently, in a study analyzing 36 patients treated with ipilimumab, an increased frequency of ICOS⁺ CD4⁺ T cells was proposed as a pharmacodynamic biomarker of CTLA-4 blockade activity at the dose of 3 mg/kg.²⁶ We were not able to confirm these observations in our cohort of 77 patients. Further investigations are therefore needed in order to confirm these data and to better characterize the phenotype of ICOS⁺ CD4⁺ T cells in terms of memory subsets.

Chemokines are critical mediators of routine immune surveillance, providing directional signals for cell trafficking.⁴² Some of these molecules orchestrate the migration of T cells to the skin. CCR4 is involved during the first step of T cell skin homing, followed by the extravasation to the skin due to CLA interaction with its ligand, E-selectin. In addition to other T cell types, CCR6 is expressed on a T cell subset with skin homing properties.⁴³ CXCR3 is a chemokine receptor that favors migration of Th1 cells and effector CD8⁺ T cells into inflamed tissues⁴⁴ and tumors.⁴⁵ Before CTLA-4 blockade, patients with advanced melanoma had a higher proportion of CD4⁺ and CD8⁺ T cells expressing CXCR3, CCR6, CCR4 and/or CLA, suggesting an increased capacity of T cells to relocate to the skin. This phenotype was observed in all patients at the baseline, regardless of clinical responses. Furthermore, ipilimumab therapy potentiated the expression of these chemokine receptors on both CD4⁺ and CD8⁺ T cells. A previous study, where patient-derived melanoma-specific T cell lines were analyzed, reported a positive association between survival and the expression of CXCR3 and CCR4 by T-cells, highlighting the importance of these molecules in the maintenance of effective *in situ* antitumor immunity.⁴⁶ Our data further emphasize the potential of CTLA-blockade in the induction of tumor infiltrating cells, already demonstrated in previous reports.^{47,48}

Upon therapy, we observed an expansion of memory subsets, with an increase of central and effector memory T cells, followed by a gradual raise in TEMRA cell counts. More importantly, our data showed for the first time a significant association between clinical benefit at week 16 and an early increase of CD4⁺ TCM and TEM followed by a delayed increase of TEMRA (Fig. 4), consistent with the stepwise differentiation pattern of memory subsets from TCM to TEMRA.⁴⁹ There was an increased proliferative state of memory T cells, and more specifically of TTM, in accordance with previous studies.⁵⁰ In contrast, the late expansion of TEMRA seemed to rather depend upon differentiation than proliferation, as suggested by the stable Ki-67 expression. Therefore, the immunological changes induced by ipilimumab may not be solely explained by enhanced proliferation. The functional development of memory T cell subsets is directed by regulatory transcription factors such as eomes and T-bet. They sustain memory CD8⁺ T cell homeostasis through the expression of CD122 and are involved

in the differentiation of memory precursor and effector CD8⁺ T cells respectively.⁵¹ In addition, they coordinately promote T cell migration to inflamed tissues via chemokine receptors and cooperate to generate cytotoxic T cells.⁵²⁻⁵⁴ In mice, CTLA-4 reduces the expression of eomes at the transcriptional level resulting in less IFN γ and granzyme B producing CD8⁺ T cells.⁵⁵ In accordance with this observation, our results showed that CTLA-4 blockade induced a higher expression of eomes in CD8⁺ T cells from patients with DC, suggesting a pivotal role of this transcriptional factor during ipilimumab therapy.

Recently, TSCM have been identified.³⁴ These cells, which are the least differentiated memory subset, are long lived, with an enhanced self-renewal capacity and multipotency to differentiate into all memory T cells. In addition, emerging evidence indicate that TSCM are involved in antitumor immunity, thus representing valuable candidates for adoptive T cell therapies. They are the most potent cells among memory subsets to trigger tumor regression in humanized mice. Moreover, some patients with metastatic melanoma have a significant fraction of MART-1-specific CD8⁺ TSCM.³⁴ Interestingly, experiments conducted in the setting of adoptive immunotherapy revealed that T cells invalidated for both T-bet and eomes were unable to trigger an antitumor response and bore characteristics consistent with TSCM. Therefore, the antitumor potential of TSCM seems to rely more on their further differentiation into effector memory cells than in their intrinsic activity.⁵⁶ After ipilimumab initiation, CD4⁺ TSCM percentages decreased despite enhanced proliferative activity suggesting a differentiation process into classical memory cells. Whether this phenomenon was driven or not by T-bet and eomes remains to be elucidated.

Factors that determine the development of IrAEs under CTLA-4 blockade are a field of intensive research. Although it was hoped in early anti-CTLA-4 trials that the induction of IrAEs,¹⁹ that are well correlated to immunotherapies, were associated with durable objective clinical responses, it is now more established that autoimmune disorders are not good predictors of improvement in anti-CTLA-4 therapy.²⁰ We were not able to confirm the overlap between patients who developed IrAEs and those who achieved durable objective clinical response (for review and references therein³⁹). In addition, we did not find any correlation between the occurrence of IrAEs and the biomarkers investigated in this study. This lack of correlation may however be related to the observation of IrAEs over a wide range of time points (7 to 186 d) which increases the complexity of the statistical analysis.

In conclusion, this study identified pharmacodynamic markers and biomarkers associated with survival and/or clinical benefit. Our data show that CTLA-4 blockade targets the early steps of memory T cell generation, inducing both differentiation and proliferation. These results suggest that ipilimumab is able to reshape the memory subset and provide new insights into the mechanisms of memory T cell subset expansion with the potential involvement of TSCM and eomes (Fig. S3). In response to ipilimumab, T cells gradually acquire skin homing and cytotoxic capacities, which may increase *in situ* antitumor responses. Identifying immune biomarkers as reliable intermediate endpoint may therefore facilitate the management of patients, providing insight into the selection of most effective therapeutic strategies.

Patients and methods

Patient characteristics and study design

Patients with unresectable stage III or IV melanoma were eligible for ipilimumab treatment if they had previously failed to one or more systemic therapy. Between June 2010 and September 2013, 77 patients were prospectively included (59 at Saint-Louis hospital and 18 at Bichat hospital, Paris, France). They received intravenous ipilimumab 3 mg/kg every 3 weeks, for an expected total course of four doses. Dose omission or discontinuation, mainly due to the occurrence of IrAEs, was discussed during multidisciplinary meetings. Blood samples were collected before each injection of ipilimumab at week 0, 3, 6, 9, and at week 12. All patients gave their informed consent for this study, which was approved by the Institutional Review Board of Saint-Louis Hospital, in accordance with the Declaration of Helsinki. Thirty samples from healthy donors were collected from the blood donor center (Etablissement Français du Sang, Hôpital Saint-Louis).

Flow cytometry and monoclonal antibodies

Lymphocyte immunophenotyping was performed on freshly collected EDTA whole blood samples, using a FACS Canto II flow cytometer and FACS DIVA software (BD Biosciences), in a laboratory that operates under GLP principles. Absolute counts were determined using the TruCount system (BD Biosciences) with anti-CD3 FITC, -CD8 PE, -CD45 PerCP, and -CD4 APC mAbs (BD Multitest, BD Biosciences). Eight colors labeling was performed with the following mAbs (all from BD unless specified): anti-CD45 FITC and PerCP, CD3 V450, -CD4 V500, -CD8 PerCP, -CD16 APCH7, -CD56 PECy7, -CD45RO PE, -CD45RA APC, -CCR7 PECy7 and BV421, -CD27 PE and APCH7, -CD28 PE, -CD25 PECy7, -HLA-DR APCH7, -CD57 FITC, -CD122 PE, -CLA FITC, -CCR4 PE, -CCR6 PECy7, -CXCR3 APC, -CD278 PE and eomesodermin (eomes) PE (e-Bioscience). Additional immunostainings were done for 20 patients from stored frozen peripheral blood lymphocytes (PBL), using anti-CD95 FITC and APC, -CTLA-4 PE, -T-bet PE, -Ki67 PECy7, -IFN γ Vio770, -TNF- α PE and granzyme B FITC (last three mAbs from Miltenyi). Intracellular staining was performed using either Foxp3 staining buffer (e-Biosciences) or inside stain buffer (Miltenyi), according to the manufacturer's instructions. These studies were performed using standard operating protocols. Acquisition was performed using a FACSCanto IITM flow cytometer. Results were expressed according to the « fluorescence minus one » (FMO) control, allowing the definition of the background signal and data were analyzed using FACS DivaTM (BD Biosciences).

Definition of tumor assessment and main clinical outcomes

Tumor assessment using clinical examination, total body scan, cerebral MRI and ultrasound lymph node scan if necessary was performed at baseline, week 12 and week 16, and then every 2 mo. Because of the atypical pattern of response to immunotherapy, week 16 evaluation was systematically maintained even if the patient exhibited tumor progression at week 12. The overall

response was assessed according to the immune-response-related criteria (irRCs) derived from time-point response assessments (based on tumor burden).

Statistical analyses

Data are described using means and standard deviation for quantitative variables and count and percentages for qualitative variables. Comparisons between patients and HD immunological status at baseline were performed using Wilcoxon rank sum test. *p* values were adjusted for multiple comparisons using the Hochberg correction.

Overall survival was defined as time between inclusion and death from any cause and was estimated using the Kaplan-Meier method. Effect of ALC at baseline on survival was assessed using Cox proportional hazard model.

Linear mixed-effect models were fitted to model the evolution of T cells between baseline and week 12. A two-slope model was used to account for a potential early and late effect of ipilimumab. The early effect was defined as the initial rise from baseline to week 3, and the late effect was modeled as a linear increase from week 3 to week 12. Random effects were added for the intercept and the two slopes. Immunological changes of T cell during the follow-up were modeled using linear mixed-effect models. To test the effect of response at week 16 on the evolution of T cells, two interaction terms between response and the two slopes were added to the model. Regarding several subsets of T cells, quantification was only performed at week 0, 3, and 6. In these cases, linear mixed-effect models were fitted with a single slope.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

J.F. is a Paris Diderot University junior fellowship. We thank all the technicians from the Immunocellular laboratory, Hôpital Saint-Louis.

Funding

This work was supported by INSERM, Société Française de Dermatologie and Institut National du Cancer, PAIR melanoma program.

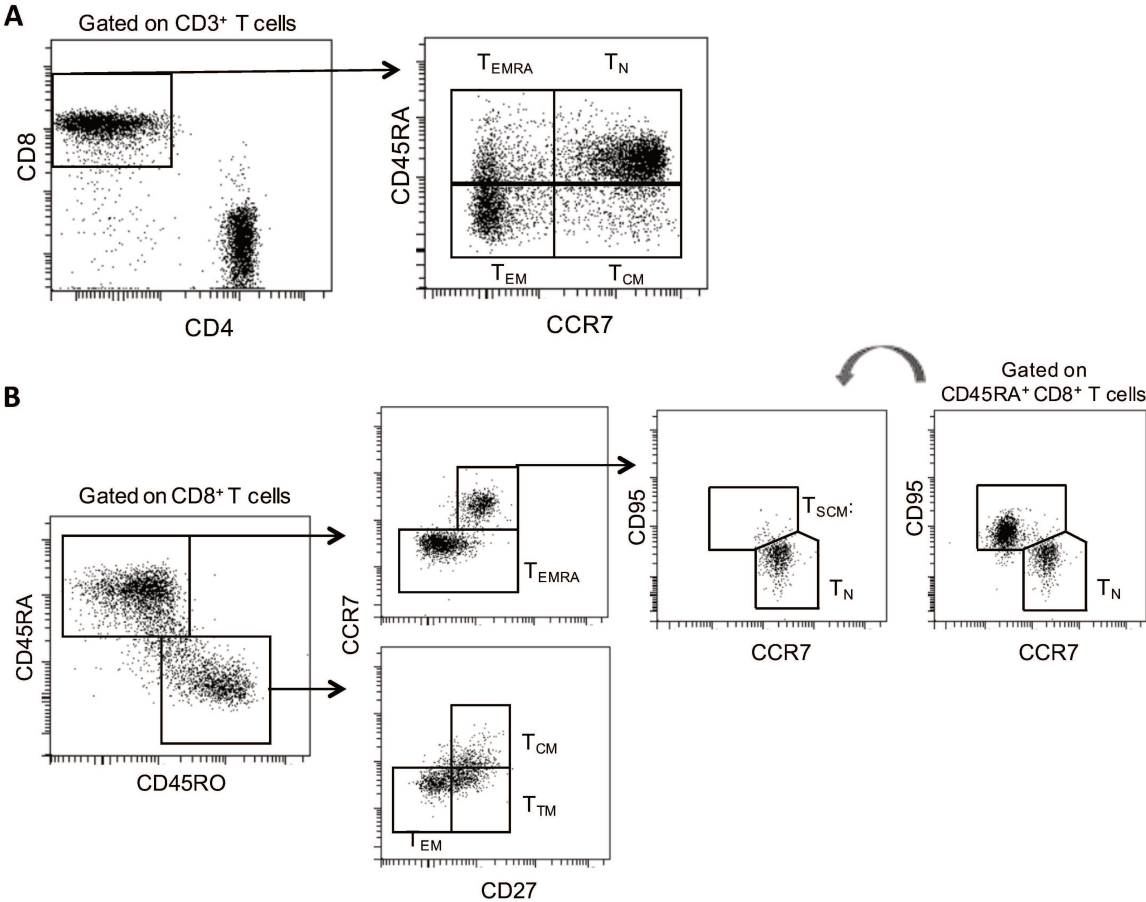
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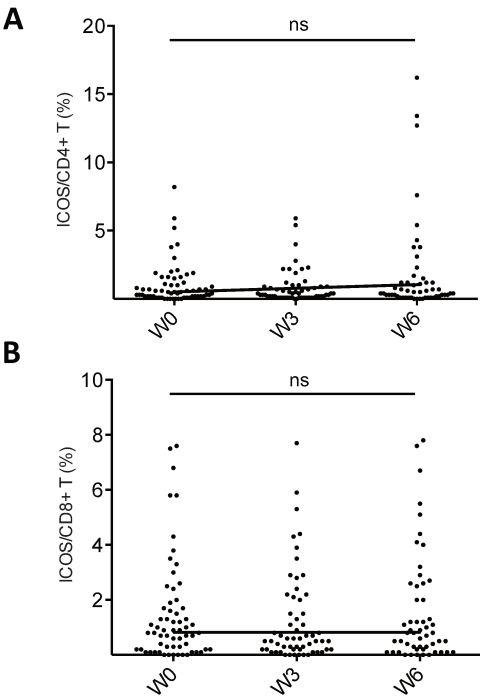
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Supplemental figure 1



Supplemental Figure 2



Article 2

Relevance of LDH, S100B and soluble MICA serum levels during follow-up of anti-CTLA-4 immunotherapy for advanced melanoma

(submitted)

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Running title: LDH, S100 and sMICA levels during Ipilimumab therapy

Conflict of interest: the authors declare that there are no conflicts to disclose

Keywords: Immunotherapy, Melanoma, CTLA-4, S100, LDH, sMICA

Purpose: To identify the utility of serum markers as early predictive biomarkers of response and survival under ipilimumab immunotherapy for metastatic melanoma or as useful markers in treatment monitoring and immune-related adverse events (irAEs) occurrence.

Experimental design: Sera of 77 consecutive stage III or IV melanoma patients submitted to ipilimumab therapy were analysed for LDH, S100B, MIA, soluble MICA (sMICA) and anti-MICA antibodies concentrations at baseline and at weeks 3 and 6 after the beginning of treatment. Statistical univariate and multivariate analysis were performed to assess the possible correlations between the analysed serum markers and response to treatment, survival and occurrence of immune-related adverse events.

Results: In univariate analysis, low levels of LDH and S100B at the baseline and at weeks 3 and 6 correlated with a better response and survival following ipilimumab treatment. After multivariate analysis, just LDH remained statistically significant, whereas S100B didn't provide additional information. A trend towards lower MIA serum levels and response to treatment was observed, with similar results for sMICA, but the relevance of these markers requires further investigation. Occurrence of adverse events didn't correlate to treatment response. However, higher sMICA serum levels in the disease-control group were associated with lower frequency of irAEs.

Conclusion: The utility of LDH as an independent biomarker of response and survival already supported by other studies was confirmed. In addition, S100B is a useful 'stand-alone' biomarker in ipilimumab monitoring, as lower concentrations correlate to treatment response and to survival before ipilimumab and after the first two doses. We provide important tools for pre-selection of patients that will more likely benefit from ipilimumab therapy. In addition, LDH and S100B are useful to monitor response in melanoma patients treated with ipilimumab. Lastly, sMICA serum levels may provide useful information in irAEs prediction.

Relevance of LDH, S100B and soluble MICA serum levels during follow-up of anti-CTLA-4 immunotherapy for advanced melanoma

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Running title: LDH, S100 and sMICA levels during Ipilimumab therapy
Conflict of interest: the authors declare that there are no conflicts to disclose
Keywords:
Immunotherapy, Melanoma, CTLA-4, S100, LDH, sMICA

Abstract

Metastatic melanoma is a rapidly spreading cancer whose prognosis remains poor although important therapy advances in recent years. Ipilimumab, an anti-CTLA-4 immunotherapy used in advanced melanoma, is an effective immunotherapy alone or combined with other agents but with few predictive biomarkers of response. Here, we sought to analyze the potential of S100B, MIA, soluble MICA, anti-MICA antibodies and LDH as serum biomarkers of response and survival in a cohort of 77 advanced melanoma patients subjected to ipilimumab. Lower levels of S100B, and LDH at baseline and at weeks 3 and 6 correlated to a better response and survival. After multivariate analysis LDH maintained its independence at baseline and week 6, whereas S100B might be a useful tool for anti-CTLA-4 treatment monitoring after the first two doses of ipilimumab (W6). In addition, higher sMICA serum levels at baseline were associated with less frequency of irAEs.

1. Introduction

Melanoma is one of the most rapidly spreading cancers in terms of worldwide incidence, with a 5-year survival rate of 16 % for patients with metastatic melanoma [1]. Recent major progresses in the understanding of molecular signaling pathways of melanoma and the complex cellular mechanisms regulating cancer immunity led to the development and approval by FDA of immune check-point inhibitors such as anti-CTLA-4 antibody ipilimumab (2011), anti-PD1 antibodies (2014) and targeted therapies such as BRAF (2011 and 2013) and MEK inhibitors (2013) for advanced melanoma [1]. CTLA-4 blockade aims to prevent the attenuation of T cell activation promoted by the CTLA-4 inhibitor receptor, potentiating tumor-specific T cell responses [2]. Ipilimumab is a fully humanized anti-CTLA-4 IgG1k monoclonal antibody approved by FDA for late stage melanoma after demonstrating to extend overall survival in two pivotal phase III trials in a subset of patients [3,4].

Ipilimumab results in long-term benefit in 20 % of patients [5], but is responsible for grade III or IV immune-related adverse events (IrAE) in around 27 % of patients [6]. Therefore, identifying biomarkers allowing a previous selection of patients or reliable intermediate endpoints that also early detect the occurrence of immune-related adverse events has become a field of intensive research in the past recent years. In this context, the identification of serologic biomarkers would be of extreme usefulness.

S100 proteins are frequently associated with inflammation and carcinogenesis due to its functions in modulation of cell proliferation and regulation of tumor suppressor p53 and apoptosis [7]. S100-beta subunit (S100B) is a 21 kDa acidic calcium-binding protein first described in cultured human malignant melanoma cells [8] and currently used in the diagnosis of both primary and metastatic melanoma [7,9]. In 2008, Mocellin and colleagues [10] demonstrated, in a multivariate survival meta-analysis including 3393 patients with stage I-IV cutaneous melanoma, the potential of S100B serum levels as a prognostic factor independent of TNM staging system, with higher levels representing a significant risk of poorer survival.

Melanoma-inhibitory-activity (MIA), a 11 kDa protein, was first isolated in the early 1990's from tissue culture supernatants purified from malignant melanoma cells [9]. Not expressed by benign melanocytes, MIA is strongly expressed by melanoma tumor cells and actively secreted in the extracellular compartment, correlating with the ability of melanoma to metastasize by allowing malignant melanocytes to detach from the extracellular matrix components [11,12]. Elevated MIA serum levels were clinically associated with advanced melanoma stage and poorer prognosis. They negatively correlated to response to chemotherapy [13,14] and to survival in stage III and IV melanoma [15]. MIA has been described as a highly specific serum marker for the detection of metastasis and suggested as a clinically useful marker in the follow-up of melanoma patients [16,17].

Until recently, the limited efficacy of treatment options in advanced melanoma made difficult for serum biomarkers such as S100B and MIA to demonstrate consistent predictive value under therapy [18,19]. The only tumor marker accepted by the American Joint Committee on Cancer (AJCC) as an independent significant predictor factor of survival in patients with stage IV melanoma is LDH [20], an ubiquitous enzyme whose activity is elevated in hypoxia, catalyzing the reversible transformation of pyruvate to lactate and rendering tumor cells less dependent on oxygen supply [21]. With the recent development of promising immune and targeted therapies, it urges to revisit the utility of these serum markers and explore their potential as predictive biomarkers in the context of new treatment response and survival.

NKG2D is a well-defined NK cell activator receptor which is also expressed by CD8 $\alpha\beta$ T and $\gamma\delta$ T cells [22,23]. Among its ligands, the major histocompatibility complex class I chain-related protein A (MICA), which has very limited expression in physiological conditions, is induced by cellular stress and broadly expressed on tumors of epithelial and hematopoietic origin [24]. Proteolytic shedding of MICA from tumor's cell surface releases a soluble form of MICA (sMICA) that potentially reduces the immunogenicity of tumor cells through down-regulation of NKG2D surface expression, favoring tumor escape [25]. Elevated sMICA serum levels have been found in patients with cancers or other malignancies, whereas low levels are found in patients with benign diseases or healthy donors [26].

In this study, we sought to elucidate the potential of LDH, S100B, MIA, sMICA and anti-MICA antibodies as serum markers for response and survival in a cohort of 77 patients with stage III and IV advanced melanoma submitted to ipilimumab therapy.

2. Materials and Methods

2.1 Patient clinical characteristics and study design

77 consecutive patients with unresectable stage IIIc or IV melanoma after failure of one or more systemic therapies were prospectively included in this study between June 2010 and September 2013 (59 at Saint-Louis hospital and 18 at Bichat hospital, Paris, France). Patients received intravenous 3 mg/kg of ipilimumab (Yervoy, Bristol-Meyers Squibb) every 3 weeks for a total course of four doses. Dose omission or discontinuation, mainly due to the occurrence of irAEs, was discussed during multidisciplinary meetings. Blood samples were collected before treatment and at weeks 3 and 6 (W3 and W6) before each injection of ipilimumab. All patients gave their informed consent for this study, which was in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Saint-Louis Hospital.

2.2 Patient sera

Patient serum samples were obtained and processed following a standardized protocol. Briefly, blood samples were centrifuged at 2000 rpm for 10 min, serum was harvested and immediately frozen at -80 °C. Serum samples were thawed for markers analysis.

2.3 ELISA

Serum levels of sMICA were determined by commercially available Human soluble MICA ELISA kit (Bamomab; Grafelfing; Germany) according to manufacturer's instructions. Briefly, plates were coated overnight at 4 °C with capture anti-MICA mAb at 5 µg/mL in PBS. Plate was then washed, blocked with 10 % FCS overnight, and incubated with the standard recombinant sMICA*04 and duplicates of patients' sera diluted 1:2 in 10 % FCS. Plates were then incubated with the detection mAb BAMO3 and IgG2a HRP-conjugated (Southern Biotechnologies, Birmingham, AL) was used for revelation. Finally, plates were

developed using the TMB Peroxidase Substrate System (Thermo Scientific, Massachusetts, New England). Absorbance was measured at 450 nm. sMICA's cut-off value was determined by calculating the 30th percentile of sMICA levels at baseline (supplemental Figure 1), according to the hypothesis that responders (approx. 30 % of patients [3]) would present lower levels of sMICA.

Serum levels of S100B and MIA were assessed respectively by S100 ELISA kit (DiaSorin; Stillwater; Minnesota) and MIA ELISA kit (Roche; Mannheim; Germany). S100B and MIA's cut-off values were chosen according to manufacturer's recommendations, being respectively 0,15 µg/L and 7,5 µg/L. LDH activity was routinely measured at the Hospital, and its cut-off value for melanoma patients was defined as 500 U/L according to previous publications [27].

2.4 Anti-MICA antibodies assessment

Anti-MICA antibodies serum levels assessment was performed by LABScreenTM assay using commercially available LABScreenTM kits and according to manufacturer's instructions (and as described in [28]). Briefly, two microbeads coated respectively with the following groups of antigens: MICA *001, *004, *012, *018, *027 (27/28) and MICA *002, *007, *009, *017, *019 were used to screen sera. The fluorescent signal was detected using LABScanTM 100 Flow Cytometry technology (LuminexTM xMAP Technology; Texas, USA) and data were analysed with HLA Fusion 2.0 software (One Lambda, Inc.). A cut-off fluorescent signal value of 2, consistent with manufacturer's recommended value, was used as the positivity threshold of anti-MICA antibodies.

2.5 Definition of tumour assessment and response evaluation

Tumour assessment using clinical examination, total body scan, cerebral MRI and ultrasound lymph node scan if necessary was performed at baseline, week 12 and week 16, and then

every two months (as described in [29]). Week 16 evaluation was systematically maintained even if the patient exhibited tumour progression at week 12 due to the atypical pattern of response to immunotherapy. The overall response was assessed according to the Immune-Response-Related Criteria (irRCs) derived from time-point response assessments (based on tumour burden).

2.6 Statistical analysis

Association of biomarkers with response at week 16 according to WHO criteria was analysed using Wilcoxon and Fisher's exact tests. Overall survival was counted from the date of initiation of ipilimumab to the date of death or last follow-up, whichever occurred first. Survival curves were estimated using Kaplan-Meier product-limit method. Follow-up was described using the reverse Kaplan-Meier method [30]. Association of biomarkers with overall survival was analysed using log-rank tests and Cox proportional hazards models. The proportional hazards assumption was checked by examination of Schoenfeld residuals and Grambsch and Therneau's lack-of-fit test [31]. For biomarkers measured at 3 and 6 weeks, a landmark analysis was used, with landmark set at 3 and 6 months, respectively. This corresponds to analyze only patients alive at the landmark time, with a new follow-up defined starting from the landmark. Analyses were performed using the R statistical software version 3.0.2 (R Core Team 2013).

Multivariate analysis for survival was performed using the Cox regression proportional hazards model with the Social Sciences (SPSS) for Windows (version 20) software.

3. Results

3.1 Patient clinical characteristics, response to treatment and immune-related adverse events

Patient's characteristics are reported in table 1. Median follow-up was 28 months with a median survival of 7. Response was assessed at week 16 after the beginning of treatment, allowing the division of patients into the disease control (DC) group and non-responders (NR) group. DC group included patients achieving complete or partial response as well as stable disease and represented 31 % of patients, whereas NR group included progression disease or death before week 16, and was observed in 69 % of patients. IrAEs occurred in 49 days (median value) after the beginning of treatment and affected 47 % of patients. The most significant IrAE was colitis (50 % of cases, reaching grade 3-4 in 13 % of patients) followed by rash/pruritis (6 % of all patients) and hepatitis (5 %). The majority of irAEs was managed with drug interruption and early intervention with corticosteroids. No association between clinical response at week 16 and the occurrence of irAEs was observed (data not shown).

3.2 Biomarkers at baseline and disease stage

Patients were divided according to unresectable stage III or IV to attempt for possible associations between serum markers at baseline and disease stage. No association was found between any of the studied markers and disease stage (data not shown). However, considering that only 8 out of 77 patients presented a stage III melanoma, and all in the subcategory of IIIC, the absence of significant differences in serum markers between the two groups was expected. The lack of differences in the baseline values of the analyzed markers between the two groups prompted us to consider the patients as a whole concerning the stage of melanoma.

3.3 Biomarkers during the course of treatment

Serum concentrations of sMICA, anti-MICA antibodies, MIA, S100B and LDH activities were determined at the baseline and at weeks 3 and 6 after the beginning of immunotherapy and are summarized in Table 2. Baseline concentrations of sMICA, MIA and S100B above

the cut-off value were observed respectively in 70 %, 82 % and 59 % of patients. By contrast, only 37 % and 9 % of patients had increased activities of LDH and anti-MICA serum levels, respectively, before the beginning of treatment. No significant differences in the serum concentrations of any of the markers were found during the course of treatment, although a trend towards an increase in MIA and S100B serum levels was observed (Table 2). No correlation was found between the kinetics of sMICA and anti-MICA serum levels (data not shown).

3.4 LDH activity and S100B serum levels are associated with better response to ipilimumab

Patients were divided in DC and NR groups according to their response to ipilimumab treatment at week 16. Serum levels at the baseline and weeks 3 and 6 of the different biomarkers and frequency of patients in the different groups below or above the upper concentration limit for each biomarker are described in Table 3. Low serum activities of LDH at baseline correlated with a better response to ipilimumab (median in DC: 384 U/L; median in NR: 496 U/L; $p = .003$). The same association was found at weeks 3 (median in DC: 382 U/L; median in NR: 475 U/L; $p = .003$) and weeks 6 (median in DC: 407 U/L; median in NR: 544 U/L; $p = .004$) after the beginning of ipilimumab treatment. At baseline, only 8 % of patients within the DC group showed LDH activities above the upper limit of 500 U/L, whereas 50 % of NR showed increased levels ($p = .0003$). This trend was also observed at weeks 3 (DC above the LDH threshold = 17 %, NR above the LDH threshold = 49 %; $p = .016$) and 6 (DC above the LDH threshold = 18 %, NR above the LDH threshold = 62 %; $p = .002$). Additionally, low S100B serum levels at baseline were also good predictors of response following ipilimumab (median in DC: 0.15 $\mu\text{g/L}$, median in NR: 0.44 $\mu\text{g/L}$; $p = .032$); and W6 (median in DC: 0.15 $\mu\text{g/L}$, median in NR: 0.74 $\mu\text{g/mL}$; $p = .001$), and a trend was observed at W3 (median in DC: 0.18 $\mu\text{g/L}$, median in NR: 0.34 $\mu\text{g/mL}$; $p = .054$). Moreover, at W6, 53 %

of patients within the DC group had serum levels of S100B below the upper limit of 0.15 µg/L, in contrast with only 17 % of non-responders ($p = .011$).

Low serum levels of MIA at W3 (median in DC: 14.4 µg/L, median in NR: 19.0) were also found associated with response to immunotherapy ($p = .049$). sMICA serum concentrations could not discriminate patients achieving disease control from non-responders, even though the proportion of patients below the cut-off value at the baseline was more likely to respond to ipilimumab ($p = .056$). No association was found between serum levels of anti-MICA antibodies and response to the immunotherapy.

Since LDH and S100B levels were associated to ipilimumab treatment response, a multivariate analysis was made to assess whether S100B improves LDH potential in predicting treatment response. After adjusting S100B levels to LDH levels at baseline, S100B lost its significance ($p = .46$), and similar observations were found at weeks 3 ($p = .57$) and 6 ($p = .094$). Therefore, S100B doesn't seem to be an LDH independent predictive factor of response to ipilimumab.

In order to assess the discrimination of LDH and S100B biomarkers in predicting patients' response, the C-index [33] was calculated for the different assessed time-points, being, for LDH at baseline, W3 and W6 respectively of 0.717, 0.728 and 0.729. S100B C-index values were of 0.661 at baseline, 0.643 at W3 and 0.763 at W6. Therefore, LDH activity seems to better discriminate DC from NR groups at baseline and W3 after the beginning of therapy, whereas S100B seems to be more accurate for outcome prediction at W6.

3.5 LDH activity and S100B serum levels correlate with survival

Log-rank tests and Cox proportional hazard models were used to analyse the association of biomarkers serum concentration with overall survival. Patients with baseline LDH serum

levels below the cut-off value had a better overall survival with a median of 9.7 months compared to 5.1 months of those with LDH activities above the cut-off ($p = .0001$) (Figure 1). Additionally, low baseline S100B serum levels were also predictive of better overall survival with patients surviving for 5 more months in median than those with S100B levels above the cut-off value (11.7 vs 6.7 months; $p = .011$). However, when a Cox multivariate analysis was performed to estimate the independence of baseline LDH and S100B values, only LDH remained independent ($p = .001$) whereas S100B lost its independence as a prediction biomarker of survival, although a trend was observed ($p = .088$). A landmark analysis was then used at weeks 3 and 6 to assess the association of biomarkers with overall survival, corresponding to analyse patients alive at the landmark time, with a new follow-up defined starting from the defined landmark (for detail see Statistical analysis). At W3, a trend towards a better survival with low LDH activities was observed ($p = .05$), whereas S100B levels below the cut-off value at W3 were significantly associated with improved survival (10.7 vs. 6.1 months; $p = .007$). After multivariate analysis, a trend towards an improved overall survival was associated with low levels of S100B ($p = .059$), although not statistically significant. Finally, low LDH serum levels at W6 led to improved overall survival (9.9 vs. 5.3 months; $p = .001$), as well as low S100B levels (10.0 vs. 5.8 months; $p = .028$). However, the multivariate analysis showed that only LDH remained as an independent marker ($p = .03$).

No association between sMICA, anti-MICA antibodies and MIA levels with overall survival was observed at the assessed time points.

3.6 Low baseline sMICA levels are associated to occurrence of IrAEs in DC group

We did not find any correlation between patients who develop IrAEs and those who achieved clinical benefit (data not shown). The analysis of potential associations between serum markers at baseline and occurrence of irAEs was performed exclusively for the DC group

(Table 4). We found an association between high serum levels of sMICA at the baseline and the absence of irAEs (sMICA baseline median in the absence of irAE: 130 µg/mL, sMICA baseline median in patients who developed irAE: 69 µg/mL, $p = .049$) (Table 4). No association was found between any other analyzed biomarkers and the occurrence of irAEs within the DC group.

4. Discussion

Ipilimumab is an effective immunotherapy for patients with metastatic melanoma. Although providing positive outcomes, this therapy can cause serious adverse events and, to date, no predictive biomarker has been identified allowing a reliable selection of the 20 % of patients with advanced melanoma that will more likely better benefit from this immunotherapy in the long term. In this study, we investigated the potential of LDH, anti-MICA Abs, S100B, MIA and sMICA serum levels as predictive biomarkers of response and survival in 77 patients with metastatic melanoma submitted to ipilimumab therapy.

The potential of MIA and S100B markers in the context of melanoma has widely been studied (for review see [19]). S100B serum levels were shown to increase with clinical stage of melanoma [34,35], and have later been suggested as useful markers to monitor response to chemo- and immuno-chemotherapy in metastatic malignant melanoma [34,36–38], with lower levels having shown to also correlate with survival [39,40]. According to ESMO guidelines, S100 is the most accurate blood test in the follow-up of melanoma patients, with a specificity for disease progression superior to LDH [41].

However, few studies have been published revisiting the utility of these markers in the context of anti-CTLA-4 blockade. A case report of a stage IV melanoma patient described a good fitting of S100B serum levels with clinical benefit observed during the course of ipilimumab treatment [42]. Various studies also reported the value of LDH baseline levels as

predictive factors of survival under ipilimumab [43–46], with LDH activities decreasing within responders [45], supporting the usefulness of LDH as a criterion for patients selection before ipilimumab therapy [46]. Another study [47] revisited the significance of high serum levels of S100B and LDH in a cohort of 113 melanoma patients treated with recent therapies including anti-BRAF, ipilimumab and anti-PD-1. Higher levels of S100B and LDH were reported in patients when compared to controls before therapy, which was followed by a decrease during therapy within responders while increased S100B levels (but not in LDH) were observed in non-responders. However, this cohort included patients submitted to different treatments, not discriminating between those receiving the different immune and/or targeted-therapies.

In our homogeneous cohort, composed uniquely of 3 mg/kg ipilimumab-treated patients, we reported that low LDH and S100B serum levels at the baseline and at W3 and W6 correlated with response after anti-CTLA-4 treatment. After multivariate analysis, LDH showed an independent association with anti-CTLA-4 outcome whereas S100B did not achieve statistical independence. This might be explained by the possible correlation between these two molecules.

Several works described the utility of S100B and LDH to predict survival, with some of them supporting the superiority of S100B to LDH in survival prediction [40,48], while others defended LDH as the most relevant parameter at least before treatment [9,49]. In our study, both LDH and S100B were predictive of overall survival before ipilimumab treatment, which was sustained when the landmark analysis was performed at W3 (for S100B) and W6 after the beginning of the immunotherapy. In the multivariate analysis, only LDH achieved statistical independence before treatment and at W6. This latter data may reflect the involvement of LDH as a marker of both tumor burden (like S100B) and, unlike S100B, of anaerobic glycolysis of rapidly expanding hypoxic tumors (Warburg effect).

Although some studies identified MIA as an independent prognostic value in advanced melanoma [50], we did not find any association between MIA serum levels and survival. However, low levels of MIA at W3 were associated to a better response to ipilimumab, indicating a potential usefulness of this molecule in treatment monitoring in the context of ipilimumab.

In 2006, a case report of a metastatic melanoma patient submitted to granulocyte-macrophage colony-stimulating factor protocol followed by anti-CTLA-4 therapy described the progressive generation of antibodies against MICA (anti-MICA antibodies) associated with a progressive decrease in sMICA, enhanced NK and CD8+ T cytotoxicity, and significant tumour destruction [51]. We have not observed similar kinetics of decreasing sMICA and increasing anti-MICA levels in any of our 77 patients. However, we found a trend towards a better response and a better overall survival in patients with decreased sMICA serum levels at baseline ($p=0.056$ and $p=.068$, respectively). A recent study shows no correlation between soluble MICA and clinical outcome in a cohort of patients treated with ipilimumab plus fotemustine [52]. Discrepancies between these studies may reflect the difficulty to measure sMICA levels in the serum and the lack of a clear cut-off value of positivity. Inner to its capacity to enhance T lymphocytes activity, the administration of anti-CTLA-4 antibodies has the potential to raise the threshold of self-tolerance. Consequently, the occurrence of irAEs during ipilimumab therapy affects a non-negligible percentage of patients and their management as well as the determination of predictive biomarkers are a subject of intensive research. The most common irAEs are usually dermatologic effects, followed by colitis/diarrhea, often associated with lymphocytic infiltrates into the epidermis or into the gastric mucosa [53]. In our cohort, irAEs affected 47 % of patients, with colitis being the most frequent manifestation, representing 50 % of all irAEs. Interestingly, our study revealed an association between high baseline levels of sMICA in the DC group and the absence of

irAEs development during ipilimumab therapy. Moreover, colitis represented 82 % of irAEs observed in the DC group. Inflammatory bowel disease development is suspected to be a consequence of breakdown of immune tolerance to intestinal antigens, resulting in a deregulated or constant activation of the immune system. The resulting epithelial damage involves non-classical MHC-I chain-related molecules, notably MICA, which is expressed in intestinal epithelium, and upregulated in some tumours including melanoma [54]. MICA engages NKG2D receptor on NK cells and CD8 $\alpha\beta$ and $\gamma\delta$ T cells and is thought to trigger an autoimmune response described in several inflammatory diseases [55]. Upregulation of MICA in the intestinal mucosa was found to play a role in the occurrence of inflammatory bowel disease by mediating the activation of NK cells and promoting IFN-g production [54,56]. The release of sMICA by proteolytic shedding of MICA from tumour's cell surface is known to be an immune escape mechanism, inducing downregulation of NKG2D expression [24]. In our study, patients in the DC group with high levels of sMICA before the beginning of treatment developed 38 % less irAEs than patients with low sMICA levels, suggesting that sMICA constitutes a protective status against auto-immune reactions possibly by downregulation of NKG2D expression and thus reduced NK and T activation, thus protecting against the development of irAEs. Although requiring further investigation, this finding provides an interesting clue on irAEs development.

In conclusion, although S100B levels did not achieve statistical independence in the course of treatment as a biomarker of response and survival, both S100B and LDH measurements proved to be pertinent in predicting treatment response and survival following ipilimumab therapy when used as 'standalone' prognostic markers. LDH is here shown to be an independent marker of response and survival prediction in the monitoring of anti-CTLA-4 treatment in metastatic melanoma patients, in accordance with a previous study [46]. Moreover, S100B appears to be a better survival biomarker at W3 and might provide a useful

tool in the monitoring of ipilimumab patients. Lastly, our data outline that the baseline sMICA serum levels might be useful in predicting patient response and survival. Moreover, high baseline sMICA serum levels were shown to confer a protective status towards the occurrence of irAEs, providing a clue in the monitoring of adverse events that will be interesting to further explore.

5. Acknowledgments

J.F. is a Paris Diderot University junior fellowship. This work was supported by INSERM, Société Française de Dermatologie, Institut National du Cancer and PAIR melanoma program.

6. References

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Table 1: Patients characteristics.

	<i>N=77</i>
Age, mean (SD) yrs	60.4 (17.4)
Women, no. (%)	35 (45)
AJCC stage at inclusion, no. (%)	
IIIc	8 (10)
IV	4 (5)
IVM1a	2 (3)
IVM1b	7 (9)
IVM1c	56 (73)
Response at week 16 (WHO), no. (%)	
CR	1 (1)
PR	4 (5)
SD	19 (25)
PD	36 (47)
Death < w16	17 (22)
Overall survival (95 % CI)	
@ 6 months	62 % (52 to 74)
@ 12 months	25 % (16 to 37)
@ 24 months	10 % (5 to 20)
Adverse events, no. (%)	
Total	36 (47)
Grade 3	16 (21)
Grade 4	2 (3)

Table 2**Table 2:** Biomarkers in the course of treatment. Data are median (first to third quartile) or frequency (percent).

Marker	Baseline	Week 3	Week 7	<i>p</i> -value
LDH, U/L	446 (342 to 562)	437 (364 to 561)	435 (368 to 624)	0.91
≥ 500 U/L	28 (37)	23 (37)	25 (45)	-
S100B, $\mu\text{g/L}$	0.26 (0.15 to 1.53)	0.28 (0.15 to 1.06)	0.35 (0.15 to 1.45)	.064
≤ 0.15 $\mu\text{g/L}$	28 (41)	23 (36)	16 (29)	-
Anti-MICA Abs	1.01 (0.76 to 1.51)	1.04 (0.71 to 1.42)	0.94 (0.67 to 1.71)	0.54
≥ 2	12 (18)	11 (18)	11 (21)	-
sMICA, $\mu\text{g/L}$	92 (31 to 239)	117 (19 to 270)	98 (0 to 265)	0.51
≤ 45 $\mu\text{g/L}$	21 (30)	21 (32)	22 (39)	-
MIA, $\mu\text{g/L}$	15.6 (8.7 to 9.2)	16.0 (7.7 to 26.6)	16.3 (8.4 to 33.8)	0.051
< 7.5 $\mu\text{g/L}$	12 (18)	15 (23)	11 (20)	-

Table 3: Association of biomarkers with response at week 16. Data are median (first to third quartile) or frequency (percent).

Variable	Disease Control	Non-responders	<i>p-value</i>
Baseline			
LDH, U/L	384 (319 to 445)	496 (396 to 649)	0.003
≥ 500 U/L	2 (8)	26 (50)	0.0003
S100B, $\mu\text{g/L}$	0.15 (0.11 to 0.45)	0.44 (0.15 to 2.13)	0.032
≤ 0.15 $\mu\text{g/L}$	13 (54)	15 (34)	0.13
Anti-MICA Abs	0.86 (0.75 to 1.31)	1.10 (0.80 to 1.51)	0.24
≥ 2	4 (17)	8 (19)	>0.99
sMICA, $\mu\text{g/L}$	71 (13 to 240)	97 (46 to 239)	0.46
≤ 45 $\mu\text{g/L}$	11 (46)	10 (22)	0.056
MIA, $\mu\text{g/L}$	12.2 (7.3 to 20.8)	19.1 (9.4 to 37.6)	0.071
< 7.5 $\mu\text{g/L}$	6 (25)	6 (14)	0.32
Week 3			
LDH, U/L	382 (338 to 438)	475 (384 to 622)	0.003
≥ 500 U/L	4 (17)	19 (49)	0.016
S100B, $\mu\text{g/L}$	0.18 (0.12 to 0.48)	0.34 (0.15 to 1.75)	0.054
≤ 0.15 $\mu\text{g/L}$	11 (48)	12 (29)	0.18
Anti-MICA Abs	0.96 (0.73 to 1.24)	1.06 (0.71 to 1.52)	0.61
≥ 2	3 (13)	9 (22)	0.51
sMICA, $\mu\text{g/L}$	146 (50 to 262)	113 (0 to 270)	0.55
≤ 45 $\mu\text{g/L}$	5 (22)	16 (37)	0.27
MIA, $\mu\text{g/L}$	14.4 (6.3 to 18.9)	19.0 (9.5 to 31.2)	0.049
< 7.5 $\mu\text{g/L}$	8 (35)	7 (17)	0.13
Week 6			
LDH, U/L	407 (342 to 430)	544 (415 to 710)	0.004
≥ 500 U/L	4 (17)	21 (62)	0.002
S100B, $\mu\text{g/L}$	0.15 (0.10 to 0.38)	0.74 (0.23 to 2.74)	0.001
≤ 0.15 $\mu\text{g/L}$	10 (53)	6 (17)	0.011
Anti-MICA Abs	0.81 (0.63 to 1.06)	1.04 (0.72 to 2.08)	0.21
≥ 2	3 (16)	9 (25)	0.51
sMICA, $\mu\text{g/L}$	68 (0 to 290)	108 (8 to 264)	0.89
≤ 45 $\mu\text{g/L}$	9 (45)	13 (36)	0.58
MIA, $\mu\text{g/L}$	13.5 (6.8 to 24.3)	20.8 (9.9 to 47.4)	0.071
< 7.5 $\mu\text{g/L}$	5 (26)	6 (17)	0.48

Table 4: Association of baseline biomarkers and response with adverse events. Data are median (first to third quartile) or frequency (percent).

Variable	No AE	AE	<i>p-value</i>
Disease control	13 (54)	11 (46)	>0.99
LDH, U/L	464 (391 to 591)	416 (323 to 531)	0.23
>= 500 U/L	15 (37)	13 (37)	>0.99
S100B, µg/L	0.27 (0.15 to 1.73)	0.25 (0.15 to 1.18)	0.77
<= 0.15 µg/L	16 (43)	12 (39)	0.81
Anti-MICA Abs	0.97 (0.76 to 1.44)	1.03 (0.77 to 1.58)	0.63
>= 2	6 (16)	6 (20)	0.76
sMICA, µg/L	130 (46 to 308)	69 (14 to 172)	0.049
<= 45 µg/L	8 (21)	13 (42)	0.071
MIA, µg/L	15.2 (8.8 to 23.8)	19.1 (8.6 to 31.6)	0.40
< 7.5 µg/L	6 (16)	6 (19)	0.76

Figure 1

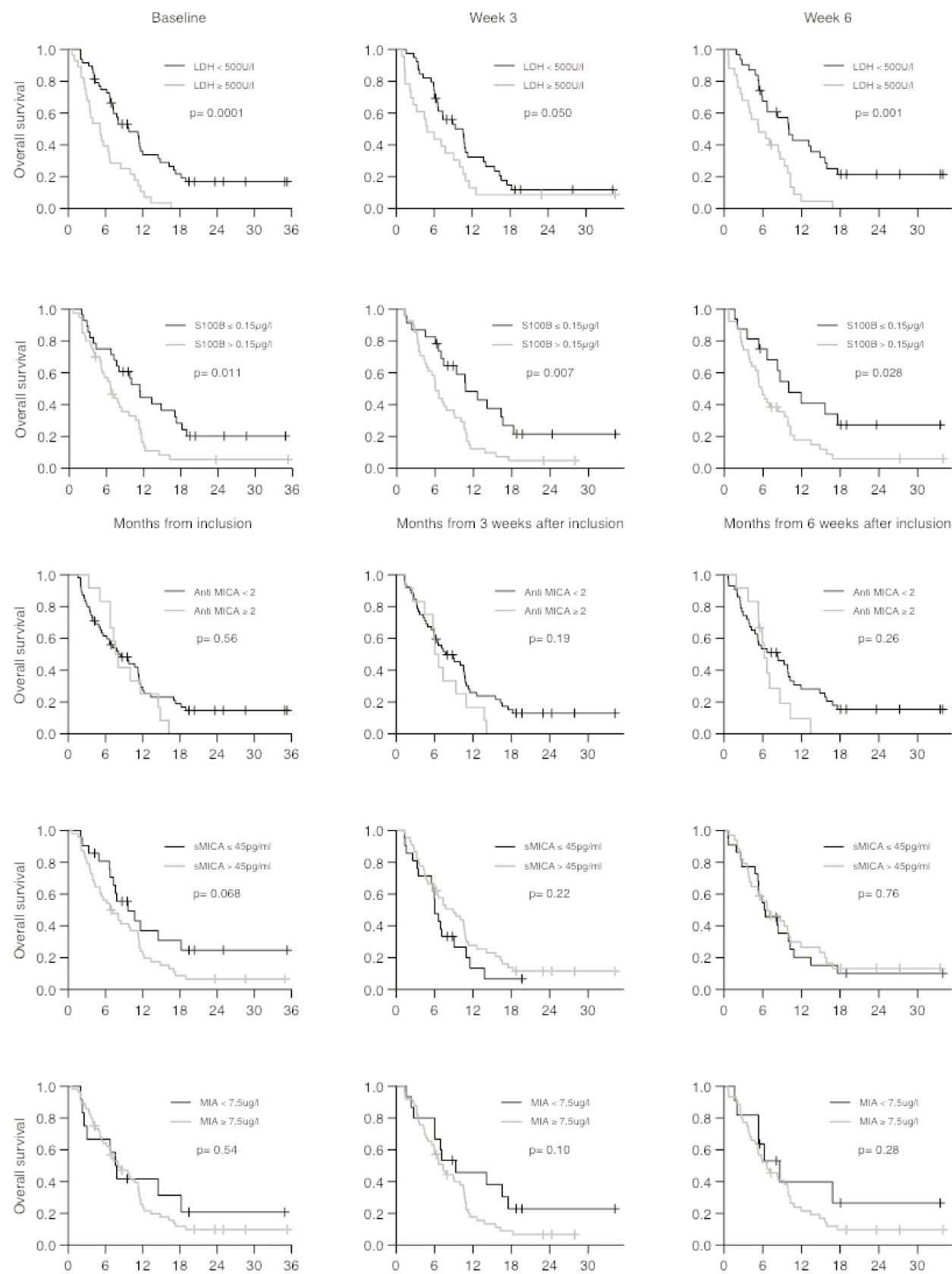


Figure 1: Association of biomarkers with overall survival.

Part III

***In vitro* differentiation of memory T cells from ipilimumab-treated patients**

Purpose

Our recent findings, showing that ipilimumab induces substantial changes within naïve and memory T cell subsets from melanoma patients, prompted us to further investigate the ability of these T cells to proliferate and differentiate in response to *in vitro* activation. We compared T cell dynamics from disease control patients (DC) and non responders (NR) before and after 2 doses of ipilimumab. In our previous study (article 1), we reported that clinical benefit was associated with a marked increase of memory subsets and of the transcriptional factor Eomes. Here, we aimed to analyse the stepwise differentiation of these T cell subsets over 5 days stimulation, with a specific focus on Eomes, and according to the capacity or not to respond to ipilimumab *in vivo*.

Methods

Frozen PBMC samples from 6 patients (3 DC and 3 NR) at baseline (before ipilimumab treatment onset) and at week 6 (W6, after two doses of ipilimumab) and from 3 healthy donors (HD) were studied.

Samples were thawed and left overnight in medium before culture in 48-well plate at 100,000 PBMCs/well in RPMI 1640 (Life Technologies) supplemented with 10 % FCS, 1 % of glutamine, 1 % of penicillin-streptomycin (all from Gibco, Life Technologies), 1 % of sodium pyruvate and 1 % of heppes buffer (Eurobio). Cells were stimulated with coated anti-CD3 antibody (0.250 µg/mL) and soluble anti-CD28 antibody (1 µg/mL) (Beckman Coulter). Lymphocyte immunophenotyping was performed before culture (D0) and every day for five days (D1-D5) using standard protocols.

Eight colour labelling was performed with the following monoclonal Abs (from BD unless specified): anti-CD3 PerCp, -CD45RA APC (Miltenyi), -CD27 APC-H7, -CCR7 BV421, anti-CD95 FITC, -Eomes PE (eBioscience), -Ki-67 PECy7 (eBioscience), Flex viability dye

eFluor 506 (eBioscience). Intracellular staining was performed using Foxp3 staining buffer according to manufacturer's instructions. Acquisition was performed using FACSCanto IITM flow cytometer. Results were expressed according to the 'fluorescence minus one' (FMO) control or isotype control (in case of intracellular staining), allowing the definition of the background signal. Data were analysed using FACS DivaTM (BD Biosciences). T_N cells were defined as expressing the CD3⁺CD45RA⁺CCR7⁺CD27⁺CD95⁻ phenotype, T_{SCM} cells as expressing the CD3⁺CD45RA⁺CCR7⁺CD27⁺CD95⁺ phenotype, T_{CM} cells as expressing the CD3⁺CD45RA⁻CCR7⁺ phenotype, T_{TM} cells as expressing the CD3⁺CD45RA⁻CCR7⁻CD27⁺ phenotype, T_{EM} cells as expressing the CD3⁺CD45RA⁻CCR7⁻CD27⁻ phenotype and T_{EMRA} cells as expressing the CD3⁺CD45RA⁺CCR7⁻ phenotype.

T_{SCM} cell sorting was performed after 3 day-culture of PBMCs in a 48-well plate in the presence of anti-CD3/CD28 antibodies as previously described. Cells were harvested and T_{SCM}, defined as the intersection of CD3⁺, CD45RA⁺, CCR7⁺, CD27⁺ and CD95⁺ expressing cells were sorted using a BDFACSAria III (BD FACSDiva 6.1.3 software). Sorted T_{SCM} were then plated for 3 days with IL-7 (25 ng/mL, as described by Lugli *et al.* (Lugli et al. 2013)) and harvested for naïve and memory T cell subsets phenotyping

Statistical analyses performed by non-parametric tests with Prism software (version 6.0).

Results

Different dynamics of T cell subsets between patients and HD during culture with potential involvement of TSCM

The Figure 6 describes the frequencies of naïve and memory T cells during 5-day culture within patients and healthy donors. Gating strategy is depicted in Supplemental Figure 1.

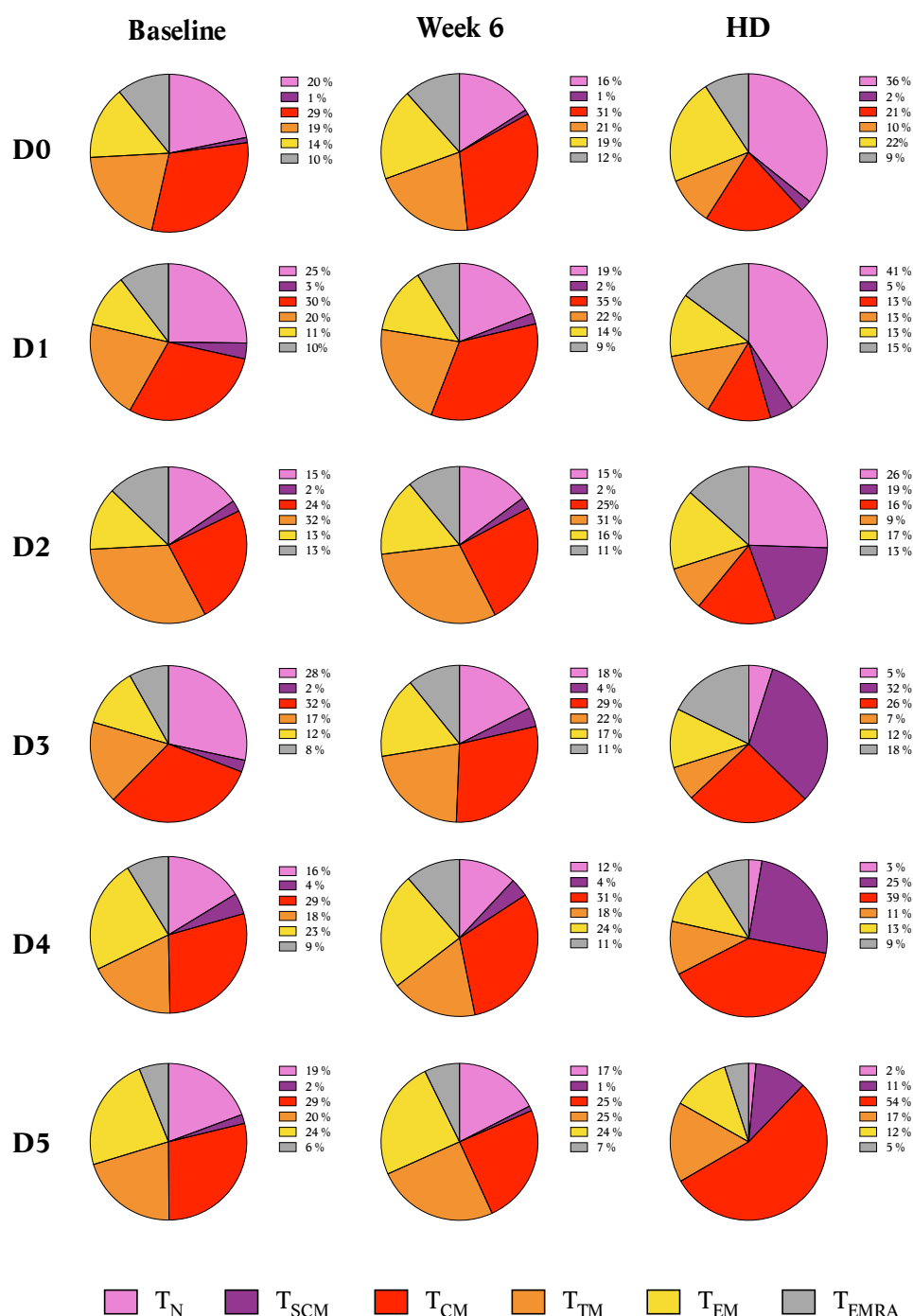


Figure 6 – Frequencies (%) of naïve and memory T cell subsets during 5-day culture *in vitro* of PBMCs from three HD and from six patients (3 DC and 3 NR) before ipilimumab treatment onset (baseline) and at W6 after the beginning of the immunotherapy. Median values are represented.

Responders and non-responders are here represented together due to lack of substantial differences concerning the frequency of different T cell subsets (data not shown). Globally, we observed no major differences in the repartition of naïve and memory T cell subsets from patients during the 5 day-stimulation, either before or after ipilimumab treatment. However, we observed that before cell culture HD presented higher levels of naïve T cells

compared to patients ($p = .004$). This suggests the occurrence of an immunological response within patients with the formation and/or expansion of memory T cells before the beginning of treatment.

In addition, we observed a striking difference in the dynamics of naïve and memory T cell subsets between HD and patients during the 5-day *in vitro* stimulation. In HD, a sustained decrease in naïve T cells was observed from D0 to D5, along with a drastic and transient increase in T_{SCM} around D3. This was followed by an increase in central memory cells from days 3 to 5 (Figure 6). Of note, the T_{SCM} compartment remained very small in patients (Figure 7).

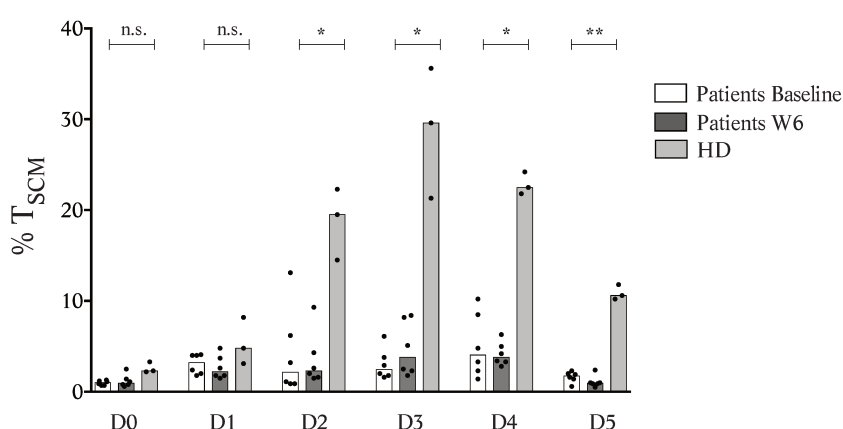


Figure 7 - Frequency (median) of stem-cell memory T cells along the 5-day culture of PBMCs from patients and healthy donors.

Increased proliferation levels in all memory T subsets

Almost all memory T cells from HD were Ki-67⁺ after 5 days of CD3/CD28 stimulation, with an onset of proliferation at D2. By contrast, the proliferative ability of memory T cells from melanoma patients was comparatively low and delayed but with a trend towards an improved proliferation after 2 rounds of ipilimumab treatment (Figure 8, gating strategy in Supplemental Figure 1). Again, no major differences between patients from DC group and NR group were observed (Supplemental Figure 2). In HD, T_{SCM} was the first subset of memory cells to proliferate with almost 80 % of Ki-67⁺T_{SCM} cells at D2, and a delayed proliferation of T_{CM}, T_{TM}, T_{EM} and T_{EMRA} at D3. Likewise, patients T_{SCM} displayed the highest level of proliferation, especially after CTLA-4 blockade.

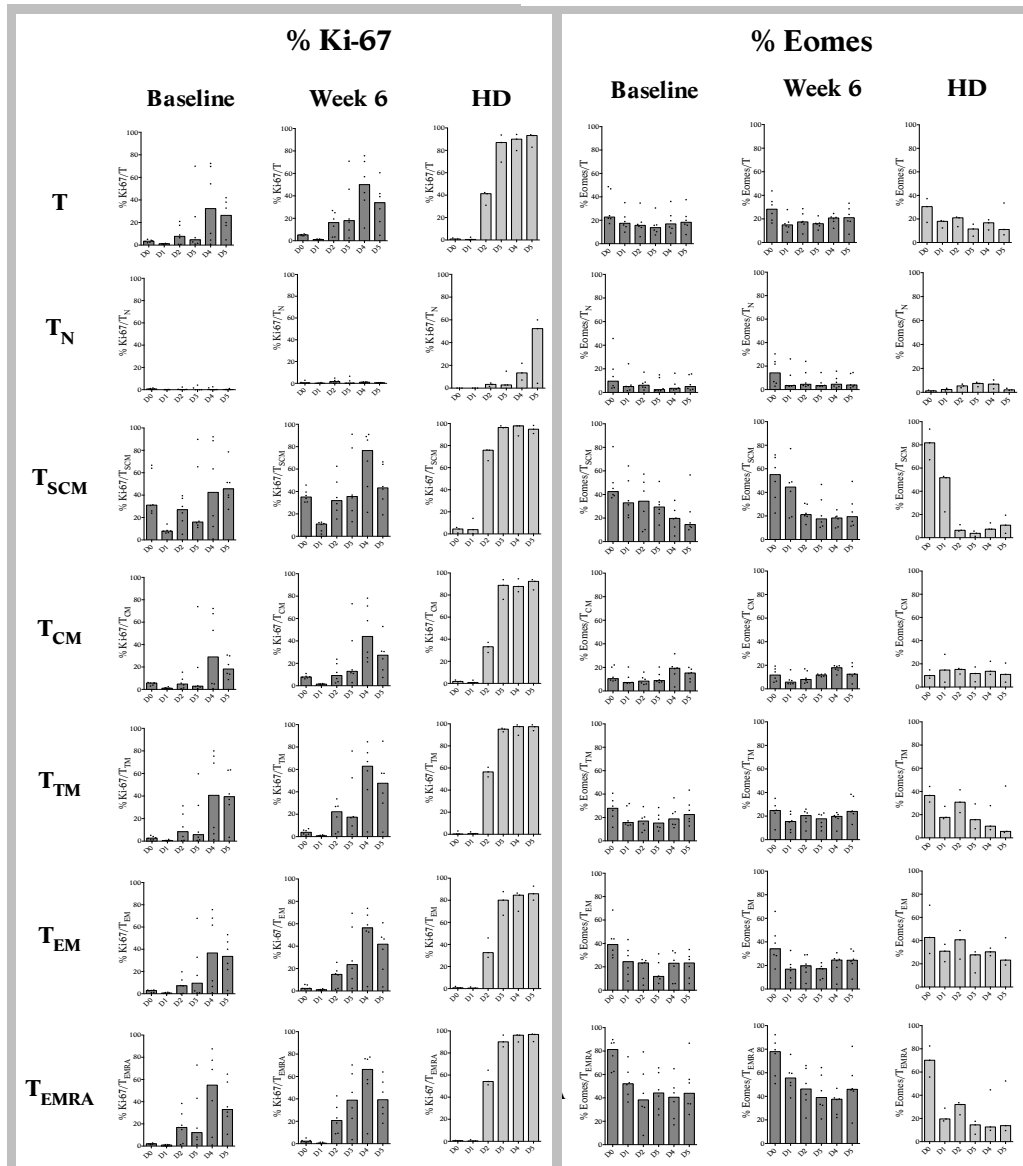


Figure 8 - Ki-67 and Eomes expression (% of T cell subsets) in naïve and memory T cell subsets of six patients, in dark grey (3 DC and 3 NR), at baseline and at W6 and of three healthy-donors (light grey). Median values are represented.

Stimulated T_{SCM} generate large numbers of T_{CM}

The elevated and transient expansion of T_{SCM} cells in healthy donors between day 2 and 4 and the delayed increase in T_{CM} may reflect either T_{SCM} differentiation into T_{CM} or the expansion of the T_{CM} compartment. We then designated the experiment described in Figure 9. HD-derived T_{SCM} were sorted after 3 days of T cell stimulation with anti-CD3/CD28 (the gating strategy of sorted T_{SCM} cells is described in Supplemental Figure 3). Sorted T_{SCM} cells were then plated in IL-7 conditions for 3 days.

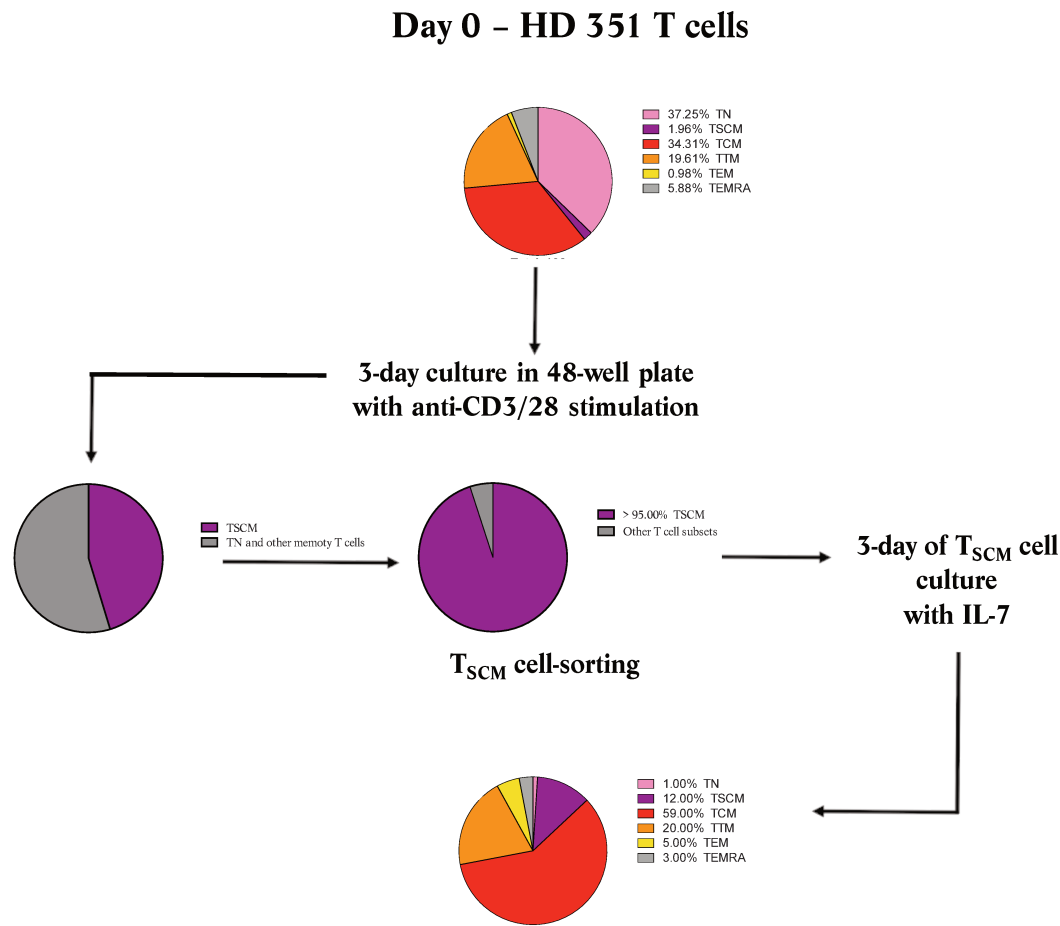


Figure 9 - Experimental design for HD 351 sorted T_{SCM} cells.

After 3-day culture, T_{SCM} cells generated a large proportion of T_{CM} cells, together with relatively high amounts of T_{TM} and small amounts of T_{EM} and T_{EMRA}. Interestingly, the proportion of different memory cells obtained after 3 days of culture seems to reflect a stepwise differentiation between memory subsets, from T_{SCM} to T_{CM} and then T_{TM} and T_{EM}. These results suggest that a process of T_{SCM} proliferation and then differentiation into T_{CM} may explain the delayed increase in T_{CM} frequencies in HD observed after 4 days of CD3/CD28 stimulation (Figure 6).

Inverse relationship between proliferation and Eomes expression in T_{SCM} and T_{EMRA} from HD and DC

Globally, no statistically significant differences were observed between HD and patients in Eomes expression at D0 (data not shown), except for T_{SCM} cells. HD's T_{SCM} cells had higher levels of Eomes than patients before ipilimumab treatment (Figure 10).

Interestingly, after 2 doses of ipilimumab, these differences were no longer significant, suggesting an effect of ipilimumab in inducing Eomes expression in T_{SCM} cells.

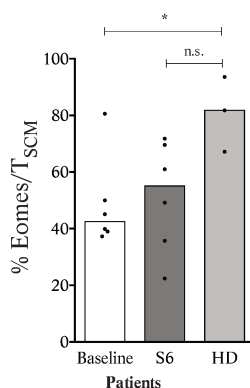


Figure 10 - Eomes expression (% of cells) at D0 in T_{SCM} cells of patients before ipilimumab treatment (white) and after two doses (dark grey) and of three healthy-donors (light grey). Median values are represented.

In addition to T_{SCM}, elevated levels of Eomes were observed in T_{EMRA} cells in both patients and HD at D0. This was followed by a decrease in Eomes expression in these memory compartments (T_{SCM} and T_{EMRA}) from D1, with a concomitant increased expression of Ki-67 (Figure 8).

Our results from HD show a significant inverse correlation between Eomes and Ki-67 expression in almost all memory T cells (Supplemental Figure 4). In patients, the inverse correlation between these markers was observed only in T_{SCM} and T_{EMRA} cells, with Eomes expression remaining relatively constant in other memory T subsets despite increased levels of Ki-67. Interestingly, when patients were divided according to clinical outcome following ipilimumab, only DC patients presented this inverse correlation between Eomes and Ki-67 in T_{SCM} and T_{EMRA} cells, while NR did not (Figure 11). Moreover, when we assessed the Eomes and Ki-67 expression in DC and NR patients according to baseline and after two doses of ipilimumab, the inverse correlation between these two markers in T_{SCM} was not observed for DC at baseline, but was potentiated with ipilimumab ($p = .086$) (Figure 12). In addition, this correlation is observed from baseline in T_{EMRA} cells of DC patients, and further exacerbated at W6. By contrast, NR patients did not present a correlation between Eomes and Ki-67 expression either before or after ipilimumab treatment, although the signal of the slope was inverted towards an inverse correlation between these two markers ($r = -0.124$ vs $r = +0.245$).

This suggests that a regulation between Eomes and Ki-67 exists in healthy donors and in patients achieving disease control after ipilimumab treatment that is absent in non-responders.

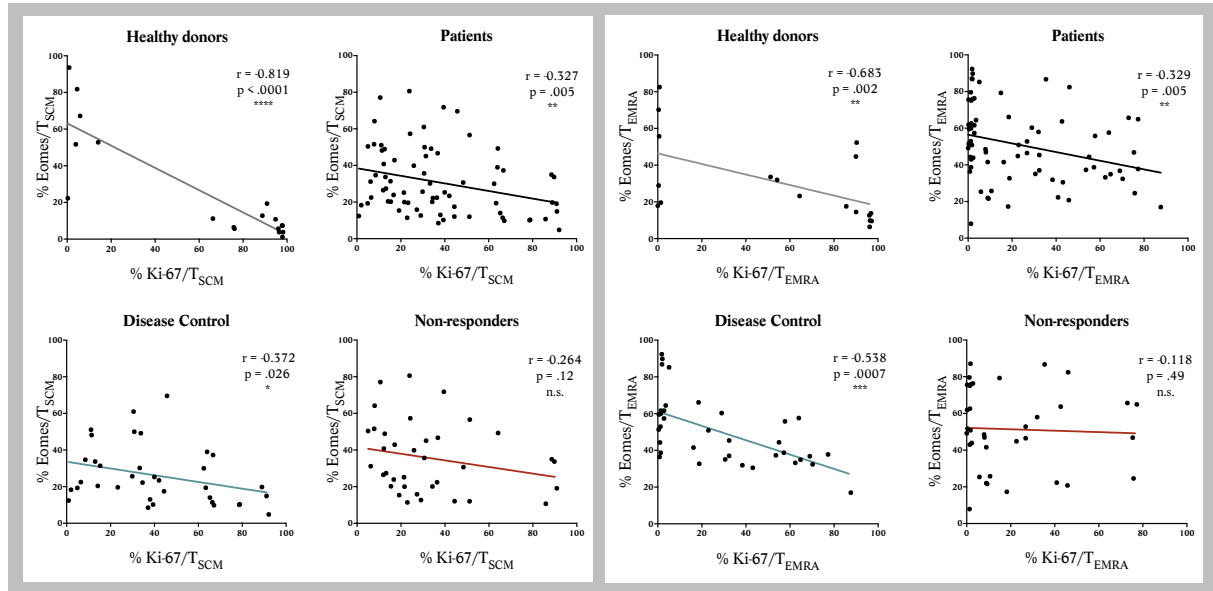


Figure 11 - Correlation between Eomes and Ki-67 expression in HD and patients for T_{SCM} (left) and T_{EMRA} cells (right). Days of *in vitro* culture are not discriminated. Correlation coefficients (R) and p values were calculated using the Spearman rank correlation test.

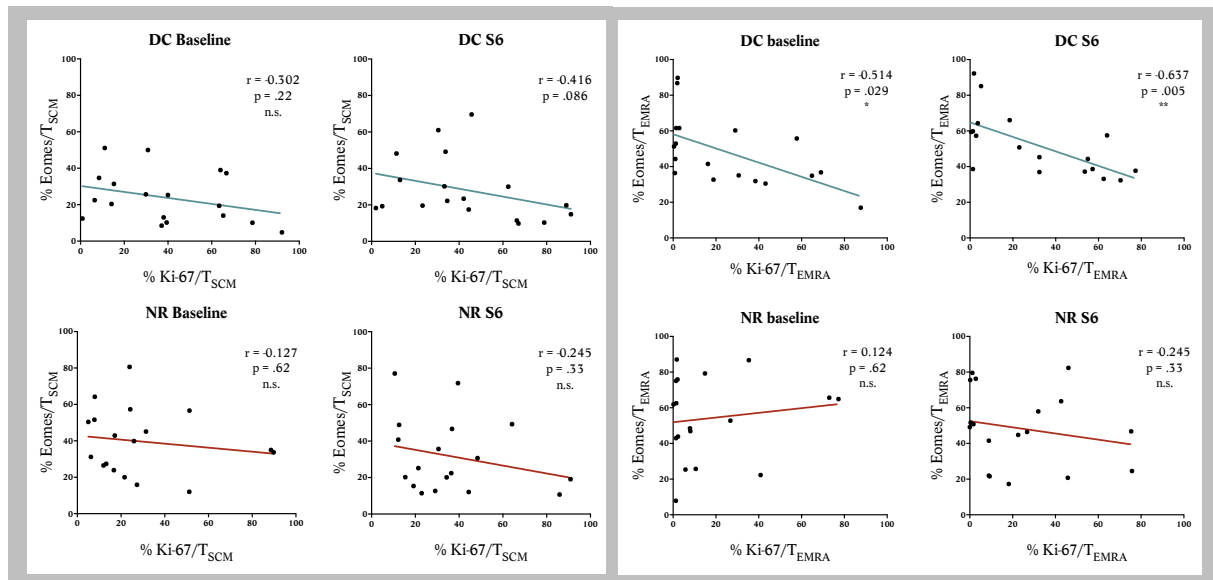
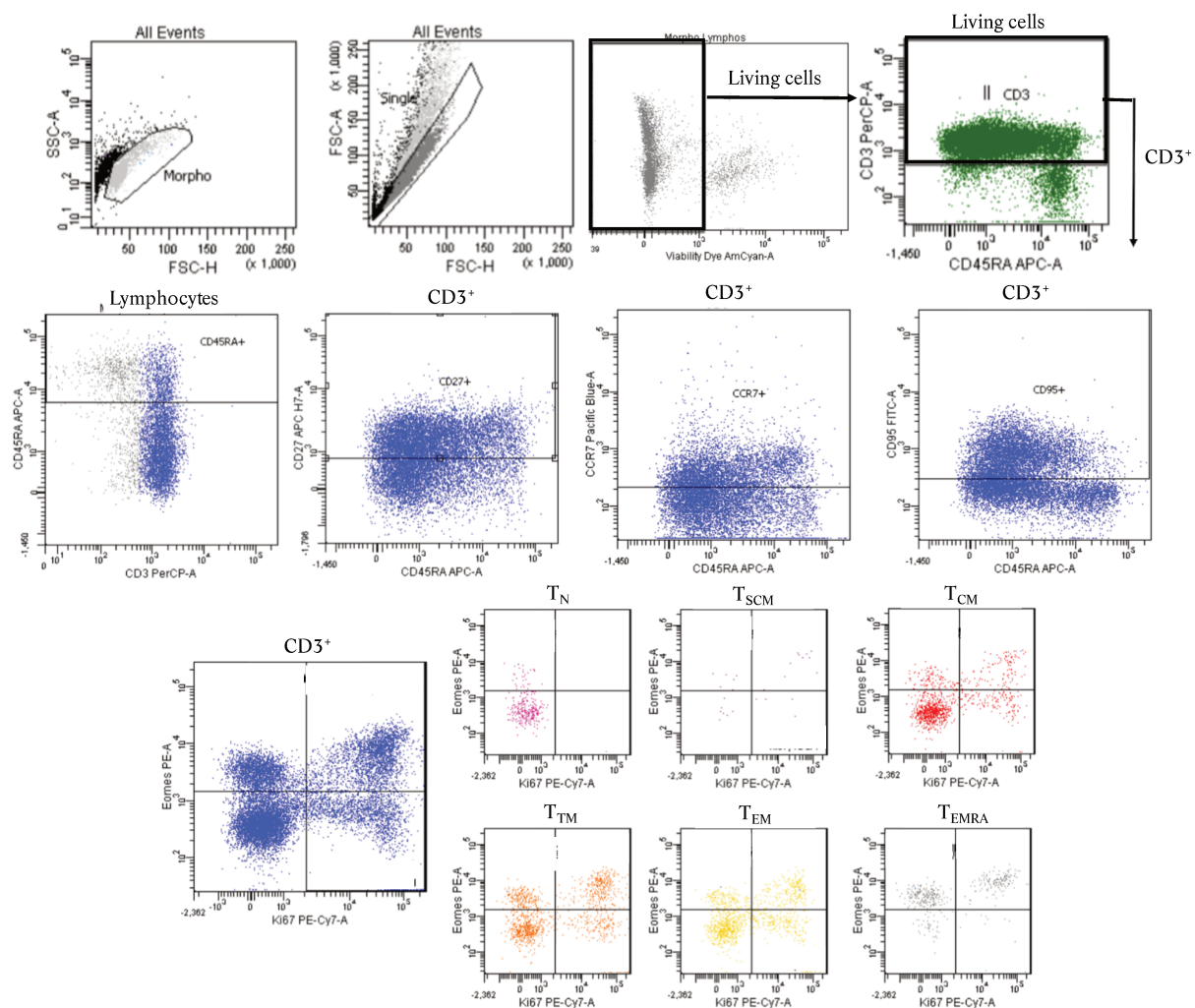


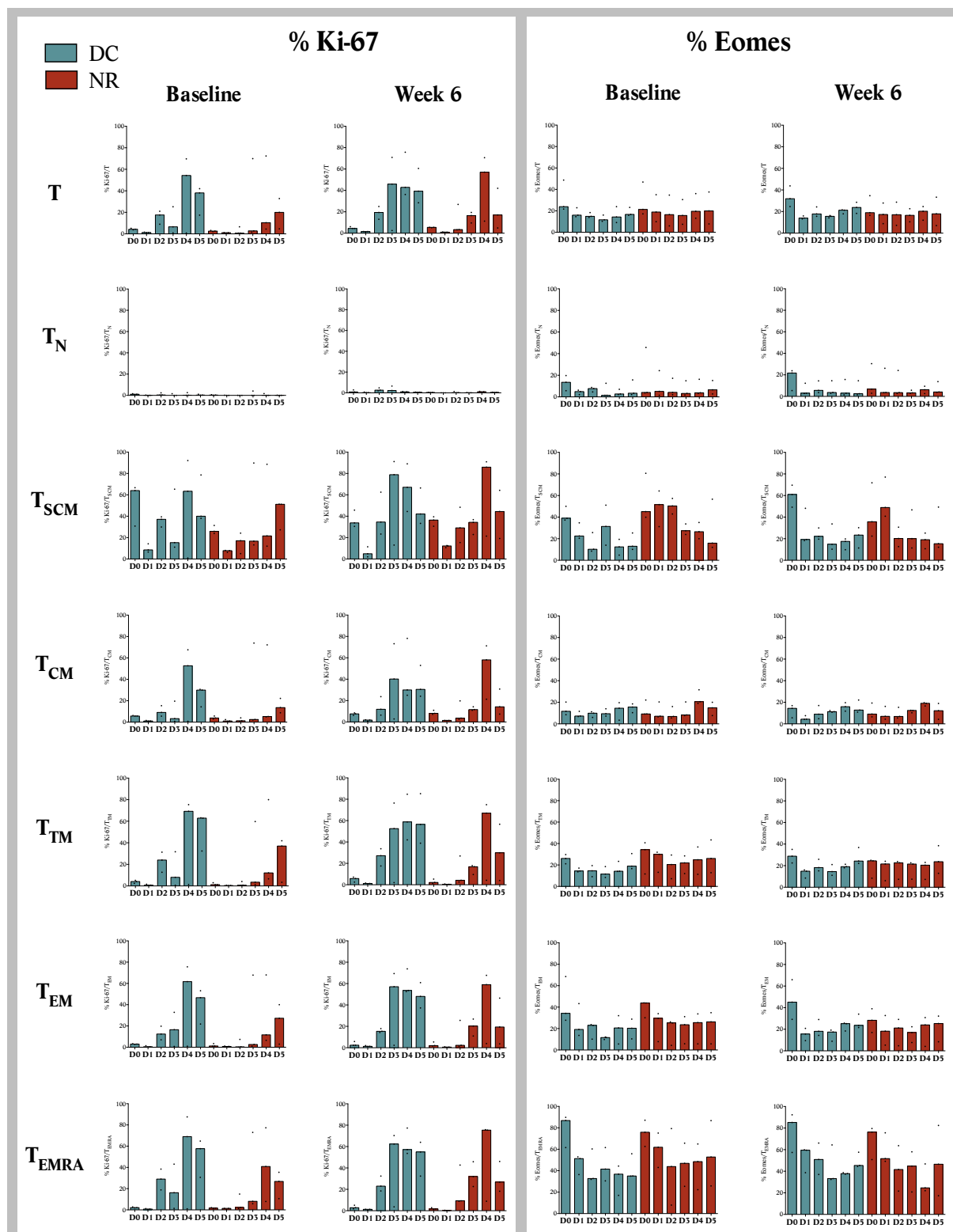
Figure 12 - Correlation between Eomes and Ki-67 expression for T_{SCM} (left) and T_{EMRA} cells (right) of DC and NR patients before ipilimumab treatment and after two doses. Days of *in vitro* culture are not discriminated. Correlation coefficients (R) and p values were calculated using the Spearman rank correlation test.

Supplemental figures

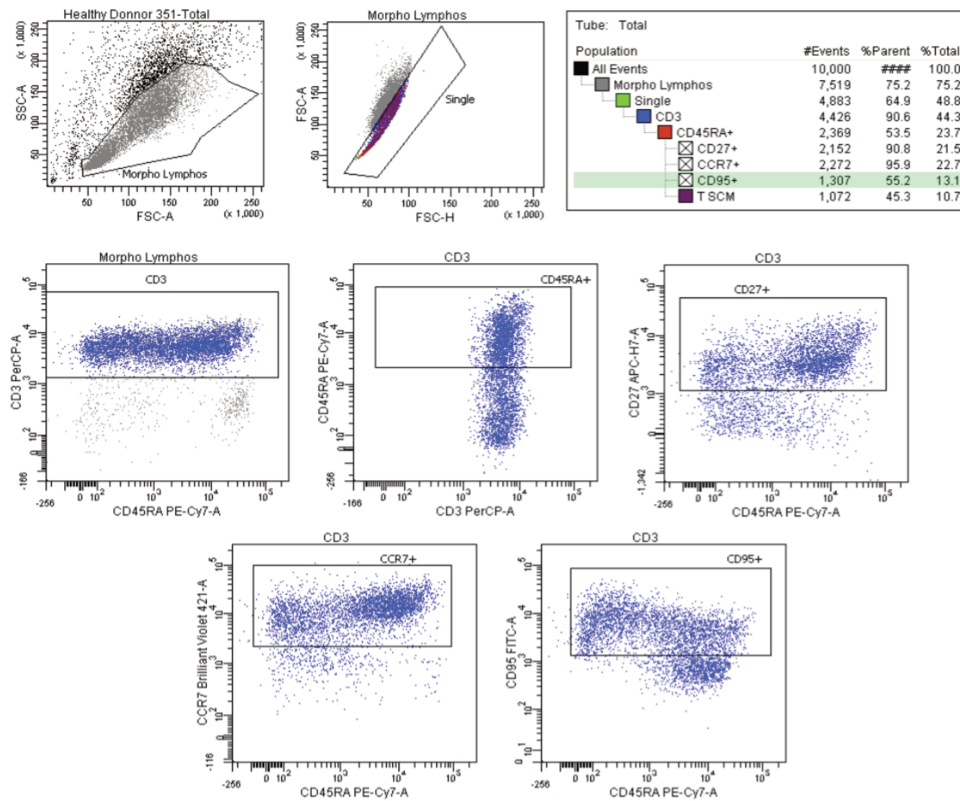


Supplemental Figure 1 - Gating strategy of naïve and memory T cell subsets and Eomes and Ki-67 expression.

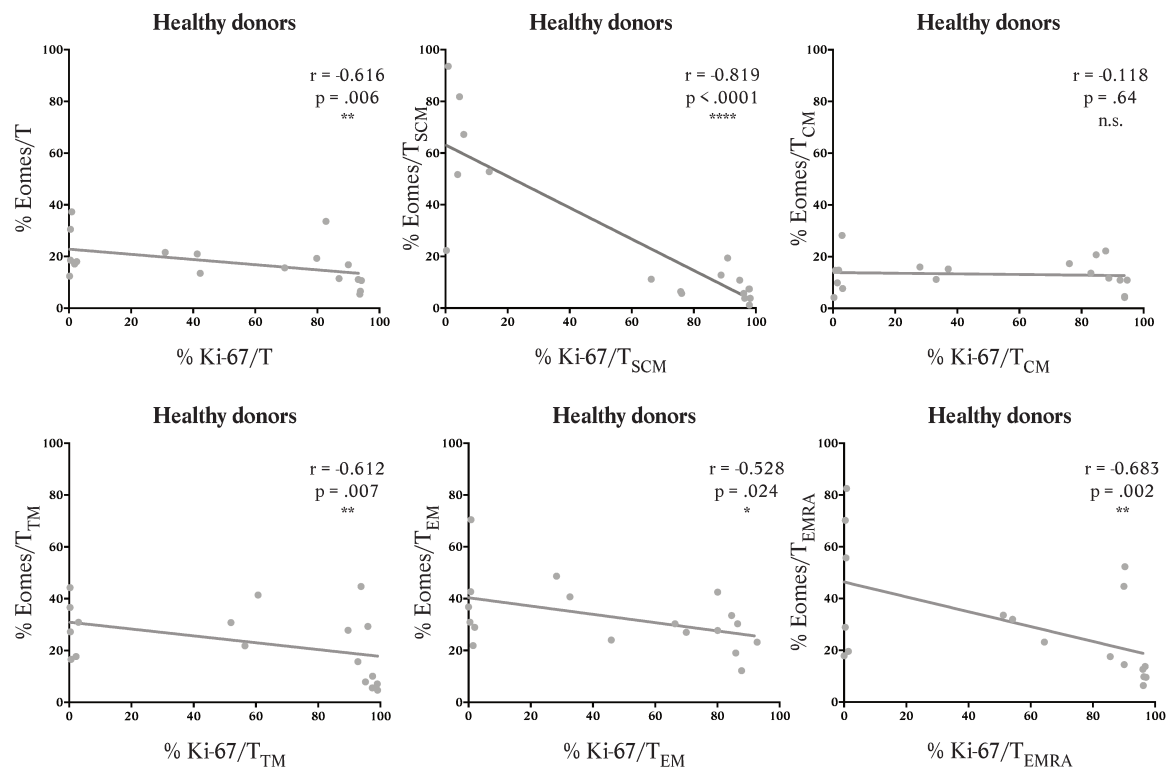
T_N cells were defined as the intersection of $CD3^+$, $CD45RA^+$, $CCR7^+$, $CD27^+$, $NOT(CD95^+)$ expressing cells, T_{SCM} cells as the intersection of $CD3^+$, $CD45RA^+$, $CCR7^+$, $CD27^+$, $CD95^+$ expressing cells, T_{CM} cells as the intersection of $CD3^+$, $NOT(CD45RA^+)$, $CCR7^+$ expressing cells, T_{TM} cells as the intersection of $CD3^+$, $NOT(CD45RA^+)$, $NOT(CCR7^+)$, $CD27^+$ expressing cells, T_{EM} cells as the intersection of $CD3^+$, $NOT(CD45RA^+)$, $NOT(CCR7^+)$, $NOT(CD27^+)$ expressing cells and T_{EMRA} cells as the intersection of $CD3^+$, $CD45RA^+$, $NOT(CCR7^+)$ expressing cells.



Supplemental Figure 2 - Ki-67 and Eomes expression (% of cells) in naïve and memory T cell subsets of six patients, 3 DC (blue) and 3 NR (red), at baseline and at W6. Median values are represented.



Supplemental Figure 3 - Gating strategy for T_{SCM} cells sorting. T_{SCM} cells were defined as the intersection CD3⁺, CD45RA⁺, CCR7⁺, CD27⁺ and CD95⁺ expressing cells.



Supplemental Figure 4 - Correlation between Eomes and Ki-67 expression in different T cell subsets in healthy donors. Levels between different days of culture are not discriminated.

GENERAL DISCUSSION

Melanoma is one of the most rapidly spreading cancers in terms of worldwide incidence. Until only five years ago, chemotherapy was still the standard treatment for metastatic melanoma. As rapid as the last half-decade, melanoma has met a striking treatment development with noticeable response ratios and survival that led to FDA approval of ipilimumab, anti-PD-1 antibodies and targeted therapies. Recently, a phase I clinical trial involving 107 metastatic melanoma patients submitted to nivolumab monotherapy reported a 5-year OS rate of 34 % (Hodi et al., AACR 2016). However, despite the impressive advances in patient management, predictive biomarkers of treatment response and survival are still lacking. It is likely that biomarker research was hampered by the context of poor response rates and survival that prevailed in melanoma treatment until recently. In this new encouraging context, potential melanoma and immunological biomarkers are to be revisited, in the view of early detection, prognostication and monitoring of malignant melanoma treatment.

In this work, we sought to identify potential biomarkers correlating with ipilimumab treatment response and survival in the context of metastatic melanoma. Peripheral biomarkers were assessed in a cohort of 77 stage IIIC/IV melanoma patients treated with ipilimumab after failure of at least one line of treatment. Serum biomarkers included both melanoma (LDH, S100B, MIA) and immune-related markers such as soluble MICA, which promotes melanoma immune-escape by targeting anti-tumour immune actors, as well as anti-MICA antibodies. Peripheral blood assessed biomarkers were focused on T lymphocytes, as they constitute the primary targets of anti-CTLA-4 therapies, with a special interest on memory T cell subsets.

Serum biomarkers of melanoma under ipilimumab

Melanoma cells release multiple compounds into circulation either by cell death or active secretion. The value of serum biomarkers relies on its easy routine measurement and relatively low cost. However, like other tumour markers, most of them show higher expression in advanced disease along with tumour load increase, making them not suitable for diagnosis due to low sensitivity especially at early disease stage (Brochez & Naeyaert 2000). The prognostic features of primary melanomas that will develop into metastatic disease have been widely studied, including parameters such as the Clark level, Breslow depth and evidence of ulceration and lymphocyte infiltration. In patients with regional nodal disease, systemic recurrence has been correlated with size and number of invaded lymph nodes (Sirott et al. 1993). However, prognosis of metastatic melanoma is less well established.

Lactate dehydrogenase is the only serum marker included in AJCC system for late stage melanoma, allowing the classification of stage IV into M categories: M1a (soft tissue metastasis), M1b (pulmonary involvement), and M1c (other visceral organs involvement or elevated LDH) (Balch et al. 2009). In stage IV melanoma, the 1- and 2- year survival rates are respectively 65 % and 40 % in patients with normal serum LDH values, compared with 32 % and 18 %, respectively, when LDH is elevated (Balch et al. 2009). Of note, LDH is not useful in predicting the sentinel lymph node status in melanoma patients (Egberts et al. 2010). However, in patients with metastatic melanoma submitted to systemic treatment, higher pretreatment LDH serum levels were shown to correlate with decreased survival (Sirott et al. 1993) and treatment response (Franzke et al. 1998; Deichmann et al. 1999a).

S100B is another potential melanoma biomarker under intensive investigation. S100B levels are correlated to clinical stage of melanoma (Bonfrer et al. 1998; Hauschild & Brenner 1999; Jury et al. 2000; Schmitz et al. 2000; Mårtenson et al. 2001; Bottoni et al. 2003), with stage IV presenting significant circulating levels. Several studies described lower survival rates in melanoma patients bearing higher serum levels of S100B (Hauschild & Brenner 1999) even in subsets of patients with stage IV melanoma (Smit et al. 2005; González Cao et al. 2007; Egberts et al. 2008), as well as poorer response to chemo- and immunotherapy (Deichmann et al. 1999b; A Hauschild et al. 1999; Djukanovic et al. 2000; Hamberg et al. 2003; Schmidt et al. 2004).

We sought to analyse the prognostic relevance of these markers in the context of ipilimumab therapy, where we describe in a cohort of 77 unresectable melanoma patients an association between low LDH and S100B serum levels at baseline, week 3 and 6 and response to ipilimumab, as well as increased overall survival, when markers are independently analysed. However, after adjustment to LDH in Cox multivariate analysis, only LDH remained statistically significant, whereas S100B did not provide additional significant information, suggesting a correlation between these two markers.

Previous studies addressing the respective involvement of S100 and LDH in multivariate analysis were performed in small cohorts ranging from 61 and 145 patients (Deichmann et al. 1999a; Axel Hauschild et al. 1999; Mohammed et al. 2001; Smit et al. 2005; Egberts et al. 2008; Egberts et al. 2012; Valpione et al. 2015). All reported a prognostic value for both markers in univariate analysis. However, after multivariate analysis, some of them (similar to our study) described LDH as the only independent prognostic marker (Deichmann et al. 1999a; Smit et al. 2005; Valpione et al. 2015), whereas only S100B remained statistically significant in other studies (Axel Hauschild et al. 1999; Mohammed et al. 2001; Egberts et al. 2008; Egberts et al. 2012).

In order to clarify this controversial question, Weide *et al.* investigated both biomarkers in a cohort of 855 stage IV melanoma patients, showing for the first time that both LDH and S100B at the time of initial stage IV diagnosis are independent prognostic factors, being useful to select patients for complete metastasectomy (Weide et al. 2012). In the same study (Weide et al. 2012), both elevated LDH and S100B levels correlated with a lower median survival (6 months) when compared to the subgroups of patients with either LDH or S100B elevated (9 months). Later, the same team assessed the prognostic significance of LDH and S100B serum levels in a cohort of 499 patients with unresectable melanoma at the time of systemic therapy onset (Weide et al. 2013). Again, S100B and LDH were found to be independent prognostic factors.

Weide's report of LDH and S100B as independent prognostic factors is not surprising, since both proteins have distinct functions. LDH is an ubiquitous enzyme present at high concentrations in liver, kidney, myocardium, skeletal muscle and red blood cells. In the absence of oxygen, LDH generates NAD^+ (required for glycolysis) through pyruvate conversion to lactate, resulting in secretion of lactate from cells as a waste product (Miao et al. 2013). This process (anaerobic glycolysis) results in a reduced net cellular gain when

compared to aerobic glycolysis, in which NAD^+ is regenerated by oxidative phosphorylation. While cells usually switch from slow aerobic to rapid anaerobic consumption of glucose, known as the Pasteur's effect, the Warburg effect consists in the avid consumption of glucose by tumour cells in the presence of oxygen, which compromises ATP yields (Jurisic et al. 2015). This phenomenon confers cancer cells significant growth advantages by attenuating the presence of reactive oxidative species generated in oxidative phosphorylation, known to induce cellular senescence and apoptosis, and by providing a source of NADH for biosynthesis of macromolecules required for rapid cell proliferation and invasion (Jurisic et al. 2015).

LDH is thus primarily expressed by cancer cells and found in a variety of cancers including melanoma (Miao et al. 2013; Díaz-Lagares et al. 2011). However, it is also elevated in a variety of tissue injuries such as hepatitis or myocardial infarction (Garnier et al. 2007). As it is not a secreted enzyme, it is thought that elevated LDH serum levels in melanoma patients is a consequence of spillage by melanoma cell necrosis, which occurs when part of a tumour outgrows its blood supply (Jurisic et al. 2015). As high turnover of tumour cells is associated with necrosis in fast-growing tumours, this results in elevated LDH serum levels (Brochez & Naeyaert 2000; Jennings & Murphy 2009).

S100B is tissue-specific, abundant in melanocytes, as well as in glial cells, Schwann cells, adipocytes and chondrocytes (Harpio & Einarsson 2004). As tissues expressing S100B are originally derived from the neural crest, it is not surprising that this protein can be found at high levels in brain tissue damage and in their associated cancers, as well as in melanoma (Ghanem et al. 2001). S100B was found to downregulate p53 and apoptosis in malignant melanoma (Lin et al. 2010), which can be reversed by S100B inhibition (Lin et al. 2004). A substantial fraction of S100B is membrane-bound. This, added to the fact that increased S100B expression is observed in melanoma cells compared to melanocytes, makes it an established immunohistochemical marker in the diagnosis of in primary and metastatic melanoma (Palmer et al. 2011). In this context, S100B might reflect the volume of the pathological tissue or an overexpression of the protein, or both.

In addition, melanoma and endothelial cells also release S100B into blood circulation following cell damage and death by apoptosis or necrosis (Ghanem et al. 2001), and serum levels of S100B were found to correlate with tumour burden in malignant melanoma (Harpio & Einarsson 2004). Due to its higher specificity for disease

progression than LDH, S100B was included in the ESMO guidelines for the follow-up of melanoma patients (Dummer et al. 2015).

Therefore, both LDH and S100B proteins are released by melanoma following tumour cell death, and elevated serum levels of both proteins seem to reflect tumour cell burden (Brochez & Naeyaert 2000; Jury et al. 2000). However, although S100B and LDH levels may be correlated, their biology is different. S100B was shown to interact with p53 and to have an active role in cell survival and proliferation, whereas LDH has an active role in hypoxia conditions. So, even though it is not surprising that they are coexpressed in a significant proportion of melanoma cells, the expression of S100B does not seem to imply LDH activity, nor vice-versa.

In our study, we report that approximately one third (32 %) of patients expressed elevated S100B levels and normal LDH at baseline (data not shown), as previously described (Weide et al. 2012; Weide et al. 2013). In addition, median survival times differed between patients with both serum markers in the normal range (11 months), patients with either LDH or S100B elevated (8 months) and patients with both markers elevated (4 months) (data not shown). Therefore, both markers seem to independently influence overall survival under ipilimumab, and the sample size of our study as well of previous studies may have accounted for the observed results.

Interestingly, both Weide's studies reported a minority of patients with isolated increase levels of LDH (≤ 5 %) before treatment (Weide et al. 2012; Weide et al. 2013). In our study, only 6 % of patients showed isolated elevated levels of LDH before ipilimumab therapy. These 4 patients had a median level of LDH of 514 U/L, i.e., near the established cut-off value (500 U/L). One would therefore speculate that LDH excretion occurs after S100B release into the circulation, as nearly one third of patients (32 %) presented elevated levels of S100B with concomitant low levels of LDH. At initial melanoma stages, S100B may interact with p53, enhancing melanoma proliferation and survival, resulting in low melanoma neoplasia and leading to S100B secretion in lower amounts. When at a higher tumour turnover (eg, at stage IV), the elevated degree of hypoxia would favour both tumour neoplasia and hypoxia-induced cell death (induced when tumour cells outgrow their blood supply), inducing both S100B and LDH release into the circulation.

We report that baseline LDH serum levels are useful in predicting OS under ipilimumab treatment in patients with metastatic melanoma, as reported by others (Di Giacomo et al.

2011; J. Delyon et al. 2013; Simeone et al. 2014; Kelderman et al. 2014; Valpione et al. 2015; A Martens et al. 2016). Moreover, LDH levels after the first and second doses of ipilimumab remain predictive of survival and ipilimumab response, which has been little reported (Simeone et al. 2014). This landmark analysis might be useful for ipilimumab immunomonitoring, as LDH serum levels might change under the course of treatment.

In addition, S100B levels at baseline, weeks 3 and 6 correlated to a better response to ipilimumab as well as an increased overall survival in univariate analysis.

Studies revisiting S100B utility in the context of new immunotherapies are few. A case report of a stage IV melanoma patient has shown a good fitting of S100B serum levels with clinical benefit in the course of ipilimumab treatment (Loquai et al. 2013). Additionally, a study grouping metastatic melanoma patients submitted to various recent therapies including immune- and targeted therapies reported increased S100B serum levels in the course of therapy(ies) within non-responders (Barak et al. 2015). However, patients were not subdivided according to different therapies. Finally, Kelderman *et al.* reported an association between low baseline S100 levels and improved OS following ipilimumab therapy, which however lost its significance after multivariate analysis (Kelderman et al. 2014). In accordance to this, we describe the utility of S100B serum levels at baseline as a ‘standalone’ prognostic marker of overall survival and response to ipilimumab. Moreover, in univariate analysis, S100B correlation with treatment response and survival remained significant after the first two doses of ipilimumab, further supporting its role in ipilimumab immunomonitoring.

With the FDA approval of anti-PD-1 antibodies and B-RAF and MEK inhibitors that lead to fast responses and enhanced survival rates, the revisit of these markers in the context of these new therapies should bring more accurate results. This should further clarify the controversial utility of S100B and LDH as predictive biomarkers of response and even a tool in patients’ treatment selection. Some studies have already been conducted in this context, including the follow-up of melanoma patients treated with BRAF-inhibitors (Abusaif et al. 2013; Sanmamed et al. 2014), and pembrolizumab (A Martens et al. 2016), that further supported a role in LDH, MIA and S100B levels in immunomonitoring.

In contrast to LDH and S100B, MIA’s expression is highly restricted to melanocytic tumours, although it can also be expressed by chondrocytes and certain advanced stage adenocarcinomas (Sandru et al. 2011). MIA is known for its active role in extravasion and

metastasis of melanoma cells by inducing cells detachment from extracellular matrix components (Bosserhoff et al. 2003), which explains its high specificity as a serum biomarker for melanoma metastasis detection (Bosserhoff & Buettner 2002; Schmidt et al. 2012). Higher serum levels of MIA were shown to correlate with melanoma clinical stage, as well as with chemotherapy response in melanoma patients (Bosserhoff et al. 1997b; Stahlecker et al. 2000). MIA was also described as a biomarker of overall survival independent of S100B and LDH (Díaz-Lagares et al. 2011), although results are inconsistent (González Cao et al. 2007).

Although lower serum levels at W3 were significantly associated to a better response to the ipilimumab in univariate analysis, we could not confirm the prognosis value of MIA in terms of response and survival. A study including 296 consecutive patients with stage II or III melanoma identified a higher sensitivity of S100B and MIA to detect new metastasis than LDH, with MIA presenting the best diagnostic accuracy (Garbe et al. 2003). Being malignant melanocytic-specific, MIA might be of better clinical utility in diagnosis and metastasis detection, that is, in the follow-up of high-risk melanoma patients, rather than in the immunomonitoring of already established late stages melanoma where melanoma metabolism (i.e., LDH) may better account for possible differences within patients.

Based on a study by Jinushi *et al.* (Jinushi et al. 2006), we sought to elucidate the potential prognostic role of soluble MICA and anti-MICA antibodies in patients submitted to ipilimumab. In this case-report of a metastatic melanoma patient responding to anti-CTLA-4 therapy, Jinushi *et al.* observed a progressive increase of anti-MICA antibodies associated with a concomitant decrease of sMICA serum levels, and an improvement of NK and CD8⁺ T lymphocytes cytotoxicity against the tumour (Jinushi et al. 2006).

sMICA is known to mediate the downregulation of NKG2D via rapid endocytosis and partial lysosomal degradation, resulting in CTL and NK cells cytotoxicity impairment (Roda-Navarro & Reyburn 2009). This phenomenon participates in tumour escape strategies, and is illustrated by the presence of high levels of soluble MICA in various types of cancer including melanoma (Paschen et al. 2009). In our cohort, composed uniquely of melanoma patients with stage IIIC (10 %) or IV patients, sMICA was significantly elevated in patients when compared to healthy donors. However, sMICA was not found to be of prognostic value of treatment response or survival during ipilimumab therapy, although a trend towards lower sMICA concentrations at baseline and better outcome was observed.

These results are in accordance with a recently published study concerning 37 metastatic melanoma patients treated with ipilimumab at 10 mg/kg and fotemustine (Maccalli et al. 2015). Finally, we were not able to find in our patients the inverse correlation of sMICA and anti-MICA serum levels described in the case report by Jinushi *et al.*

Although resulting in long-term benefit in 20 % of patients (Schadendorf et al. 2015), ipilimumab is responsible for the occurrence of IrAEs in 64 % of cases, with severe IrAEs (grade 3 or 4) affecting 27 % of patients (Larkin et al. 2015; Ibrahim et al. 2011). This can be expected since anti-CTLA-4-blockade promotes enhanced cytotoxicity, which might result in weaker self-tolerance. IrAEs induction was shown to correlate with objective response rates in metastatic melanoma patients submitted to ipilimumab in early studies (Downey et al. 2007; Beck et al. 2006; Blansfield et al. 2005; Attia et al. 2005), but they are not so far established as predictive markers of response. Here, we report the occurrence of IrAEs in 47 % of patients. However, IrAEs occurrence did not correlate with clinical benefit, in accordance with a recent study (A Martens et al. 2016).

We sought to test whether the occurrence of IrAEs was associated with baseline biomarkers related to melanoma or to immunologic markers. No association was found between IrAEs and the assessed immunological markers such as lymphocytes activation status (assessed by CD25, HLA-DR and ICOS expression). We have also analysed the mucosal-associated invariant T (MAIT) cells in our cohort, a non-conventional subset of T cells abundant in human blood and intestinal mucosa (Le Bourhis et al. 2010) that plays a potential role in ulcerative colitis (Treiner 2015). We did not find any association between this population and the occurrence of IrAEs. Tumour-related biomarkers such as LDH, S100B and MIA were also not associated to the occurrence of IrAEs during treatment.

However, our results suggest an association between the absence of IrAEs and the presence of higher levels of sMICA at baseline in disease control group. As colitis affected 82 % of patients in this group, we speculate that sMICA, shed by tumour cells, might confer a protective status to patients from IrAEs occurrence. Actually, besides being upregulated in stress conditions and in various malignancies including melanoma, MICA is also constitutively expressed at low levels on intestinal epithelium of the gut (Groh et al. 1996). Ulcerative colitis, an inflammatory disorder of the gastrointestinal tract, is suspected to be a consequence of a breakdown of immune tolerance to intestinal antigens, resulting in a deregulated or constant activation of the immune system (Ge et al. 2011).

The resulting epithelial damage involves non-classical MHC-I molecules including MICA. MICA was shown to be upregulated in the colonic mucosa of ulcerative colitis patients, mediating the activation of NK cells through interaction with NKG2D and inducing IFN- γ production (Ge et al. 2011). We hypothesize that, although CTLA-4-blockade might enhance cytotoxicity throughout the body, sMICA shed by tumour cells might promote NKG2D downregulation in cytotoxic lymphocytes (CD8⁺ T and NK), counter-balancing MICA-dependent cytotoxicity and resulting in fewer inflammation in the gastrointestinal tract that could otherwise develop into colitis. Soluble MICA serum levels might thus constitute a clue in immune-related adverse events prediction during ipilimumab treatment, which will be interesting to explore.

Peripheral biomarkers of immune response under ipilimumab

We report here that patients presented lower levels of absolute lymphocyte counts (ALC) at baseline compared to healthy donors. In addition, patients with lymphopenia (lymphocyte count lower than $10^9/L$) before treatment had a lower median survival (5 months) than patients with higher lymphocyte counts, who had an extended overall survival of 11 months. The latter group included a higher proportion of patients achieving disease control. These results suggest that potentially immune-competent patients are more prone to respond to a checkpoint inhibitor. The reported lymphopenia may be caused by chemotherapy (Mignot et al. 2014) or by tumour-derived factors that have been shown to induce immune cells death both at the tumour site and in peripheral circulation (Whiteside 2013). In addition, we observed an early increase in ALC between baseline and week 3 after the beginning of ipilimumab treatment, involving CD4⁺ and CD8⁺ subsets, in both DC and NR groups. Interestingly, this increase was sustained between weeks 3 and 12 in DC group, whereas a decrease was observed in NR. Therefore, an increase in ALC during the first weeks of ipilimumab treatment may reflect an immune response capable of prolonging patients survival. Our results are consistent with previous studies (Ku et al. 2010; Di Giacomo et al. 2011; J. Delyon et al. 2013; Bjoern et al. 2016; A. Martens et al. 2016). Accordingly, results from a meta-analysis including 6 ipilimumab studies reported a significant ALC increase over time in patients receiving ipilimumab with or without chemotherapy, whereas no increase in ALC was observed in patients receiving gp100 or BRAF-inhibitor (Postow et al. 2013). To the best of our knowledge, we

identified for the first time baseline levels of ALC as a predictive marker of clinical response and overall survival in patients submitted to ipilimumab.

We also observed an increase in the frequency of circulating T_{reg} (defined as $CD4^+CD25^+CD127^{low}$) following ipilimumab therapy (data not shown). Conflicting results have been reported concerning ipilimumab modulation of T_{reg} compartment. Due to their constitutive expression of CTLA-4, T_{reg} represent potential direct targets of ipilimumab. Previous studies reported that higher baseline levels of circulating T_{reg} or decreased levels during ipilimumab therapy correlated to a favourable outcome (A Martens et al. 2016; Simeone et al. 2014). Furthermore, it was described that anti-CTLA-4-mediated activity on T_{reg} compartment is mediated by Fc-dependent depletion of tumour-infiltrating (but not lymph node-infiltrating) T_{regs} (Simpson et al. 2013; Bulliard et al. 2013). Previous studies reported a decrease of intratumoural effector-to-regulatory T cells ratio associated to anti-CTLA-4 response (Hodi et al. 2008; Liakou et al. 2008). However, these changes observed in the tumour microenvironment don't seem to be clearly reflected in blood (Quezada et al. 2006), and CTLA-4-expressing T-cell-depletion does not seem to occur in the periphery (Selby et al. 2013). In accordance with our results, a significant increase in circulating T_{reg} was observed in a cohort of 35 stage III/IV melanoma patients receiving ipilimumab, that was even correlated with progression-free survival (Tarhini et al. 2014). However, in the same study, the opposite change in T_{reg} was found in the tumour microenvironment. In addition, T cells have been shown to become entirely resistant to circulating T_{reg} -inhibitory effects following tremelimumab therapy (Ménard et al. 2008a). Although we did not find any association between T_{reg} levels and either treatment response or IrAEs, these conflicting results raise the question of whether these cells are functional. In any case, changes in the frequency of regulatory T cells in the systemic circulation after ipilimumab do not seem to provide a reliable marker of immunological impact.

Our results further emphasize the accepted ipilimumab mode of action, that is, to prevent inhibitory control of T lymphocytes by CTLA-4 and boosting specific T-cell responses. This can occur either by amplifying previously induced T-cell responses, as reported by Tarhini *et al.* (Tarhini et al. 2014), or by inducing new tumour-specific T cell reactivity, which was supported by studies reporting an evolution and diversification of TCR repertoire under ipilimumab therapy (Kvistborg et al. 2014; Cha et al. 2014). It was also reported that OS positively correlated with the maintenance of high-frequency-clones present at baseline

(Cha et al. 2014), further underlining the importance of pre-existing high-avidity T cells for antitumour response.

Prior to ipilimumab, we reported that melanoma patients exhibited an activated immune status, as illustrated by increased HLA-DR and granzyme B expression in CD8⁺ T cells. In addition, we found elevated chemokine receptors expression such as CXCR3, CCR4, CCR6 and CLA. These features are characteristic of an enhanced ability for skin migration of activated T cells in normal conditions. However, in the context of melanoma, these phenotypes only indicate the presence of an existent but failed immune reaction against the melanoma.

Ipilimumab treatment resulted in an expansion of activated (HLA-DR⁺) CD4⁺ and CD8⁺ T cells which was evident after the first dose of ipilimumab and persisted during treatment, and which was frequently observed by other groups (Bjoern et al. 2016; Weber et al. 2012; Maker et al. 2007; Maker et al. 2005; Attia et al. 2005; Phan et al. 2003).

More importantly, we report that patient memory subsets are reshaped during ipilimumab treatment. CD4⁺ and CD8⁺ central and effector memory compartments expanded both in absolute number and percentage in the first three weeks of treatment, reflecting an early effect of ipilimumab. This was followed by a late increase of T_{EMRA} CD8⁺ T cells between weeks 3 and 12. As the lineage relationship amidst different memory CD8 T subsets is not clear (see Terminally Differentiated Effector Memory T cells (TEMRA) (p.49) of Introduction), it is difficult to suggest that the delayed increase in T_{EMRA} results from a stepwise differentiation from T_{CM} to T_{EM} to T_{EMRA} (Mahnke et al. 2013), or from homeostatic cytokine response promoting a direct differentiation of T_{CM} to T_{EMRA} (Sallusto et al. 2004; Willinger et al. 2005). In either cases, the observed delayed increase in T_{EMRA} seems to originate from less differentiated memory T cells rather than from self proliferation, as Ki-67 expression remained unchanged. By contrast, the proliferative capacity of T_{CM} and T_{EM} in both CD4⁺ and CD8⁺ compartments was enhanced from baseline to W6, which might explain their expansion during therapy.

As one might expect, the main effect of CTLA-4 blockade is to unleash pre-existing antitumour-specific effector and memory T cells from inhibition. In fact, although not significant, we have observed at baseline a higher expression of CTLA-4 in the memory cell compartment CD8⁺ T cells (including T_{SCM}, T_{CM}, T_{TM} and T_{EM}) when compared to healthy donors. This suggests an ‘arrested’ state of these cells promoted by CTLA-4

inhibitory signals that might favour tumour progression before treatment, and probably account for the observed expansion of these subsets during anti-CTLA-4 therapy.

It has been shown that CTLA-4-blockade in mice during *in vivo* primary antigen exposure as well as in recall memory CD8⁺ T cell responses to *Listeria monocytogenes* infection increases the expansion and enhances the effector functions of antigen-specific memory CD8⁺ T cells, resulting in a greater accumulation of memory T cells with improved ability to eliminate subsequent infections (Pedicord et al. 2011). Before anti-CTLA-4 administration, patients are likely to harbour a heterogeneous pool of naïve, effector and memory T cells. In our study, Ipilimumab was shown to expand memory T cell subsets with concomitant decrease in percentage but not absolute counts of naïve T cells (data not shown). This data suggest that the expanded memory compartment resulted from memory T cell expansion and possibly differentiation. However, if these expanded cells are melanoma-specific remains elusive.

We reported that the early increase in CD4⁺ T_{CM} and T_{EM} cells was significantly higher for patients with better clinical outcome. This was followed by a significant increase of both CD4⁺ and CD8⁺ T_{EM} cells and of CD8⁺ T_{EMRA} between W3 and W12, which also correlated with DC. Moreover, this was accompanied by an enhanced proliferative potential of central and effector memory CD8⁺ T cells in responders.

Previous studies of advanced melanoma patients submitted to ipilimumab reported the induction of memory (CD45RO⁺) TILs in tumour biopsies (Tarhini et al. 2014). In addition, a restoration of the memory CD4⁺ and CD8⁺ T compartment has been observed in tremelimumab-treated patients (Ménard et al. 2008b). Several studies reported an increase of CD45RO⁺ T cells following ipilimumab therapy (Phan et al. 2003; Attia et al. 2005; Maker et al. 2005; Maker et al. 2007), or more precisely in T_{CM} (Weber et al. 2012) and T_{EM} cells (Bjoern et al. 2016; Weber et al. 2012) but no correlation with treatment response was described. Recently, a study including 31 stage III/IV melanoma patients treated with ipilimumab plus fotemustine reported an increase in circulating CD4⁺ and CD8⁺ activated T_{CM} cells following treatment (W12), which correlated with clinical outcome (Maccalli et al. 2015).

We have followed the expression of the transcription factors Eomes and T-bet, and reported an increase of Eomes expression in both CD4⁺ and CD8⁺ T cells and of T-bet in the CD8⁺ T cell compartment from baseline to W6. This was accompanied by an increase

in CXCR3, a chemokine receptor involved in migration to tumour and inflamed tissues (Li et al. 2013). T-bet and Eomes have been shown to be expressed by colorectal TILs, with increased expression correlating with survival (Atreya et al. 2007). In addition, mice germline-deletion of both Eomes and T-bet results in a drastic decrease in CXCR3 expression with limited numbers of tumour-infiltrating cells following transfer of B16 melanoma cells (Y. Zhu et al. 2010). In our cohort, patients achieving DC showed a higher increase of Eomes-expressing CD8⁺ T cells following the first two doses of ipilimumab, as compared with non-responders. The enhanced expression of Eomes and T-bet under ipilimumab might promote T cell migration to tumour by enhancing CXCR3 expression. Another study reported an association between extended survival and the expression of CXCR3 by melanoma-specific memory (CD45RO⁺) CD8⁺ T cells derived from the peripheral blood and tumour-involved lymph nodes of metastatic melanoma patients (Mullins 2004). Moreover, CXCR3 was highly coexpressed with CCR4. We report enhanced percentages of CCR6 and CCR4-expressing CD4⁺ and CD8⁺ T cells, as well as of CLA⁺CD8⁺ T cells following the first two doses of ipilimumab. These chemokine receptors promote specific interactions between T cells and selectins and integrins on the vascular epithelium, as well as T-cell interactions with tumour-derived soluble chemokines (Mullins 2004).

Since ectopic expression of Eomes reverses the CTLA-4-mediated inhibition of effector molecules production, consequently enhancing the cytotoxicity of these cells *in vivo* (Hegel et al. 2009), one could hypothesize that CTLA-4 mediates its inhibitory effect on CD8⁺ T cells by repressing Eomes. In addition, low expression of T-bet and Eomes were shown to define dysfunctional T cells rendered tolerant *in vivo* after encounter with tumour cells (Berrien-Elliott et al. 2014). Finally, a combined checkpoint blockade (with anti-CTLA-4, -PD-1 and -LAG-3) was shown to induce T-bet and Eomes in responding T cells (Berrien-Elliott et al. 2014). In accordance with this, we have shown that anti-CTLA-4 blockade in melanoma patients results in an increased expression of both T-bet and Eomes in CD8⁺ T cells with increased effector functions such as TNF- α and granzyme B production. The fact that T-bet promotes short-lived effector CD8 T cells while Eomes promotes the generation of long-lived memory T cells (Kaeck & Cui 2012) is consistent with the expansion of the central and effector memory cell compartments and of terminally differentiated T cells.

Consistent with the increased expression of Eomes and T-bet by CD8⁺ T cells, we observed an expansion of CD122-expressing CD4⁺ and CD8⁺ T cells. This was not

surprising, since these transcription factors sustain CD8⁺ memory T cell homeostasis through the expression of CD122, the β -subunit of IL-15 receptor (Li et al. 2013).

We have then followed the recently described T_{SCM} memory compartment. After two doses of ipilimumab, the percentage of these cells decreased, with absolute values remaining unchanged. This observation, associated to increased levels of Ki-67⁺T_{SCM} cells, suggested an asymmetric division, characteristic of these cells and involving both expansion and differentiation (Gattinoni et al. 2011). T_{SCM} cells have been shown to be endowed with great proliferative potential and capacity to self-renew and to differentiate into all other memory subsets (Gattinoni et al. 2009; Gattinoni et al. 2011; Cieri et al. 2013). In addition, studies have characterized their clinical potential in murine models, showing a superior ability to confer immunity than T_{CM} and T_{EM} cells in adoptive T-cell therapy (Gattinoni et al. 2009; Gattinoni et al. 2011). We did not analyse the specificity of the expanded and differentiated T_{SCM} cells in our study. However, we suggest that T_{SCM} differentiation might account for the observed expansion in central and effector memory T cell compartments. We are currently examining the effect of CTLA-4 blockade *ex vivo* on the dynamics of melanoma-specific T cells by using MHC class I and II tetramers (collaboration with Danila Valmori, Nantes).

A recent study reported that combined T-bet- and Eomes-deficiency in adoptively transferred T cells in mice generated a severe reduction in effector and central memory cells compartments with a concomitant increase in antigen-specific T_{SCM}-like cells and impaired immune responses (Li et al. 2013). This suggests a role of these transcription factors in regulating the balance between effector/central and stem-cell memory T-cell fates, highlighting their importance in the generation and maintenance of effector and central memory T cell compartment. In addition, this data suggests a repressive or non-essential role of T-bet and Eomes in T_{SCM} formation (Li et al. 2013). However, and in contrast to previous studies (Gattinoni et al. 2009), the generated T_{SCM}-like cells in double-KO mice showed compromised anti-tumour functions, suggesting a crucial role for T-bet and Eomes in the differentiation of T_{SCM} cells into effector and memory T cells.

Globally, we suggest a model (Figure 13) where CTLA-4 blockade unleashes the arrested state of pre-existing tumour-specific T cells, promoting increased expression of T-bet and Eomes. The fact that CTLA-4-blockade is expected to increase IL-2 levels and that Eomes expression is amplified by IL-2 secretion (Li et al. 2013; Kaeck & Cui 2012) might

account for this. In turn, Eomes and T-bet promote CXCR3 expression, favouring T cell migration to tumours (J. Zhu et al. 2010). Moreover, these transcription factors might endow CD8⁺ T cells with increased effector functions such as TNF- α and granzyme B, as observed in our study. Furthermore, they might promote effective T_{SCM} differentiation into central, memory and terminally differentiated T cells, these later endowed with effector capacities and ability to migrate to tumour. At the same time, Eomes and T-bet induce the expression of CD122, required for effector and memory T cells homeostasis and survival through IL-15 signalling.

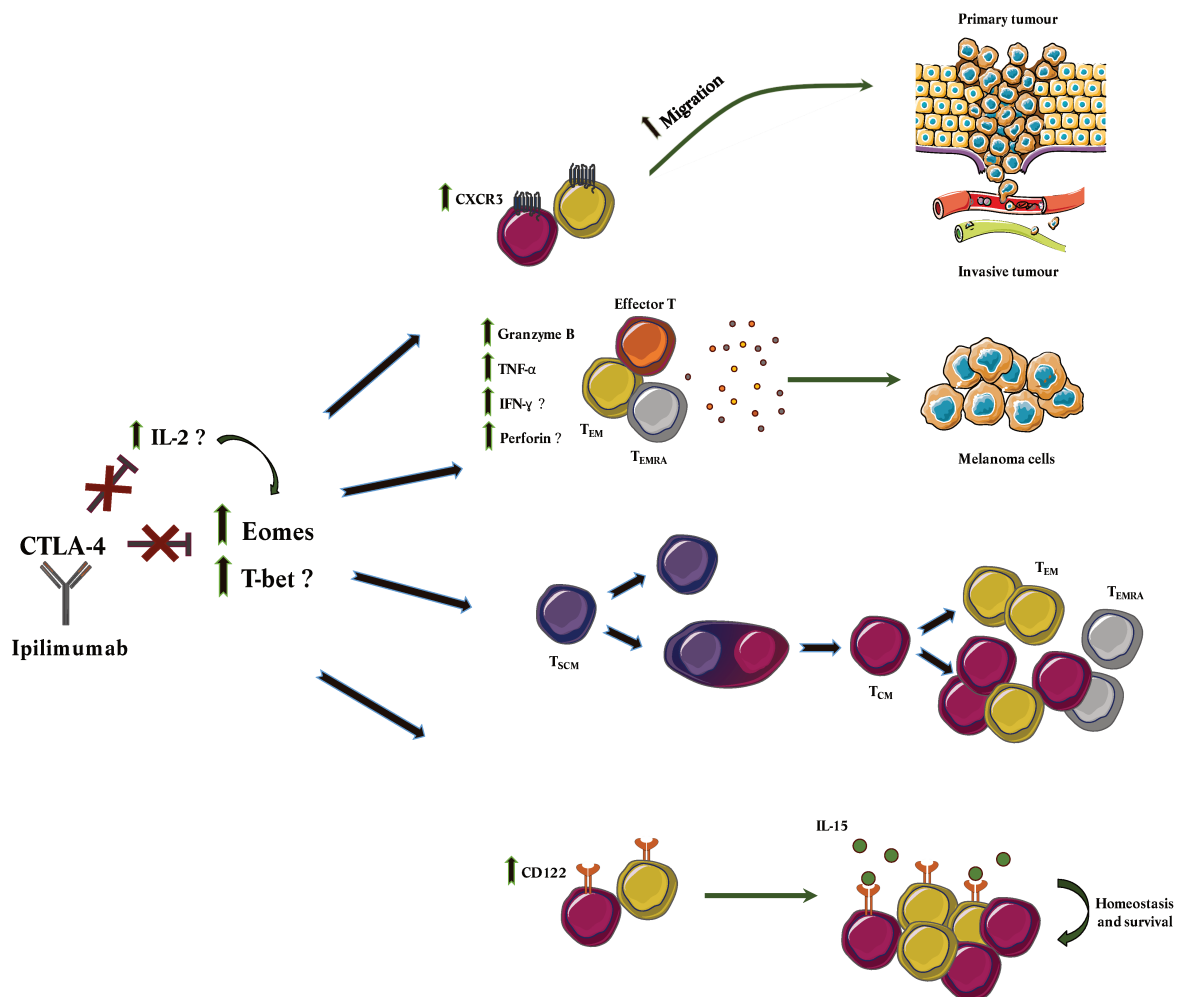


Figure 13 - Model for modulation of the immune system following CTLA-4-blockade in the context of advanced melanoma.

In order to analyse *in vitro* the behaviour of patients' memory T cells before and after ipilimumab treatment, we randomly selected 3 patients achieving disease control, 3 non-responders and 3 healthy donors to assess the ability of these cells to respond to anti-CD3/C28 stimulation. Memory T cell populations as well as Eomes and Ki-67 expression

were followed everyday for 5 days. Although we did not find significant differences in the evolution of T cell subsets between responders and non-responders, the differences between patients and healthy controls were striking.

HD showed a drastic and transient increase in the frequency of T_{SCM} cells from the second day of culture. This was accompanied by a delayed increase in the frequency of T_{CM} cells. Although T_{SCM} cells represent 2-3 % of all circulating T cells in healthy donors (Gattinoni et al. 2011), which is consistent to our results showing frequencies of 1-2 %, we observed a significant expansion of these cells following *in vitro* stimulation, which was not observed in T cells from metastatic melanoma patients. In addition, when T_{SCM} cells from HD were sorted at D3 and cultured with IL-7 stimulation, they were able to generate all other memory T cells, with higher frequencies in T_{CM} and progressive lower levels of T_{TM}, T_{EM} and T_{EMRA}. This is consistent with previous results showing a restrictive differentiation potential *in vitro* from T_{SCM} to T_{CM} to T_{EM}, with T_{SCM} being able to generate both other memory subsets, T_{CM} generating T_{EM} cells but not T_{SCM}, and T_{EM} not being able to differentiate in the previous memory subsets (Gattinoni et al. 2009). This result suggests a stepwise differentiation of memory T cells in the following order: T_{SCM}-T_{CM}-T_{EM}-T_{EMRA}. Most of all, these data suggest that a process of T_{SCM} expansion and differentiation might explain the delayed increased frequencies of T_{CM} in HD observed *in vitro*.

We are not able to assess if the observed expansion of T_{SCM} resulted from T_N differentiation or from T_{SCM} expansion. The higher levels of Ki-67 observed in T_{SCM} surely accounts for their increased levels. However, a previous study reported the generation of high levels CD8⁺ T_{SCM}-like cells from naïve precursors from healthy donors following *in vitro* stimulation with anti-CD3/CD28, IL-7 and IL-15 (Cieri et al. 2013), also supporting a T naïve-origin of these cells.

Overall, the observed expansion of T_{SCM} in HD followed by an expansion in T_{CM}, in contrast to the relatively constant frequencies of different memory T cells observed in patients, suggests that a defect of T_{SCM} cells exists in patients. These cells might have limited ability to either expand or differentiate into other memory subsets.

In our previous studies, suggesting a role of Eomes under ipilimumab treatment, the expression of this transcription factor was assessed in CD4⁺ and CD8⁺ T cells but not in different memory T cell subsets. Interestingly, here we describe that Eomes is highly expressed *in vivo* on T_{SCM} cells, and that ipilimumab seems to promote Eomes expression

in patients' T_{SCM}, as showed by the increased levels between baseline and W6. In addition, Eomes levels progressively increased from T_{CM} to T_{EM} and to T_{EMRA}. This have been previously suggested by gene hierarchical clustering, showing an increase in Eomes expression, as well as in cytotoxic molecules, with differentiation (Takeshita et al. 2015; Gattinoni et al. 2009).

Interestingly, we found an inverse correlation between Eomes and Ki-67 in memory T-cell subsets from healthy donors. This inverse correlation was also found in DC patients for T_{SCM} and T_{EMRA} cells, which was accentuated following 2 doses of ipilimumab therapy. By contrast, non-responders did not show this inverse correlation in any of the memory T cell subsets before and after therapy. This suggests that HD and DC patients may possess a mechanism of regulation between Eomes and Ki-67 that might be perturbed in NR. Eomes and T-bet are known for not being required for memory T cell proliferation (Li et al. 2013). However, they play an important role in memory T cells fate, homeostasis and effector functions (Kaech & Cui 2012). Under memory T cells homeostasis, memory T cell proliferation rates might be low compared with potent anti-CD3/CD28 *in vitro* stimulation. Under potent stimulation, the high Ki-67 expression in HD might be accompanied by a process of memory cells differentiation, which might be driven by Eomes that is probably progressively down-regulated as proliferation and differentiation occurs. Interestingly, a previous study found the same inverse correlation between Eomes and proliferation capacity of CMV-specific CD8⁺ T cells during primary infection following solid organ transplantation, which differentiated the capacity for durable immune control. Accordingly, relapsers showed significantly reduced proliferative capacity along with higher Eomes expression levels, resulting in poor differentiation, lower T-bet levels and reduced acquisition of effector function (Popescu et al. 2014). In addition, a study by Li *et al.* reports that a deficiency in both T-bet and Eomes promotes the accumulation of T_{SCM} cells with high differentiation defects (Li et al. 2013). This suggests that Eomes and T-bet expression in T_{SCM} might be essential for their effective differentiation into effector and memory T cells.

The high Eomes expression in T_{SCM} during the first two days of culture is also consistent with the suggested role of Eomes in counter-regulating the terminal differentiation of T cells (Kaech & Cui 2012). These cells, as well as T_{EMRA} cells, progressively lost Eomes expression during *in vitro* stimulation, consistent with onset of proliferation and differentiation. In differentiated states, effector functions of T cells are generally assured

by the expression of T-bet (Kaech & Cui 2012). It would be interesting to explore the role of this transcription factor in our experiments.

This *in vitro* study provides very interesting clues but still requires confirmation with a larger cohort of patients and donors, with the inclusion of other known markers of T_{SCM} phenotype such as CXCR3 and CD122, and with different cytokine stimulation conditions such as IL-7 and/or IL-15, known to be important in T_{SCM} homeostasis. Furthermore, assays assessing the ability of sorted T_{SCM} cells from patients to effectively differentiate into conventional memory subsets would be of great interest. Such experiments should give further insight in the cellular and molecular mechanisms improving or not memory T cells differentiation and proliferation after CTLA-4 blockade.

We propose here a model allowing the early detection of T_{SCM} cells' differentiation into conventional memory T subsets under ipilimumab treatment with the potential role of Eomes transcription factor.

CONCLUSION & PERSPECTIVES

Ipilimumab was the first therapy showing to improve overall survival compared to dacarbazine in patients bearing metastatic melanoma (Hodi et al. 2010), broadening treatment options and bringing new hope for innovative immune and targeted therapies, that emerged soon after. However, just a subset of patients appears to benefit from immune- and targeted-therapies and, despite clinical advances, a proportion of patients with advanced metastatic melanoma still have a high risk of mortality.

We have shown that melanoma biomarkers such as LDH and S100B might be of useful in the clinical management of patients under ipilimumab therapy, or even in patients' pre-selection for this treatment. In addition, soluble MICA serum levels might prove to be of clinical utility in the setting of frequent immune-related adverse events under ipilimumab.

We have also reported several immunological changes induced by ipilimumab in melanoma patients. These include the reshaping of the memory T cell compartment, with potential involvement of T_{SCM} cells. In addition, ipilimumab induced the expression of several chemokine receptors, important for T-cell migration, and of Eomes and T-bet, master transcription factors of fate and effector functions of T lymphocytes. The enhanced expression of these transcription factors might play a role in the observed dynamics of memory T cells.

Finally, we reported a defect of patients' T_{SCM} cells expansion and/or differentiation *in vitro*, which might be related with a perturbed regulation of Eomes and Ki-67.

We suggest that T_{SCM} cells might play an important role in melanoma patients immune status, which might be potentiated with ipilimumab. The monitoring of these cells within patients with a more complete phenotype including Eomes, Ki-67 (as performed in our *in vitro* studies) and T-bet expression might provide better understanding of the mechanisms

underlying their preferential expansion or differentiation, in light of the recently identified role of Eomes and T-bet. We are currently analysing the *ex vivo* effect of CTLA-4 blockade on the dynamics of MHC class I- and II-tetramer-sorted melanoma-specific T cells, in collaboration with Danila Valmori, of Nantes. In addition, memory T cells' *in vitro* expansion from available PBMCs from our cohort of ipilimumab-submitted melanoma patients is being assessed in order to complete our study.

Since their identification in 2009, T_{SCM} cells have been the focus of important research, with studies assessing their ability in adoptive therapy. Long-term persistence of transferred T cells is fundamental for the success of adoptive immunotherapies. Interesting results demonstrated high antitumour efficacy of T_{SCM}, with enhanced survival and efficient reconstitution of the full memory and effector T lymphocytes following adoptive transfer, when compared to conventional memory T cells (Gattinoni et al. 2009; Gattinoni et al. 2011; Cieri et al. 2013). As a result, tumour-specific T_{SCM} cells are actively pursued for clinical translation.

With the recent advances in immunotherapy as well as in targeted therapies, the landscape of metastatic melanoma is dramatically and positively changing. In a near future, chemotherapy will hopefully be outdated. However, the diversified treatments comprising different mechanisms of action arise along with the urgent necessity of reliable procedures allowing a preselection of patients that will more likely benefit from one or more treatments in combination. Randomized studies directly comparing different therapies and assessing their resulting immunological changes should allow a better insight of benefits and potential biomarkers associated with each therapy.

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