CONTRIBUTION OF SYSTEMIC T CELL IMMUNITY TO CLINICAL EFFICACY OF ANTI-PD-L1/PD-1 IMMUNOTHERAPIES IN LUNG CANCER

Memoria presentada por

Miren Zuazo Ibarra

para optar al grado de doctor por la Universidad Pública de Navarra

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El Dr. David Escors Murugarren informa que la presente memoria de tesis doctoral elaborada por Miren Zuazo Ibarra ha sido realizada bajo su dirección y cumple con las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firma la presente en Pamplona, 11 de Noviembre de 2019.

David Escors Murugarren

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A CONTINUACIÓN, SE EXPONEN BREVEMENTE LAS AYUDAS Y FINANCIACIÓN QUE HAN PERMITIDO EL DESARROLLO DE ESTA TESIS DOCTORAL:

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A mis aitas, Kepa y Laura, por su amor y dedicación

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ABSTRACT

A high percentage of lung cancer patients progressing from conventional therapies are refractory to PD-L1/PD-1 blockade monotherapy. Here, we show that the proportion of highly differentiated (T_{HD}) CD4 T cell population can identify potential responders to PD-L1/PD-1 blockade therapy as quantified from peripheral blood samples before treatment initiation. Indeed, a baseline high proportion of CD4 T_{HD} (>40%) is an indicator of functional systemic CD4 immunity which turned to be a differential factor for clinical responses. In these patients, CD4 T cells possessed significant proliferative capacities and low co-expression of PD-1/LAG-3 following activation, and were responsive to PD-1 blockade *ex vivo* and *in vivo*. In addition, quantification of highly differentiated CD4 T cells in combination with PD-L1 tumor positivity identified a group of patients with response rates to immunotherapy of about 70%. In contrast, patients with low percentages of CD4 T_{HD} (<40%) did not respond even though they had lung cancer-specific T cells. Although proficient in cytokine production, CD4 T cells in these patients exhibited proliferative dysfunctionality, strongly co-upregulated PD-1/LAG-3 and were largely refractory to PD-1 monoblockade. Systemic CD8 immunity only recovered by PD-L1/PD-1 blockade therapy in patients who had baseline functional CD4 immunity. In contrast, baseline systemic T cell proliferative dysfunctionality in patients refractory to PD-1/PD-L1 monoblockade strategies could be reverted by PD-1/LAG-3 co-blockade confirming that PD-1/LAG-3 co-expression was a contributor to T cell dysfunctionality. These results provide a strong rationale for the combination of PD-L1/PD-1 and LAG-3 blockade therapies in patients exhibiting baseline CD4 T cell dysfunctionality.

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RESUMEN

Un alto porcentaje de pacientes con cáncer de pulmón resistentes a terapias convencionales son refractarios a la inmunoterapia con anticuerpos bloqueadores de la interacción PD-L1/PD-1. En la presente tesis doctoral se ha demostrado que la cuantificación de la proporción de linfocitos T CD4 altamente diferenciados (T_{HD}) en sangre periférica antes de comenzar el tratamiento identifica a potenciales respondedores a la inmunoterapia anti-PD-L1/PD-1. En efecto, una alta proporción de CD4 T_{HD} (>40%) pretratamiento es un indicador de la funcionalidad sistémica CD4 que resulta ser un factor diferencial para obtener respuestas clínicas. En estos pacientes, las células T CD4 son funcionales a nivel de capacidades proliferativas y presentan una baja co-expresión de PD-1/LAG-3 bajo estimulación, además de ser receptivos al bloqueo de PD-1 *ex vivo* e *in vivo*. Además, la cuantificación de los linfocitos T CD4 altamente diferenciados en combinación con la expresión positiva de PD-L1 tumoral identifica a un grupo de pacientes con una tasa de respuesta alrededor del 70%. En cambio, los pacientes con porcentajes bajos de CD4 T_{HD} (<40%) antes de comenzar el tratamiento no respondieron al bloqueo anti-PD-L1/PD-1, a pesar de presentar linfocitos T específicos de cáncer de pulmón. Aunque las células T CD4 en estos pacientes son competentes a la hora de producir de citoquinas, son disfuncionales a nivel de proliferación, co-expresan altos niveles de PD-1/LAG-3 y son refractarios al monobloqueo de PD-1. Así, la inmunidad sistémica CD8 solo pudo ser revertida a través del bloqueo de PD-L1/PD-1 en aquellos pacientes que presentaban una inmunidad CD4 basal funcional. En cambio, la disfuncionalidad proliferativa observada en las células T de pacientes refractarios a la inmunoterapia anti-PD-L1/PD-1 pudo revertirse a través del doble bloqueo de PD-1/LAG-3. De esta manera, mediante los presentes datos se ha confirmado que la co-expresión de PD-1/LAG-3 contribuye a la disfuncionalidad de las células T en pacientes con cáncer de pulmón resistentes a terapias convencionales. Estos resultados proporcionan el fundamento experimental para la combinación de las terapias bloqueadoras de PD-L1/PD-1 y LAG-3 en pacientes que manifiestan una inmunidad CD4 basal disfuncional.

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LIST OF ABBREVIATIONS

AE adverse events

AEC absolute eosinophil count

ALC absolute lymphocyte count

ALK anaplastic lymphoma kinase

ANC absolute neutrophil count

ANOVA analysis of variance

APC antigen presenting cell

ATCC American Type Culture Collection

BATF basic leucine zipper ATF-like transcription factor

BDNF brain-derived neurotrophic factor

CD40L CD40 ligand

CEA carcinoembryonic antigen

CHO Chinese hamster ovary

CK cytokeratin

CM central memory

CT computed tomography

CTC circular tumor cell

ctDNA circulating tumor DNA

CTL cytotoxic T lymphocyte

CTLA-4 cytotoxic T lymphocyte-associated protein 4

DC dendritic cell

dNRL derived neutrophil to lymphocyte ratio

EGFR endothelial growth factor receptor

EM effector memory

EMA European Medicine Agency

EMRA effector memory cells re-expressing CD45RA

FasL Fas ligand

FBS fetal bovine serum

FC flow cytometry

FDA Food and and Drug Administration

FLT3LG fms-like tyrosine 3 ligand

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GRIm score The Gustave Roussy Immune Score

GM-CSF granulocytic macrophage colony stimulation factor

HEK human embryonic kidney

HER2 human epidermal growth factor receptor 2

HLA human leukocyte antigen

HR hazard ratio

H2AX H2A histone family, member X

IDO idoleamine 2,3-dioxygenase I

IFN-γ interferon gamma

IFNR1/2 interferon receptor 1/2

Ig immunoglobulin

IgV variable-like immunoglobulin domain

IHC immunohistochemistry

IL interleukin

IRF interferon regulatory factor

ITIM immunoreceptor tyrosine-based inhibitory motif

ITISM immunoreceptor tyrosine-based switch motif

HPD hyperprogression dissease

JAK1 Janus kinase 1

JAK2 Janus kinase 2

KLRG1 killer cell lectin-like receptor subfamily G member 1

LAG-3 lymphocyte-activation gene 3

LDH lactate dehydrogenase

LTR long terminal repeat

LV lentivector

MAGE-3 melanoma associated gene A3

MAPK mitogen-activated protein kinase

M-CSF macrophage colony stimulation factor

MDC macrophage derived chemokine

MDSC myeloid derived suppressor cell

M-MDSC monocytic myeloid derived suppressor cell

MHC major histocompatibility cluster

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MMR DNA mismatch repair

mOS median overall survival

mPFS median progression free survival

MSK-IMPACT The Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets

MUC-1 mucin-1

NA naïve

NGS next generation sequencing

NFAT nuclear factor of activated T cells

NK natural killer

NRA4 nuclear receptor subfamily 4 group A

NRL neutrophil to lymphocyte ratio

NSCLC non-small cell lung carcinoma

NTRK1 neurotrophic tyrosine receptor kinase 1

NY-ESO-1 New York esophageal squamous cell carcinoma-1

ORR overall response rate

OS overall survival

PBMC peripheral blood monocytes

PD-1 programmed cell death protein 1

PD-L1 programmed cell death ligand 1

PFS progression free survival

PI3K phosphatidylinositol 3-kinase

PLR platelet-to-lymphocyte ratio

p-MHC peptide-MHC

PMN-MDSC granulocytic myeloid derived suppressor cell

RECIST Response Evaluation Criteria in Solid Tumors

SCC squamous cell carcinoma

SCLC small cell lung carcinoma

SCM stem cell memory

SFFV spleen focus-forming virus promoter

SHP1 Src homology region 2 domain-containing phosphatase 1

SHP2 Src homology region 2 domain-containing phosphatase 2

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SIN U3-deleted self-inactivating LTR

sPD-L1 soluble PD-L1

TAA tumor associated antigens

TAM tumor associated macrophages

TAP transporters associated with antigen presentation

TCGA Cancer Genome Atlas

TCR T cell receptor

TTF-1 thyroid transcription factor 1

TGF-β transforming growth factor β

TGK tumor growth kinetics

TGR tumor growth rate

Th T helper

THD highly differentiated T cells

TIL tumor infiltrating lymphocyte

TIM-3 T cell immunoglobulin and mucin domain containing 3

TKI tyrosine Kinase inhibitor

TMB tumor mutational burden

TMD transmembrane domain

TNF-α tumor necrosis factor α

TOX thymocyte selection-associated high mobility group box protein

Treg regulatory T cell

TTF-1 thyroid transcription factor

ULN upper limit of normal

US unstained

VEGF vascular endothelial growth factor

VH variable heavy chain

VL variable light chain

WHO World Health Organization

WES whole exome sequencing

WGS whole genome sequencing

ZAP70 Zeta-chain associated protein 70

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INTRODUCTION

1. LUNG CANCER

Cancer comprises a large group of heterogeneous diseases which have in common the transformation of somatic cells into cells that proliferate without control with pathological consequences. Apart from uncontrolled growth, malignant cancer cells acquire the capacity for invading tissues and organs leading to disruption of their proper functioning. Cancer cells can also induce angiogenesis, which facilitate the formation of tumor masses and propagation routes for metastases through the formation of new blood vessels.

The malignant transformation of cancer cells is a multistage process driven by genetic and environmental factors, of which mutagens usually play a prominent role. This has been extensively reviewed elsewhere (Hanahan and Weinberg 2000). Briefly, mutagens cause genetic damage into the genomic DNA (mutations) that modify genes controlling the cell cycle, proliferation, survival pathways and apoptosis. These factors can be classified as environmental (UV radiation and oxidative damage), biological (certain viruses or bacteria), chemical (such as asbestos and radioactive materials) and lifestyle-related (diet, alcohol, tobacco). Certain inherited genetic mutations through the germline may also favor the development of pre-malignant lesions by dysregulating the cell cycle (familial retinoblastoma) or interfering with DNA repair or physiological apoptosis (mutant variants of the MRC1 gene for melanoma).

The cell cycle is highly regulated and includes mechanisms to repair DNA and ensure chromosomal replication and stability while minimizing mutational events. Nevertheless, many mutations are in fact accumulated in the genomic DNA during the lifespan of an individual (Moskalev et al. 2013). Thus, ageing is the most important risk factor for cancer. The time-dependent accumulation of cellular damage can eventually lead to genomic instability, epigenetic alterations, telomere shortening, altered metabolism and cellular senescence, which in turn results in a significant decline in physiological organ function (Aunan, Cho, and Søreide 2018). Hence, a lifespan exposure to mutagens combined with a less effective DNA repair machinery reinforces genomic instability, accumulation of mutations and malignant cell transformation if anti-oncogenes are inactivated, or if oncogenes arise from mutations in cell cycle-regulating genes (Jackson and Bartek 2009). Low grade chronic inflammation associated to aging (inflammaging) is also closely related to cancer development and progression. Hence, the constant production of reactive oxygen/nitrogen molecular species represents an important source of mutagens for DNA damage in tissues (Ohnishi et al. 2013)(Pinlaor et al. 2004). The immune system is also affected by aging-dependent deleterious effects, a process known as immunosenescence

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(Fulop et al. 2010). Consequently, immune cell populations progressively lose the ability to detect and eradicate tumors and infections. All in all, it is not unexpected that half of cancers occur in individual older than 65 (Hsu 2016).

Lung cancer is the most common neoplastic malignancy in men and the third most common in women worldwide, and it is the major cause of cancer-related death in developed countries. 2.1 million cases were diagnosed in 2018 with 1.8 million deaths, being among the worst 5-year survival cancer types (Vachani, Sequist, and Spira 2017). Smoking remains the main risk factor, responsible for more than 71% of cases (Ordóñez-Mena et al. 2016). Other risk factors include exposure to asbestos, arsenic, radon, non-tobacco-related polycyclic compounds, and indoor air pollution (Malhotra et al. 2016). The average age at the time of diagnosis is around 65-70 years old, while lung cancer in young individuals is mainly caused by driver mutations. Lung cancer is widely classified in two types from a histological perspective; non-small cell lung carcinoma (NSCLC) and small-cell lung carcinoma (SCLC).

2. NON-SMALL CELL LUNG CARCINOMA

2.1. PATHOLOGY AND MOLECULAR BIOLOGY

NSCLC is the most prevalent lung cancer type, accounting from 85% to 90% of the cases. Due to the absence of symptoms during disease development and its metastatic nature, most patients are diagnosed at advanced stages when tumors are unresectable. Hence, histological diagnosis is carried out from small biopsy or cytology samples from the primary tumor or accessible metastases. Histological diagnosis is based upon the World Health Organization (WHO) classification (Travis et al. 2013). The main histological NSCLC subtypes are adenocarcinoma, squamous cell carcinoma, large cell carcinoma and sarcomatoid carcinoma **(Figure 1)**. Adenocarcinoma and squamous cell carcinoma (SCC) are the most common worldwide accounting for 50% and 30% of cases, respectively. Lung adenocarcinomas consist of epithelial tumors with glandular differentiation and usually with mucin production. In contrast, SCC arises from bronchial epithelial cells with a solid nest growth pattern (Perez-Moreno et al. 2012). These tumors lack glandular differentiation and mucin production and can be further classified into keratinizing, non-keratinizing, and basaloid subtypes. However, morphological characterization might not be precise enough and immunohistochemistry (IHC) is usually required to confirm the diagnosis (Travis et al. 2013)(Travis et al. 2015). Adenocarcinoma cells frequently express pneumocytic markers. Thus, thyroid transcription factor 1 (TTF-1) and NapsinA can serve as diagnostic biomarkers and for establishing the differentiation stage (Mukhopadhyay and Katzenstein 2011). In contrast, SCC are generally TTF-1 negative and can be

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differentiated by the expression of several markers such as p40, cytokeratin (CK) 5/6, CK5, and p63 (Conde et al. 2013)(Hayashi et al. 2013).

In addition, NSCLC can be also classified by disease stage or anatomical spreading by the TNM classification in four categories, stage I to IV (Lim et al. 2018). This classification considers tumor size (T), affected adenopathic nodes (N) and presence of metastases (M). Stage I tumors are <2 cm size and found exclusively within the lung, while in stage II and III the disease is locally advanced with or without hiliar, mediastinal and supraclavicular lymph node metastases, respectively. Stage IV tumors are diagnosed when further organ metastases are present.

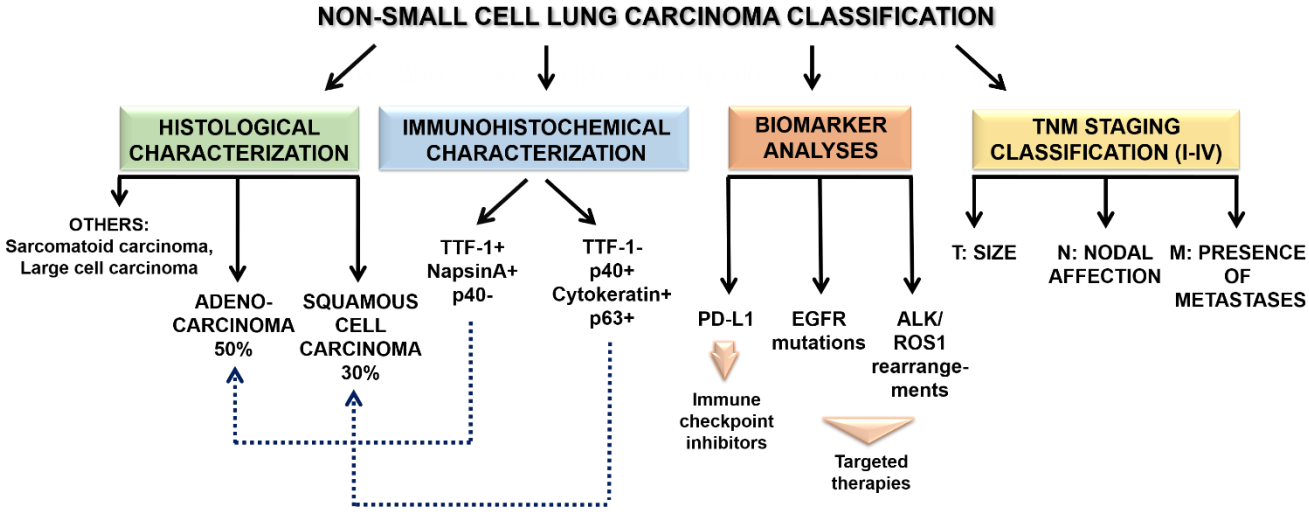

Figure 1. Classification of NSCLC tumors. *Diagnosis of NSCLC is performed by different complementary approaches including evaluation of growth pattern by histology, expression of specific markers by IHC and TNM staging classification. Additional analyses of biomarker expression are also carried out for medical decisions on specific therapeutic interventions. Identification of mutations in EGFR, ALK and ROS1 allows patient selection for targeted therapies with inhibitors of EGFR and ALK tyrosine kinase receptors, while PD-L1 tumor expression serves as a biomarker for PD-L1/PD-1 blockade immunotherapy.*

After morphological diagnosis, molecular identification of driver mutations is also of clinical routine. The most common driver mutation genes for NSCLC affect the endothelial growth factor receptor (EGFR), KRAS, BRAF, anaplastic lymphoma kinase (ALK) and ROS1 (Pao and Hutchinson 2012). Mutations affecting EGFR, ALK and ROS are mostly encountered in non-smoker young patients, although they can also be found in patients who smoke. Most of lung tumors harboring these mutations are adenocarcinomas. *EGFR* mutations are identified in 10% of lung adenocarcinomas in Western Europe, while *ALK* and *ROS1* rearrangements in 5% and 1%, respectively. These serve as efficient biomarkers for patient selection to targeted therapies with inhibitors of EGFR, ALK and ROS1 tyrosine kinase receptors. As BRAF/MEK inhibitors are being widely approved, *BRAF* V600E mutations are also being evaluated as predictive biomarkers (Planchard et al. 2019). More recently, others driver

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mutations including human epidermal growth factor receptor 2 (*HER 2*), *MET* exon 14 mutations and *RET* and neurotrophic tyrosine receptor kinase 1 (*NTRK1*) rearrangements are emerging as therapeutic targets and predictive biomarkers (Lindeman et al. 2018)(Planchard et al. 2018)

2.2. THERAPIES FOR NSCLC

Lung cancer has the lowest survival rate among the most frequent cancer types with an overall 5-year survival below 25%, which is strongly related to disease stage at diagnosis **(Figure 2)**. For localized NSCLC the 5-year survival rate is about 33%, while for advanced NSCLC is around 6%. Currently, the therapeutic approaches to treat NSCLC patients include surgery, radiotherapy, chemotherapy and immunotherapy, depending on the staging at diagnosis. Surgery and radiotherapy are only beneficial when tumors are resectable or can be stereotactically targeted. In contrast, NSCLC patients with advanced disease are treated with systemic therapies such as cytotoxic platinium-based chemotherapy with very poor clinical outcomes and numerous side-effects. One-year survival is reached only by 33% of the patients (Schiller et al. 2002). Targeted therapies with kinase inhibitors have considerably increased survival rates for adenocarcinoma subtypes harboring the specific mutations conferring susceptibility for these therapies (National Comprehensive Cancer Network 2017). However, it has to be remarked that these patients represent a minority. Hence, there is still an urgent need for more clinical approaches with improved efficacy.

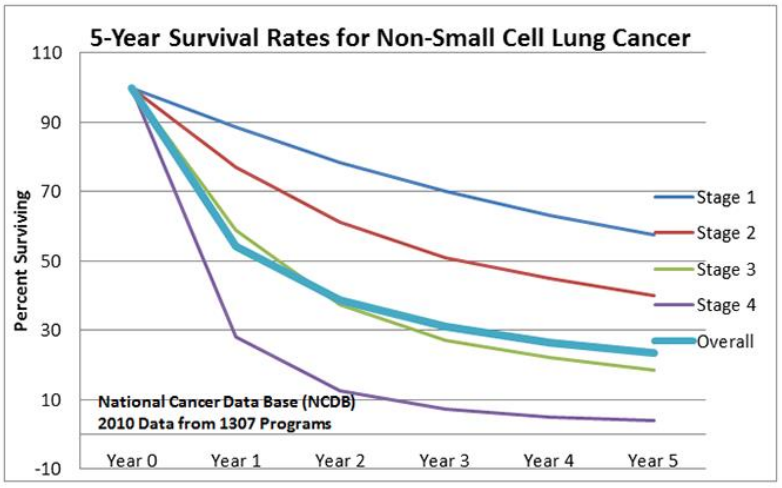

Figure 2. Survival rates of NSCLC according to disease staging. *Data from the National Cancer DataBase (NCDB) on 5-year survival rates as a function of staging (I-IV). Survival is strongly influenced by the stage at the time of diagnosis. There is a clear advantage at an early stage, while survival rates at advanced stages are very low (https://www.phoebehealth.com/cancer/non-small-cell-lung-cancer).*

Recently, immunotherapies based on immune checkpoint inhibitors have revolutionized oncology, including the treatment of NSCLC. Immune checkpoints consist on a system of receptor-ligand interactions that regulate immune responses in physiological conditions, especially T cell activities.

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These interactions play a key role in the induction/maintenance of immunological tolerance and in the restriction of collateral inflammatory damage. Cancer cells frequently utilize this strategy to inhibit the natural anti-tumor response of the immune system. The development of immune checkpoint inhibitors has radically changed the approach to NSCLC treatment due to durable responses and increase in survival compared to conventional cytotoxic agents (Carbone et al. 2015)(Borghaei et al. 2015)(Rizvi, Mazières, et al. 2015)(Herbst et al. 2016)(Fehrenbacher et al. 2016)(Antonia et al. 2017). These inhibitors consist on recombinant antibodies that bind immune checkpoints, blocking their interactions and disrupting their inhibitory activities over effector immune cells. Although immune checkpoint inhibitors exert some adverse events (AEs), these are clinically less aggressive and severe than previously anticipated and certainly less toxic than conventional treatments.

In the last years, the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) have approved nivolumab and pembrolizumab as programmed cell death protein 1 (PD-1) blockers, and atezolizumab and durvalumab as programmed cell death ligand 1 (PD-L1) blockers for the treatment of advanced NSCLC patients progressing from cytotoxic agents **(Table 1)**. In addition, the anti-PD-L1 antibody avelumab is not yet EMA or FDA-approved, but it is currently being evaluated in clinical trials (Gulley et al. 2017) together with several others under development.

Pembrolizumab and nivolumab were accepted by the FDA in 2015 for advanced NSCLC patients progressing from platinum-based chemotherapy. Nivolumab was developed by immunizing humanized mice with a PD-1-Fc-PD-1 human hybrid protein. Nivolumab is an IgG4 antibody that interacts poorly with Fc receptors (FcγRII and FcγRIII) reducing antibody-dependent cell-mediated cytotoxicity with high *in vivo* half-life (Davies et al. 2014). Phase III clinical trials CheckMate 057 (Borghaei et al. 2015) and CheckMate 017 (Brahmer et al. 2015) led to its approval. In contrast, pembrolizumab was generated by immunizing mice with human PD-1 and isolating a cDNA from an antibody binding PD-1. The PD-1 binding region was cloned into an IgG4-encoding-expression vector and produced from Chinese Hamster Ovary (CHO)-transfected cell lines. Pembrolizumab is therefore a humanized IgG4 anti-PD-1 antibody. It was accepted based on results from the phase III KEYNOTE 010 clinical trial. Patients with PD-L1 tumor expression equal or superior to 50% showed most benefit. Pembrolizumab was also approved in 2016 for first line treatment of NSCLC based on results from the Phase III clinical trial KEYNOTE-024 (Reck et al. 2016), with inclusion criteria being PD-L1 tumor expression ≥50%. In contrast, atezolizumab is a PD-L1-specific humanized IgG1 antibody with mutations in the Fc domain to reduce its affinity to Fc receptors, and consequently, antibody-dependent cell-mediated cytotoxicity and antibody-dependent cellular phagocytosis. It was approved by the FDA in 2016 for advanced NSCLC as a second line treatment based on results from phase II trial POPLAR (Fehrenbacher et al. 2016) and phase III trial OAK (Rittmeyer et al. 2017). Later in 2018, Durvalumab

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was approved by the FDA for unresectable locally advanced (stage III) NSCLC progressing from platinum-based chemo-radiotherapy based on the results of the phase III PACIFIC trial (Antonia et al. 2017).

Table 1. Clinical trials on immune checkpoint inhibitors in NSCLC as a second line treatment

Immunotherapy	Trial Reference	Phase	No	Histology/Line of treatment	Results
Nivolumab	CheckMate 017	III	272	Squamous/second	ORR vs. docetaxel: 20% vs 9% (P=0.008). Improvement in OS (median, 9.2 vs 6 months; HR, 0.59; p<0.001). PD-L1 expression neither predictive nor prognostic.
	CheckMate 057	III	582	Non-squamous/second	ORR vs. docetaxel: 19% vs 12% (p=0.02). Improvement in OS (median, 12.2 vs 9.4 months; HR, 0.73; p=0.002). PD-L1 expression associated with great efficacy at all levels (≥1, ≥5, ≥10).
Pembrolizumab	Keynote 010	II/ III	1.034	NSCLC/second/PD-L1 positive	ORR vs. docetaxel: 18% for low dose and 18% for high dose vs 9% (P=0.0005 and 0.0002). Improvement in OS with low dose (median, 10.4 vs 8.5 months; HR, 0.71; p=0.0008) and high dose (median, 12.7 vs 8.5 months; HR, 0.61; p<0.001). Efficacy was greater in patients with PD-L1 ≥50%.
Atezolizumab	POPLAR	II	287	NSCLC/second	ORR vs. docetaxel: 14.6% vs 14.7. Improvement in OS (median, 12.6 vs 9.7 months; HR, 0.73; P=0.04). Efficacy was greater in patients with highest levels of PD-L1 on tumor and immune cells.
	OAK	III	850	NSCLC/second	ORR vs. docetaxel: 14% vs 13.8. Improvement in OS (median, 13.8 vs 9.6 months; HR, 0.73; P=0.0003). OS was improved regardless of PD-L1 expression. Patients with tumors expressing high levels of PD-L1 in both tumor cells and immune cells exhibited the greatest benefit from atezolizumab.
Durvalumab	PACIFIC	III	709	Stage III NSCLC with no disease progression after ≥2 cycles of chemoradiotherapy/second	ORR vs. placebo: 30% vs 17.8% (p<0.001). Improvement in OS (median, 66.3 vs 55.6 months; HR, 0.52; p=0.0001). PFS and OS benefits with durvalumab were observed in all subgroups, including PD-L1 expression ≥25% or <25%.

Abbreviations: ORR, overall response rate; PFS, progression free survival; OS, overall survival; NSCLC, non–small cell lung cancer; HR, hazard ratio.

4. CELLULAR AND MOLECULAR BASES OF IMMUNOTHERAPY

The main goal of immunotherapy is to stimulate the immune system of the patients to re-activate or induce strong anti-tumor responses. Different types of cancer immunotherapies have been developed

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in the last century. These therapies can be classified as passive or active depending on the degree of modulation and the status of the host immune system towards cancer cells (Lesterhuis, Haanen, and Punt 2011). Passive immunotherapies include administration of anti-tumor antibodies, adoptive transfer of T cells and cytokine administration to incorporate the missing anti-tumor immunity in patients. In contrast, active immunotherapy seeks to enhance the intrinsic anti-tumor responses (Papaioannou et al. 2016). These strategies include vaccines based on administration of nucleic acids, peptides/proteins, antigen-presenting dendritic cell (DC), oncolytic viruses, whole tumor cell-based vaccines, immunostimulatory monoclonal antibodies and immune checkpoint inhibitors. The efficacy of the latter does not only rely on the status of immune system but also in the tumor immunogenicity and the susceptibility of the tumor to be recognized by the host immune system.

3.1 Immunogenicity of lung cancer cells

NSCLC was classically considered a non-immunogenic cancer. However, the experimental evidence indicates that this is not quite the case (Dammeijer et al. 2016). NSCLC is most frequently caused by chronic exposure to carcinogens in tobacco smoke, and as a consequence NSCLC tumors possess a high tumor mutational burden (TMB) that increases the generation of immunogenic tumor-associated antigens (TAA) (Schumacher and Schreiber 2015). These TAAs can be recognized by the immune system, leading to T cell reactivity. Indeed, several studies have shown that the degree of tumor infiltration with CD8 and CD4 T cells is associated with improved survival (Kawai et al. 2008)(Suzuki et al. 2013)(Al-Shibli et al. 2008)(Brambilla et al. 2016)(Geng et al. 2015). Thus, the active role of the immune system in keeping NSCLC at bay has been confirmed by increased lung cancer rates within immunosuppressed individuals (Tartour and Zitvogel 2013).

TAAs represent a large heterogeneous group of proteins produced by the tumor. These include mutated self-proteins, neoantigens, oncofetal proteins or tissue-specific proteins. TAAs can be originated by acquired mutations during oncogenesis or from aberrant expression of non-characteristic proteins for the tissue of origin. TAAs play a central role in triggering adaptive immunity, tumor-specific T cell responses and tumor cell elimination. Several immunogenic TAAs have been identified in lung cancer including mucin-1 (MUC-1), New York esophageal squamous cell carcinoma-1 (NY-ESO-1), melanoma associated antigen (MAGE-3), carcinoembryonic antigen (CEA) and human epidermal growth factor receptor 2 (HER-2)/neu among others. The ability of these and other TAAs as vaccines to stimulate autologous T lymphocytes is under investigation (Itoh et al. 2002)(Disis et al. 2002)(Vansteenkiste et al. 2016)(Butts et al. 2005)(Butts et al. 2014).

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4.2. Cancer cell recognition by the immune system

The recognition of tumor cells and development of specific anti-tumor responses constitutes a complex process. Tumors are initially identified in a non-specific manner by innate immune cells including neutrophils, NK (natural killer) cells, γδ T cells and macrophages. These cells operate very early in cancer immune surveillance promoting local inflammation by secretion of pro-inflammatory cytokines and chemokines. Activated NK and γδ T cells secrete interferon gamma (IFN-γ), perforin and granzyme B which eventually kill tumor cells. These cells can also express apoptosis-inducing ligands such as Fas ligand (FasL) and TRAIL which cause cancer cell death after interacting with their receptors (Cheng et al. 2013)(Kondo et al. 2008). Eventually, tumor killing releases TAAs that are captured by professional antigen-presenting cells (APCs) which serve as a bridge with the adaptive immune system. Of these, DCs are considered the major professional APCs that can trigger anti-tumor responses. DCs and macrophages can also phagocytose whole necrotic cells that in the presence of appropriate signals induce their maturation and migration into secondary lymphoid organs such as draining lymph nodes. Once there, TAA-derived peptides bind to major histocompatibility complex molecules (MHC) forming TAA-MHC complexes which are exported to the DC surface to be presented to naïve CD4 and CD8 T cells. If these T cells have specific T cell receptors (TCRs) to these TAA-MHC complexes, they can potentially be activated in the proper immunological context (T cell priming) (Chen and Mellman 2013). In general terms, peptide-loaded MHC class I molecules (MHC-I) present endogenous antigens to CD8 T cells, while MHC class II molecules (MHC-II) are loaded with peptides from exogenous antigens taken up by phagocytosis and presented to CD4 T cells. There is an additional mechanism whereby exogenous antigens captured by phagocytosis can be presented in the context of MHC-I molecules to CD8 T cells. This mechanism is called cross-presentation and plays a key role in anti-tumor immunity (Joffre et al. 2012)(Sánchez-Paulete et al. 2017).

4.2.1. Antigen presentation and T cell priming

Antigen presentation is highly regulated at multiple levels by cell-to-cell interactions between APCs and T cells to ensure proper T cell activation while preventing autoreactive responses **(Figure 3)**. These interactions provide three main types of signals to the T cell that will regulate their activation, clonal expansion and differentiation towards effector and memory phenotypes. Signal 1 is given to the T cell following the binding of the TCR to the peptide-MHC (p-MHC) complex. This binding triggers a signaling kinase cascade (Zuazo et al. 2017). However, T cell stimulation with only signal 1 leads to a limited wave of T cell clonal expansion, but these T cells will fail to proliferate after a second antigen re-encounter. These cells are called “anergic” and represent a physiological central tolerogenic mechanism towards autoantigens (Crespo et al. 2013). Most TAAs can be considered quasi-autoantigens, and in general tumor-specific T cells are frequently anergic. For T cells to be properly

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activated at the required degree and the appropriate differentiation stage, they need to receive additional signals (Curtsinger, Johnson, and Mescher 2003). These signals integrate positive and negative co-stimulation in what is called the "signal 2". The main co-stimulatory interaction is driven by CD80 on the surface of the APC binding to CD28 on the T cell. Both signals promote production and secretion of IL-2 by T cells, which is required to support the proliferation and survival of T cells during priming (Zhu, Yamane, and Paul 2010). On the other hand, negative regulatory signals modulate T cell activation to ensure protection against exacerbated immune responses (Nurieva et al. 2006), and thus are physiological immune checkpoints. T cells express multiple co-inhibitory receptors such as PD-1, lymphocyte-activation gene 3 (LAG-3), cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and T-cell immunoglobulin and mucin domain-containing 3 (TIM-3), especially after activation. These inhibitory receptors interact with their corresponding ligands on the APC to regulate by inhibition (or fine-tuning) either T cell activation or their effector capacities. Deletion of either PD-L1/PD-1 or CTLA-4 in murine models leads to spontaneous autoimmune disorders, and therefore a loss of central tolerance (Nishimura et al. 1999)(Tai et al. 2007).

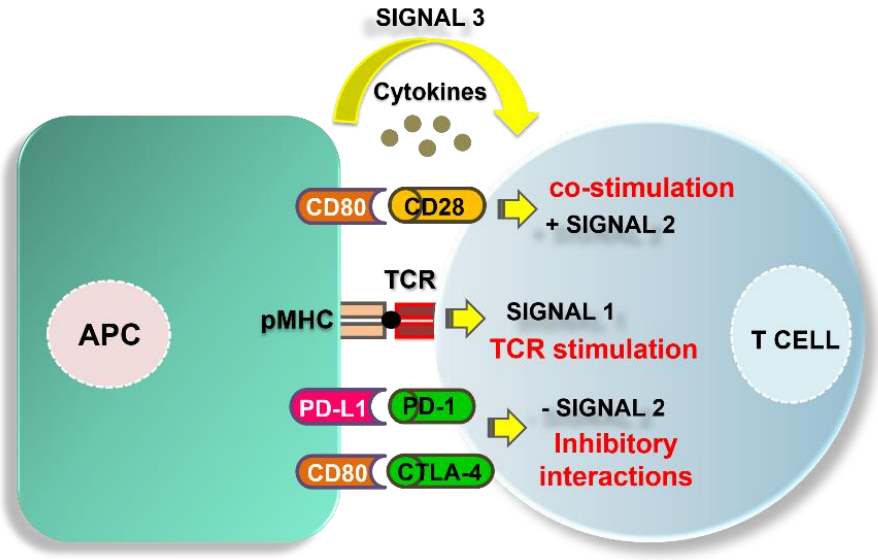

Figure 3. Three-signal model of antigen presentation in the immunological synapse. *T cells get activated and expand exponentially after encountering antigenic peptides specific for their cognate TCRs. Antigenic peptides are presented by professional APCs (left) to T cells (right). There, peptide-MHC complexes bind to the TCR within the immunological synapse to initiate signal 1 (TCR stimulation). Co-stimulatory (CD28) or co-inhibitory receptors (PD-1, LAG-3, CTLA-4) on T cells bind to their ligands on APCs (CD80, PD-L1, MHC-II), representing signal 2. Integration of stimulatory and inhibitory signals determine de degree of activation of T cells. Furthermore, cytokine secretion (Signal 3) by APCs regulates T cell differentiation into effector T cells.*

Chronic stimulation with highly immunogenic antigens provokes a sustained high-level surface expression of immune checkpoints in T cells such as PD-1, LAG-3 or TIM-3. These chronically stimulated T cells sequentially lose the capacity for multi-cytokine production, and therefore their proliferative capacities and effector functions. This T cell stage is called exhaustion and was described

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first in chronic viral infections (Crespo et al. 2013). This T cell inhibitory mechanism also takes place in highly-immunogenic cancers, particularly within the tumor microenvironment (Baitsch et al. 2011). Finally, T cells receive a third type of signal, or signal 3, provided by cytokine stimulation most frequently from the APC (Curtsinger, Lins, and Mescher 2003). This signal is important to direct T cell differentiation towards different effector types. This is exemplified for CD4 T cells which differentiate into multiple T helper (Th) subsets depending on the cytokine milieu. IFN-γ and interleukin (IL)-12 (IL-12) induce Th1 differentiation; IL-10 and IL-4 induce Th2 differentiation; Combinations of transforming growth factor beta (TGF-β), IL-17, IL-23 and IL-6 induce Th17 differentiation; and inducible regulatory T cells (Tregs) are differentiated by IL-10 and TGF-β in the absence of pro-inflammatory cytokines (Luckheeram et al. 2012). CD8 T cells differentiate into cytotoxic T cells (CTL) in the presence of IFN-γ, IL-2 and IL-12.

4.3. Differentiation and senescence of human T cells

Human T cells can also be divided into functionally different subsets based on the expression of different combinations of cell surface receptors. The first main classification is based on membrane-bound tyrosine phosphatase CD45RA and L-selectin CD62L expression profiles. Naïve (NA) and stem cell memory (SCM) T cells co-express both CD62L and CD45RA. These T cells exit the thymus and migrate to secondary lymphoid organs driven by CD62L (Sallusto et al. 1999).When they recognize an antigen in secondary lymph organs they proliferate and differentiate into effector cells which migrate to the infection or tumor site. Effector T cells are short-lived cells, from which following antigen clearance, a small proportion differentiate into long-lived memory T cells.

Memory T cells undergo fast activation and strong effector responses upon antigen re-encounter (McKinstry, Strutt, and Swain 2010)(Taylor and Jenkins 2011)(Strutt et al. 2012). Memory T cells express high levels of CD40 ligand (CD40L) which binds to its receptor CD40 on the DC surface to engage rapid T cell re-expansion (Grewal and Flavell 2006)(Johnson et al. 2009). Simplifying, memory T cell compartments are located in secondary lymphoid organs (central memory) or in inflamed tissues (effector memory). Central memory (CM) T cells express CD62L but not CD45RA. In contrast, effector memory (EM) T cells are tissue-resident and do not need CD62L nor CD45RA. EM T cells express high levels of chemokine and cytokine receptors to reach inflamed tissues. Peripheral CD4 populations are enriched in CM T cells while in CD8 populations EM are the predominant subset (Taylor and Jenkins 2011). Finally, the effector population which re-expresses CD45RA (EMRA) is considered a terminally differentiated phenotype which accumulates during lifetime. Indeed, elderly individuals with a lifetime exposure to variety of infections and diseases present a higher proportion of memory and effector T cell subsets compared to young individuals (Simon, Hollander, and McMichael 2015).

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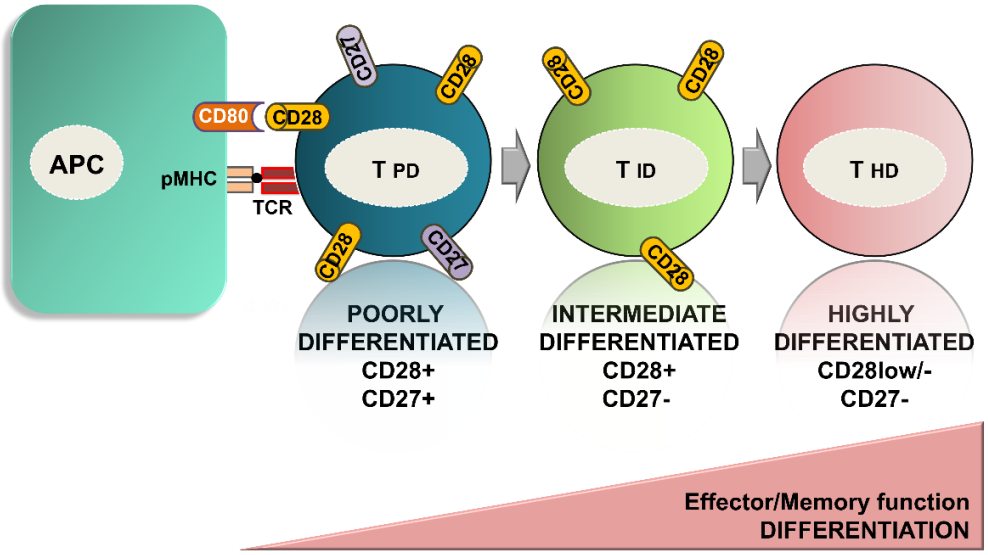

Figure 4. T cell differentiation according to their CD27/CD28 expression profiles. *Upon antigen recognition, T cells progressively lose CD28/CD27 expression and differentiate into poorly differentiated ($CD27^{+}$ $CD28^{+}$, T_{PD}), intermediately differentiated ($CD27^{negative}$ $CD28^{+}$, T_{ID}) and highly differentiated ($CD27^{negative}$ $CD28^{low/negative}$, T_{HD}) T subsets during effector/memory phenotype differentiation.*

The second main classification is based on CD27/CD28 expression profiles. Following the initial antigen recognition, T cell differentiation advances through the progressive loss of CD27 and CD28 co-stimulatory receptors (Lanna et al. 2014)(Lanna et al. 2017). Hence, human T cells can be classified according to their CD27/CD28 expression profiles into poorly-differentiated ($CD27^{+}$ $CD28^{+}$), intermediately-differentiated ($CD27^{negative}$ $CD28^{+}$) and highly-differentiated ($CD27^{negative}$ $CD28^{low/negative}$, T_{HD}) subsets **(Figure 4)** (Lanna et al. 2014)(Amyes et al. 2003). In humans, T_{HD} cells are largely composed of memory, effector and senescent T cells. Senescence is associated with natural ageing and it was first described in fibroblasts (Campisi and D'Adda Di Fagagna 2007). Age-dependent chronic DNA damage activates signaling pathways regulating T cell senescence through AMPK-dependent p38 MAPK activation (Lanna et al. 2014). In addition, sestrin proteins maintain T cell senescence through an alternative MAPK activation pathway (Lanna et al. 2017). Senescent T cells are characterized by telomere shortening, low telomerase activity, loss of surface co-receptors and reduced proliferative capacity (Effros 2002). They also express surface inhibitory/cytotoxic natural killer receptors such as CD57 and lectin-like receptor subfamily G member 1 (KLRG1) which are involved in impaired proliferative capacities (Brenchley et al. 2003)(Henson et al. 2009). Nevertheless, senescent T cells maintain the ability to exhibit effector functions (Libri et al. 2011)(Vallejo 2005).

4.4. CD4 T cells in anti-tumor immunity

CD8 T cells play a direct role in anti-tumor responses due to their potent cytotoxicity mediated by IFN-γ production, secretion of cytotoxic granules and induction of apoptosis through cell-to-cell

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interactions (Zhang and Bevan 2011). Upon antigen recognition CD8 T cells differentiate into CTL that infiltrate tumors where they exert cytotoxic activities. Some of these cells will differentiate into memory subsets. Hence, tumors can prevent elimination by immune system down-regulating MHC-I expression and therefore inhibiting anti-tumor CD8 T cell recognition (Garrido, Cabrera, and Aptsiauri 2010).

In contrast, the importance of CD4 immunity for anti-tumor responses is less recognized due to limited studies. Nevertheless, there is compelling experimental evidence supporting an important role of CD4 immunity by promoting and coordinating innate and adaptive responses in anti-tumor immunity **(Figure 5)** (Pardoll and Topalian 1998)(Blattman and Greenberg 2004)(Kennedy and Celis 2008)(Muranski and Restifo 2009)(Hung et al. 2002). For example, CD4 Th1 cells prime and license CD8 T cells during antigen presentation to become tumor-specific CTLs by producing IFN-γ and IL-2 (Bos and Sherman 2010)(Wong, Bos, and Sherman 2008). They also contribute to the maturation and activation of DCs in a process called "DC licensing" by engaging CD40L with CD40 on DCs which enhances their ability to prime naïve $CD8^+$ T cells into CTLs in a IL-12-dependent manner (Schoenberger et al. 1998)(Nesbeth et al. 2010)(Baxevanis et al. 2014). Moreover, CD4 cells are required to generate long-lasting CD8 memory responses (Janssen et al. 2003)(Laidlaw, Craft, and Kaech 2016)(Sun and Bevan 2003). This CD4 T cell subset also activates innate anti-tumor responses by NK and type-1 anti-inflammatory macrophages (M1) (Eisel et al. 2019). The fact that mutations in MHC-II-restricted neoantigens are exposed to a stronger selective pressure than MHC-I-restricted neoantigens during tumorigenesis also support the key contribution of CD4 T cells in cancer immunosurveillance (Marty et al. 2018). In addition, tumor infiltration with CD4 Th1 cells was associated with better prognosis in patients along several cancer types including NSCLC (Fridman et al. 2011).

In contrast, other CD4 T helper cells including Th2, Th17 and regulatory T cells (Tregs) have been generally associated with tumor progression and poor prognosis (Becht et al. 2016). For example, several studies support the role of Th2 effector T cells in carcinogenesis and tumor progression (Ochi et al. 2012); For instance, they secrete IL-4 and IL-13 which favor type-2 macrophage (M2) polarization that in turn produce immunosuppressive cytokines and suppress inflammation (Mantovani and Locati 2013). Likewise, Th17 cells have been associated with carcinogenesis, tumor progression and angiogenesis (Numasaki et al. 2003)(Akbay et al. 2017). Nevertheless, several studies also show the contrary. For example, Th2 cells may contribute to efficacious anti-tumor responses (Nishimura et al. 2002)(Mattes et al. 2003). Recent studies have brought evidences for the role of this effector population establishing long-term anti-tumor memory responses (Lorvik et al. 2016). Likewise, Th17 responses have been reported to also induce potent anti-tumor responses (Kryczek et al.

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2014)(Kryczek, Wei, et al. 2009)(Kryczek, Banerjee, et al. 2009)(Wilke et al. 2011)(Martin-Orozco et al. 2009)(Muranski et al. 2008). The duality of responses observed for these effector helper populations is likely context dependent. Finally, Tregs, key contributors of tolerance, exert potent suppressor activities towards the other immune cell populations by several means (Sasada et al. 2003)(Curiel et al. 2004)(Sato et al. 2005)(Bates et al. 2006), including cell-to-cell contact and production of anti-inflammatory cytokines such as IL-10 and TGF-β (Larmonier et al. 2007)(Jarnicki et al. 2006)(Liu et al. 2007).

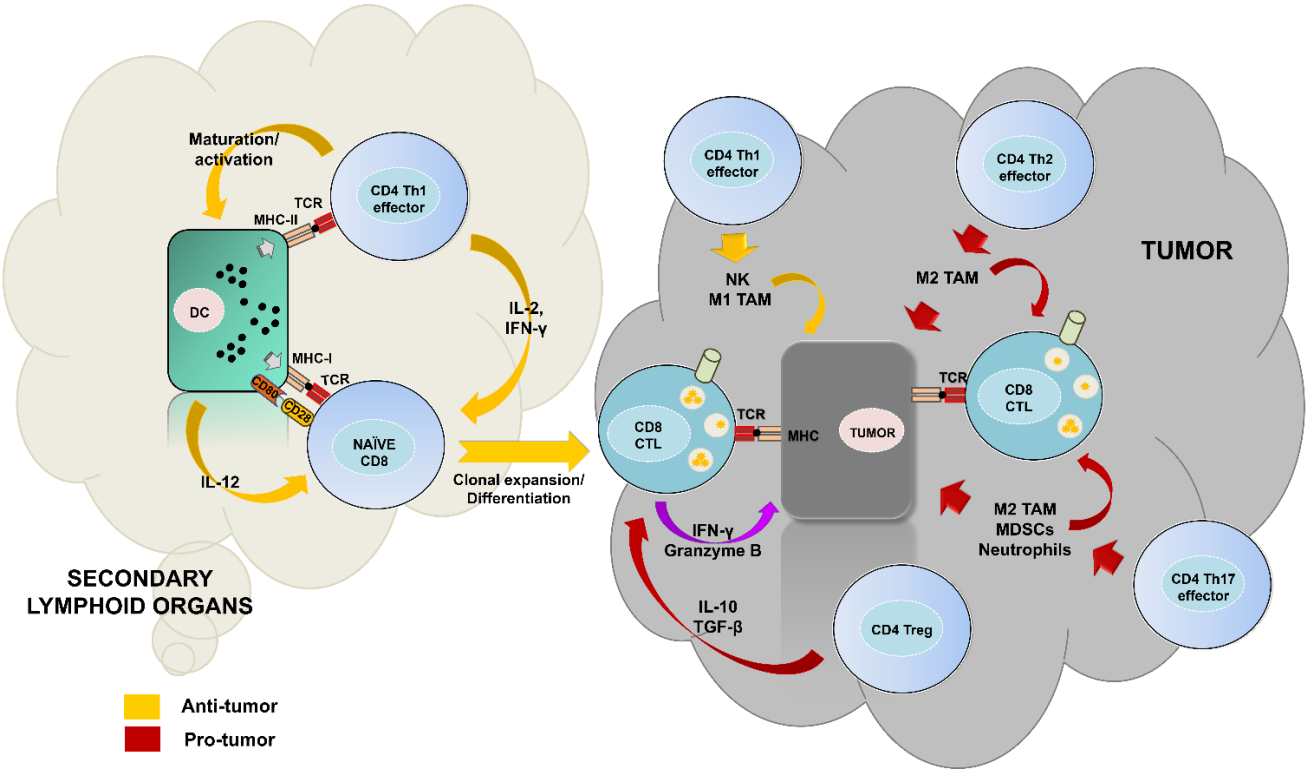

Figure 5. The contribution of CD4 T helper subsets in anti-tumor immunity. *The figure summarizes the well-known roles of CD4 T helper subsets in anti-tumor responses. In the right, CD4 Th1 cells allow the correct priming and differentiation of naïve CD8 T into CTLs by secretion of IFN-γ and IL-2 within the secondary lymphoid organs. They also contribute to DC maturation and activation for correct T cell priming. CD8 CTLs infiltrate tumors and exert cytotoxic responses against tumor cells after TAA recognition. Within the tumors, CD4 Th1 cells activate NK and M1-macrophages enhancing anti-tumor innate responses. In contrast, tumor infiltrating CD4 Th2, Th17 and Tregs contribute mainly to tumor progression by several mechanisms including induction of M2-macrophages, myeloid-derived suppressor cells (MDSCs), neutrophils, and secretion of pro-angiogenic and immunosuppressor cytokines. Yellow arrows indicate anti-tumor activities, and red arrows pro-tumor activities. Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; Treg, regulatory T; CTL, cytotoxic T lymphocytes; DC, dendritic cell.*

Besides a subset of CD4 T cells can also exert direct cytotoxic responses (Hung et al. 2002)(Pardoll and Topalian 1998)(Quezada et al. 2010). Both cytotoxic CD4 and CD8 T cells also express ligand of tumor necrosis factor (TNF) superfamily including FasL or TRAIL which binds to its death receptors in tumor cells and induce its death (Knutson and Disis 2005)(Cullen and Martin 2008).

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3.5. Immunosuppressive mechanisms in cancer

Tumors utilize a wide array of mechanisms during progression to evade the immune system and survive therapies. Possibly, the first one is immunoediting. This process is based on the selection of less immunogenic cancer cell variants that have acquired mutations or epigenetic alterations to avoid immunosurveillance. Immunoediting can select cancer cells with down-modulated MHC molecules to avoid T cell recognition (Zitvogel, Tesniere, and Kroemer 2006)(Campoli, Chang, and Ferrone 2002). Other mechanisms also include the selection of cancer cells with impaired interferon signaling with inactivating janus kinase 1 (JAK1) and 2 (JAK2) mutations, or by eliminating the expression of β-microglobulin (Zaretsky et al. 2016)(Garcia-Diaz et al. 2017).

A second main mechanism caused by progressing tumors is the induction of systemic immune dysfunctionality (tumor-induced immunosuppression), especially inactivation of T immunity through several direct and indirect mechanisms. Direct mechanisms include T cell exhaustion, anergy and CD3 downmodulation **(Figure 6)** (Crespo et al. 2013)(Grywalska et al. 2018). Hence, continuous TCR stimulation ends up in sustained expression of immune checkpoint receptors by T cells, and loss of multi-cytokine production and proliferative capacities when stimulated. These T cells acquire an "exhausted" phenotype which is characterized by an altered transcriptional program involving the NFAT, BATF, IRF4, NR4A and TOX among others, and a unique epigenetic signature (McLane, Abdel-Hakeem, and Wherry 2019)(Khan et al. 2019)(Scott et al. 2019)(Seo et al. 2019)(Man et al. 2017)(Martinez et al. 2015). Hence, the tumor microenvironment is frequently infiltrated with exhausted PD-1^{+} T cells with increased simultaneous expression of multiple inhibitory receptors (Ahmadzadeh et al. 2009)(Matsuzaki et al. 2010)(Fourcade et al. 2009)(Sfanos et al. 2009)(Zhang et al. 2010). Many tumor cells upregulate immune checkpoint ligands which bind to their receptors (CTLA-4, PD-1, TIM-3, and LAG-3) on tumor infiltrating T cells (TILs) leading to inhibition of T cell responses. For example, CTLA-4 binds to CD80 and CD86, PD-1 binds to PD-L1/PD-L2 and LAG-3 with MHC-II molecules amongst other recently described ligands (Freeman et al. 2000)(Azuma et al. 1993)(Huard et al. 1997).

T cell anergy is frequently observed in cancer patients due to the low immunogenicity of most TAAs. Therefore, TAA-derived peptides are usually presented by APCs in the absence of positive co-stimulation (signal-1-only stimulation), while inhibitory signals are prevalent (Schwartz 2003). These T cells undergo one round of expansion but lose the capacity to respond to a second antigen encounter. These anergic T cells are hyporesponsive, with reduced proliferative capacities and loss of IL-2 production amongst other cytokines (Crespo et al. 2013). Anergic T cells do not constitutively express high surface levels of immune checkpoints.

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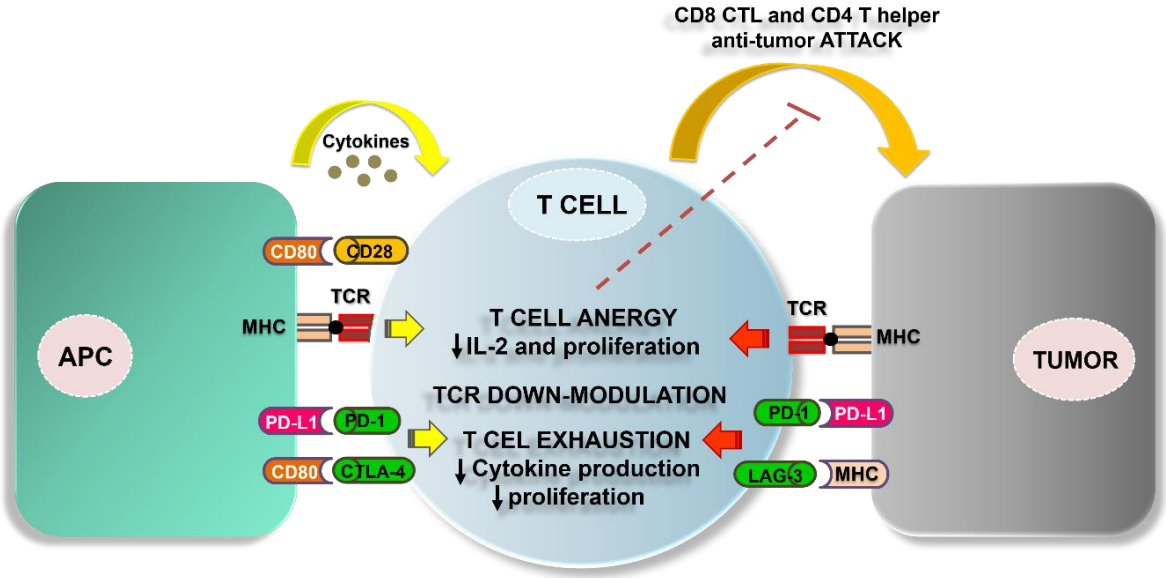

Figure 6. Tumor-induced T cell dysfunctionality. *The figure represents the main tumor-induced T cell dysfunctional states. Continuous tumor antigen recognition induces upregulation of immune checkpoint receptors by T cells (middle) which acquire an exhausted phenotype characterized by the loss of multi-cytokine production and proliferative capacities. In contrast to DCs (left), tumor cells (right) usually present TAA-derived peptides in the absence of co-stimulatory interactions, which will cause T cell anergy. Moreover, tumor cells upregulate immune checkpoint ligands (right) which interact with their receptors on T cells making the inhibitory interactions the most prevalent and consequently inducing T cell anergy and inhibition. Anergic T cells are hyporesponsive with reduced proliferative capacities and loss of IL-2 production. PD-L1/PD-1 interaction induces a sustained TCR-downmodulation and impaired T cell antigen recognition. As indicated in the figure, all these dysfunctional states inhibit anti-tumor CD4 and CD8 effector T cell responses allowing tumor cells to escape.*

The third direct inhibitory mechanism over T cells relies of down-modulation of CD3 molecules, particularly in TILs (Eleftheriadis et al. 2008). CD3 intracellular domains transduce TCR signals to the T cell. However, cancer-induced PD-L1/PD-1 interactions trigger TCR down-modulation with the consequent impairment of T cell responses (Karwacz et al. 2011). TCR down-modulation is also a feature associated with T cell exhaustion, anergy and apoptosis (Zhang et al. 2012)(Cornwell and Rogers 2010).

T cell immunity is impaired through indirect mechanisms as well. Tumor cells deliver “instructions” through the secretion of soluble factors, including cytokines and chemokines, or exosomes which alter locally and systemically the functionality of immune cell populations to support tumor progression. For example, a high tumor load leads to increased systemic levels of IL-10 and TGF-β that promote the recruitment of suppressor immune cell populations including Tregs, macrophages and myeloid derived suppressor cells (MDSCs) which generate an immunosuppressive microenvironment and inhibit effector immune cells (Shimizu et al. 2010)(Srivastava et al. 2012). Tumors frequently secrete high levels of granulocyte macrophage colony stimulation factor (GM-CSF), macrophage colony stimulated factor (M-CSF) and IL-6, which favor the differentiation and mobilization of MDSCs from

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the bone marrow (Gabrilovich and Nagaraj 2009). The role of MDSCs and other immunosuppressive myeloid cell types over T cell inhibition has been extensively addressed elsewhere (Tcyganov et al. 2018). The tumor microenvironment can also be highly hypoxic and poor in nutrients, which inactivates T cells and promotes angiogenesis and tumor escape (Chang et al. 2015).

Lung tumors are particularly enriched in Tregs which inhibit T cells, DCs and NK cells through IL-10 secretion and promoting tolerogenic DCs (Baecher-Allan, Viglietta, and Hafler 2004)(Bettelli et al. 2006)(Elpek et al. 2014)(Steinbrink et al. 1999). Likewise, lung tumors also produce high levels of TGF-β and vascular endothelial growth factor (VEGF) which promote an immature DC phenotype (Zong et al. 2016)(Brown et al. 2001)(Gabrilovich et al. 1998). Thus, an insufficient priming of T cells might also induce low immunogenicity against tumors (Fu and Jiang 2018). In addition, increased IL-17 levels is also a characteristic of lung tumors as an important contributor to immunosuppression by supporting tumor progression and promoting angiogenesis (Chen et al. 2010)(Fridlender and Albelda 2012). Indeed, IL-17 has been showed to correlate with advanced stage of NSCLC and overall survival (OS) (Zhang et al. 2014)(Xu et al. 2014). IL-17 induces the production of pro-inflammatory cytokines such as IL-6 from the stromal and tumor cells, and recruits tumor-associated myeloid derived populations including MDSCs, tumor associated macrophages (TAMs) and neutrophils which have been demonstrated to support lung cancer progression by nitric oxide (NO) and reactive oxygen species (ROS) production (Liu et al. 2012)(Li et al. 2014) (Akbay et al. 2017). Indeed, tumor high neutrophil/T cell ratio is associated to poor prognosis in lung cancer patients (Takahashi et al. 2016).

4. PD-L1 / PD-1 INTERACTION, A KEY IMMUNE CHECKPOINT IN CANCER

4.1. PD-1 structure and signal transduction

In the last decade, PD-1 has become one of the most studied inhibitory receptors due to its clinical relevance in oncology. PD-1 is a type 1 transmembrane glycoprotein from the B7-CD28 superfamily. It is expressed on T cells after activation, but it is also expressed on B cells, myeloid-derived populations, DCs and NK cells. Although some studies have reported PD-1 expression by cancer cells (Yao et al. 2018), this remains a highly controversial issue. The structure of PD-1 consists on an extracellular amino-terminal variable-like immunoglobulin domain (IgV), a stalk region separating the IgV domain from the plasma membrane, and a transmembrane domain followed by an intracellular cytoplasmic tail containing tyrosine-based signaling motifs. These motifs include the immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) which regulate PD-1-dependent inhibitory functions (Chemnitz et al. 2014). PD-1 expression can also

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be induced by several cytokines such as IL-2, IL-21, IL-15, IL-7, and type 1 IFN upon TCR activation (Wong et al. 2009).

PD-1 engagement with its ligands interferes with TCR signal transduction by recruiting phosphatases containing SH2 domains (SHP1 and SHP2) to its ITIM and ITSM tyrosine-based motifs **(Figure 7)** (Neel, Gu, and Pao 2003). These phosphatases de-phosphorylate TCR-associated kinases such as ZAP70, PI3K, AKT, ERK and PK0 (Plas et al. 1996). Nevertheless, recent studies have brought evidence of CD28 as the major target of the dephosphorylating activity of PD-1, suggesting that PD-1 inhibits T cell function by inactivating the co-stimulatory signaling (Hui et al. 2017)(Kamphorst, Wieland, et al. 2017). In addition, PD-1 engagement upregulates E3-ubiquitin ligases of the CBL family, that downregulate TCRs from the T cell surface possibly after ubiquitination of CD3 and PI3K **(Figure 7)** (Karwacz et al. 2011)(Naramura et al. 2002)(Prelaj et al. 2019)(Nurieva et al. 2006).

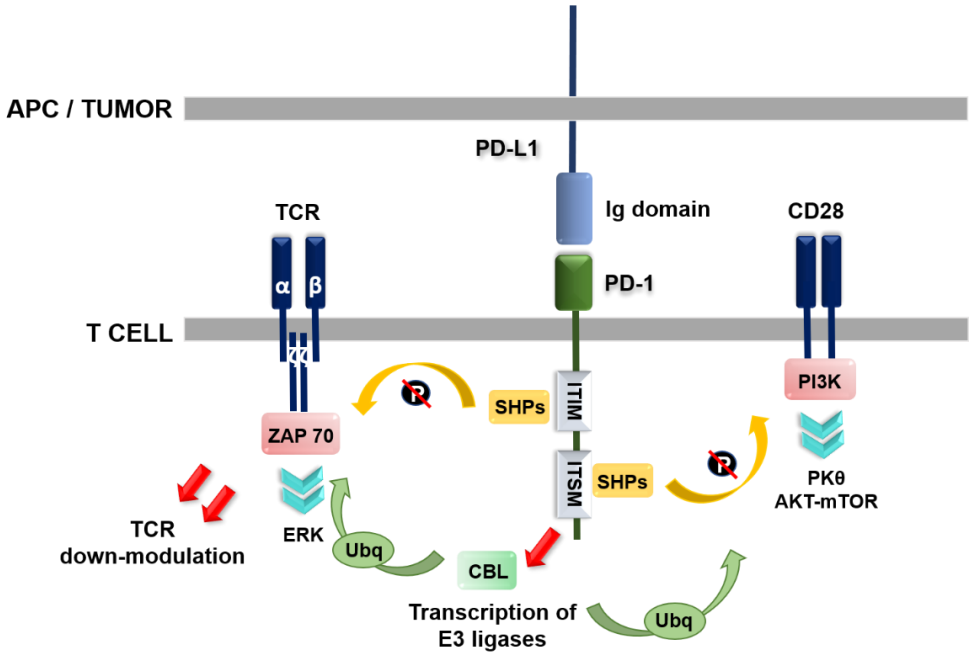

Figure 7. PD-1-dependent T cell inhibitory mechanisms. *The TCR complex including the co-stimulatory molecule CD28 is associated closely to PD-1, which is upregulated following antigen presentation. PD-1 engagement induces the recruitment of SHP phosphatases into the phosphorylated ITIM and ITSM motifs in the cytoplasmic domain of PD-1, as shown in the figure. These proteins de-phosphorylate and inhibit kinases mediating TCR signal transduction including ZAP70 and PI3K (yellow arrows). The second major mechanism involves the transcriptional upregulation of CBL E3 ubiquitin ligases (red arrow) that trigger ubiquitination of CD3 and PI3K leading to TCR down-modulation. ITM, immunoreceptor tyrosine-based activation motif; ITSM, immunoreceptor tyrosine-based switch motif; TCR, T cell receptor; CBL, casitas B lineage lymphoma protein; Ubq, ubiquitination; SHPs, phosphatases containing SH2 domains.*

Moreover, PD-1 engagement causes a shift on the T cell metabolic reprogramming. As PI3K and ERK induce the expression of glycolytic genes, PD-1-depedent engagement inhibits these signaling axes

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inducing the suppression of oxygen consume and aerobic glycolysis (Patsoukis et al. 2015). Therefore, fatty lipid oxidation become the main source of energy promoting the production of reactive oxidative species and creating an oxidative microenvironment (Tkachev et al. 2015). These metabolic changes might have an important contribution in PD-1 dependent T cell suppression in cancer (Boussiotis 2016).

4.2. PD-L1 structure and signal transduction

PD-1 binds to ligands PD-L1 and PD-L2, both type-I transmembrane glycoprotein of the B7 family of co-stimulatory/inhibitory molecules expressed by many cell types (Freeman et al. 2000). PD-L1 is constitutively expressed on professional APCs and its expression increases after activation (Liechtenstein et al. 2012). It is also expressed in a wide variety of tissues including vascular endothelial cells, pancreatic islet cells, lung cells, muscle cells among others (Pauken et al. 2013)(E., D.V., and A. 2013). Both PD-L1 and PD-L2 are expressed by many other cell types, including in cells within immune-privileged tissues such as placenta and testicles supporting their important role in immune self-tolerance. PD-L1 consists on an immunoglobulin-like extracellular domain composed of an Ig variable (IgV) distal region followed by an Ig constant domain, a transmembrane domain and a short intracytoplasmic domain. The intracytoplasmic domains contains phylogenetically-conserved non-canonical intracellular motifs involved in intrinsic signaling, namely RMLDVEKC, DTSSK and QFEET (Gato-Cañas et al. 2017). Two of these motifs (RMLDVEKC and DTSSK) regulate the crosstalk with signal transduction by the type I interferon receptor **(Figure 8).** While RMLDVEKC interferes with the STAT-3 branch of the IFN pro-apoptotic pathway, the DTSSK motif and lysines 271 and 280 act as negative regulators of PD-L1 anti-IFN functions (Gato-Cañas et al. 2017). Therefore, PD-L1 constitutes a functional protective barrier against IFN cytotoxicity. Recent evidence has shown that PD-L1 ubiquitination controls the stability and functions of PD-L1. Although the specific lysine residues that get ubiquitylated are currently unknown, these may be lysines 271 and 280. Recently, the USP22 deubiquitinase has been demonstrated to bind to the QFEET motif and participate in the stabilization of PD-L1 (Huang et al. 2019).

Many tumor cells upregulate PD-L1 which in turns inhibits T cell responses by engaging with PD-1 (Saunders et al. 2005)(Pardoll 2012)(Zhang et al. 2010). PD-L1 expression can be induced by pro-inflammatory cytokines such as type I and type II IFNs, and tumor necrosis factor alpha (TNF-α) secreted by TILs and other immune cell types (Garcia-Diaz et al. 2017)(Karwacz et al. 2011). In addition, PD-L1 expression is also regulated by oncogenic pathways including AKT-mTOR and PTEN (Parsa et al. 2007).

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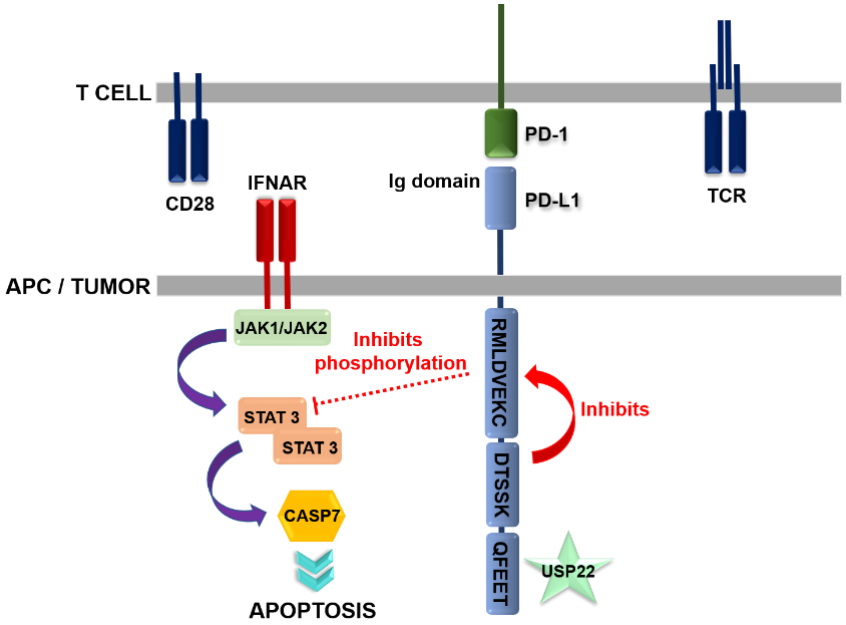

Figure 8. Protective function of PD-L1 intracellular signaling against IFN cytotoxicity. *IFN binding to its receptor on cancer cells activates the JAK1/JAK2-STAT signal transduction pathways, culminating in tumor cell apoptosis. The figure represents the main mechanisms by which PD-L1 intracellular motifs protect tumor cells from IFN cytotoxicity. The RMLDVEKC motif interferes with the STAT-3 branch of the IFN pro-apoptotic pathway (dotted red arrow) and the DTSSK motif acts as negative regulator of PD-L1 anti-IFN functions (red arrow). Ig domain, Immunoglobulin-like domain; IFNAR, Interferon I and II Receptor; Casp7, Caspase 7; JAK1/JAK2; janus kinase 1 and 2; USP22, ubiquitin carboxyl-terminal hydrolase 22.*

4.3. PD-L1/PD-1 blockade as a strategy to recover T cells from dysfunctionality

In 2012, Suzanne Topalian and collaborators demonstrated that PD-L1/PD-1 blockade was efficacious for the treatment of a wide variety of different cancers in a clinical context (Brahmer et al. 2010)(Brahmer et al. 2012). Since then, the use of monoclonal antibodies blocking this interaction has revolutionized the treatments of oncological patients. These treatments have been approved by the FDA and EMA for many cancer types including metastatic melanoma, NSCLC, renal cell carcinoma (RCC), bladder cancer, head and neck cancer and Merkel carcinoma.

The specific mechanisms supporting the efficacy of PD-L1/PD-1 blockade have not been fully elucidated yet. When efficacious, these therapies seem to counteract tumor-induced T cell dysfunctionality by interfering with PD-1 and PD-L1 signals and unleashing activating pathways (Topalian, Drake, and Pardoll 2015)(Anagnostou et al. 2017)(Pardoll 2012). Although most of the experimental evidence has been shown in chronic infection models, T cell anergy and exhaustion seem to be reversed using PD-L1/PD-1 inhibitors, restoring proliferative and effector T functions and reducing viral and tumor load **(Figure 9)** (Wherry and Kurachi 2015)(Pauken and Wherry 2015)(Chinai et al. 2015)(Kamphorst, Wieland, et al. 2017). Indeed, various groups have identified

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both systemic and intra-tumoral exhausted $CD8^+$ $PD\text{-}1^+$ T cell populations that experience a proliferative burst after PD-L1/PD-1 treatment (K. H. Kim et al. 2019)(Kamphorst, Pillai, et al. 2017)(A. C. Huang et al. 2017)(Siddiqui et al. 2019). However, emerging studies are demonstrating that exhausted T cell subsets are highly heterogeneous and differ in the susceptibility to be reinvigorated by PD-L1/PD-1 pathway blockade. The stem cell-like and memory-like $PD\text{-}1^+$ $TCF1^+$ CD8 T cell subset seems to be the responsible for the proliferative and effector responses following PD-L1/PD-1 blockade (Siddiqui et al. 2019)(Miller et al. 2019)(Im et al. 2016). In contrast, epigenetic reprogramming of severely exhausted and terminally differentiated $PD\text{-}1^+$ TCF^- CD8 T cell subset derived from progenitors might not be reversible after PD-L1/PD-1 blockade therapy (Jadhav et al. 2019)(Pauken et al. 2016). Nevertheless, since most of the studies have been mostly carried out in chronic infection models, further investigation using tumor models are required to deeply understand whether there are indeed tumor-specific progenitor TIL populations targetable by PD-L1/PD-1 blockade.

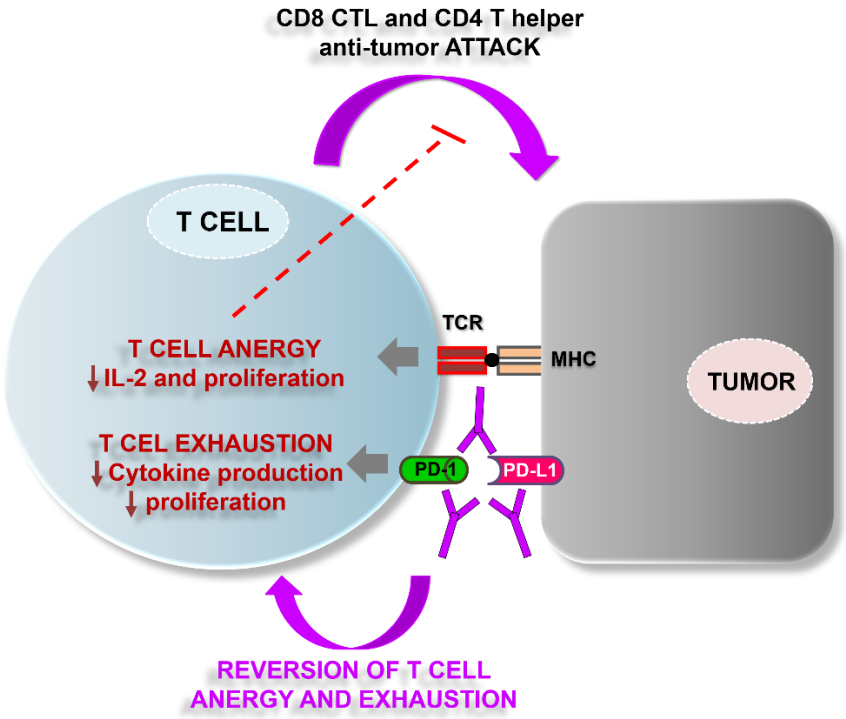

Figure 9. PD-L1/PD-1 blockade counteracts tumor-induced T cell dysfunctionality. *The figure represents the effects of anti-PD-L1/PD-1 antibodies on the reversion of T cells from anergy and exhaustion. Consequently, T cells recover their capacities of exerting efficient anti-tumor effector responses.*

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4.4. Role of T cell immunity in the efficacy of PD-L1/PD-1 blockade

Most of the experimental research supports T cells as the major target of anti-PD-L1/PD-1 immunotherapy. However, the specific mechanisms leading to efficacious clinical responses and whether it is mediated by reinvigorated dysfunctional TILs or newly recruited tumor-specific T cells from the periphery remain incompletely understood.

PD-1 blockade expands tumor-infiltrating CD8 memory T cells with a concurrent increase in INF-γ production, which positively correlates with therapeutic efficacy (Ribas et al. 2016). As mentioned before, recent studies have demonstrated that the proliferative burst of CD8 T cells observed after PD-1 blockade comes exclusively from PD-1^+ $TCF1^+$ stem-like and memory-like CD8 T cell subsets (Im et al. 2016)(Sade-Feldman et al. 2018)(Siddiqui et al. 2019)(Miller et al. 2019). In contrast, the epigenetic reprogramming observed in exhausted terminally differentiated TIL seems to be preserved during immune checkpoint blockade (Ghoneim et al. 2017)(Pauken et al. 2016). Hence, these studies suggest that PD-L1/PD-1 inhibitors might only target less-differentiated memory-like T cells creating an effector pool of cells, rather than reinvigorating terminally differentiated exhausted T cells within the tumor. In contrast, several studies have demonstrated that disrupting the molecular pathways controlling T cell exhaustion such as TOX and IRF4 reduced the survival of exhausted TIL populations suggesting that PD-L1/PD-1 blockade might also induce terminal differentiation and apoptosis of reinvigorated exhausted T cell pools (Khan et al. 2019)(Alfei et al. 2019)(Scott et al. 2019)(Man et al. 2017).

Nevertheless, PD-L1/PD-1 blockade also alters the systemic dynamics of immune cell populations (Kamphorst, Pillai, et al. 2017)(Kamphorst, Wieland, et al. 2017)(Hui et al. 2017). Indeed, a functional systemic immune system was shown to be a requirement for the efficacy of treatments based on administration of anti-cancer cell immunoglobulins in murine models (Spitzer et al. 2017). Some studies in NSCLC patients have shown that PD-1^+ CD8 T cells expand systemically following PD-1 blockade therapy (Kamphorst, Pillai, et al. 2017). These cells had an effector phenotype and were predominantly $CD28^+$ supporting the contribution of CD28 co-stimulation (possibly during antigen presentation by APCs) for reversion from dysfunctionality (Hui et al. 2017)(Kamphorst, Wieland, et al. 2017). The authors of these studies suggested that this expanding population might be tumor specific. Consistent with the previous results, a similar study with melanoma patients treated with pembrolizumab also identified a systemic $CD8^+$ PD-1^+ exhausted population that expanded after treatment. These T cell clones were equivalent to those in TILs (A. C. Huang et al. 2017). In addition, data published by K.E. Yost and colleagues showed that the ability of PD-1 blockade to rescue pre-

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existing TILs from exhaustion might be limited (Yost et al. 2019) and they demonstrated that T cell responses to checkpoint blockade were derived from novel tumor-specific T cell clones recruited from peripheral sources, again supporting the importance of systemic immunity for clinical responses to immune checkpoint blockade (Yost et al. 2019).

Particularly, the contribution of CD4 T cell immunity to the efficacy of PD-L1/PD-1 blockade therapy remains poorly understood. Some pre-clinical murine models have provided evidence for their requirement (Spitzer et al. 2017)(Markowitz et al. 2018)(Moreno et al. 2016). The systemic expansion of a specific murine CD4 T cell subtype was the main correlator with efficacy following administration of anti-cancer cell immunoglobulins (Spitzer et al. 2017). Other studies have previously shown that CD4 T cells recognizing tumor neoepitopes contribute significantly to the efficacy of several types of immunotherapies in murine models and in cancer patients (Kreiter et al. 2015)(Knocke et al. 2016)(Sahin et al. 2017). Therefore, the presence of functional CD4 T cell immunity might be of importance for the efficacy of several types of immunotherapies.

5. RESISTANCE TO PD-L1/PD-1 BLOCKADE

Despite all the successes, a significant proportion of patients do not benefit from PD-L1/PD-1 blockade. Only a minority (20%-30%) of advanced NSCLC patients receiving anti-PD-1 or anti-PD-L1 antibodies as a second line of treatment benefit from these therapies, while the remaining patients are intrinsically resistant. Moreover, some early responders eventually progress due to acquired resistance (Restifo, Smyth, and Snyder 2016)(Pitt et al. 2016)(Sharma et al. 2017). The mechanisms underlying primary and acquired resistances are varied and probably driven by tumor intrinsic and extrinsic factors (Sharma et al. 2017).

5.1. Tumor cell-intrinsic factors contributing to resistance to PD-L1/PD-1 blockade

Tumor-intrinsic mechanisms include genetic and epigenetic alterations which alter TMB, neoantigen formation, antigen processing/presentation and sensitivity to cytotoxic T cell responses. It is thought that elevated TMB correlates with a higher number of expressed TAAs (Schumacher and Schreiber 2015) which in turns confers an increased degree of immunogenicity to the tumor. This would favor the generation of a pool of tumor-specific T cells, thought to be the main target for PD-L1/PD-1 blockade therapy. Hence, a reduced pool of expressed TAAs will result in poorly immunogenic ("cold") tumors and a failure to raise strong T cell responses by PD-L1/PD-1 blockade therapy (Gubin

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et al. 2014). Indeed, there is a correlation between TMB and clinical response to PD-L1/PD-1 blockade in several tumor types (Yarchoan, Hopkins, and Jaffee 2017)(Rizvi, Hellmann, et al. 2015).

Highly immunogenic tumors can also develop mechanisms to impair antigen presentation through selection of mutations that interfere with the proteasome and with other components regulating antigen presentation. For example, down-modulation of MHC molecules, transporters associated with antigen presentation (TAP) or beta-2-microglobulin (Gettinger et al. 2017)(Marincola et al. 2000)(Sucker et al. 2014)(Restifo et al. 1996)(Zaretsky et al. 2016). Expression of genes encoding proteins that regulate antigen presentation can also be altered by epigenetic changes such as histone acetylation and hypermethylation (Kim and Bae 2011)(Karpf and Jones 2002). Consequently, tumor cells lose the ability to present TAAs on their surface and become invisible to TAA-specific T cells. Moreover, downregulation or loss of TAA expression during PD-L1/PD-1 blockade therapy has been also observed. Hence, the loss of multiple TAAs during the course of treatment does take place in NSCLC patients, with an eventual acquired resistance to PD-L1/PD-1 blockade (Anagnostou et al. 2017).

Inhibiting T cell trafficking and infiltration into the tumor can also contribute to resistance to PD-L1/PD-1 blockade. Mutations in oncogenic pathways such as the MAPK (Hu-Lieskovan et al. 2015) and WNT/β-catenin (Spranger, Bao, and Gajewski 2015) together with PTEN loss contribute to the inhibition of T cell recruitment into the tumors (Peng et al. 2016). Several studies are demonstrating the implication of tumor dedifferentiation or stemness in resistance to immune checkpoint therapies, which is also linked to WNT signaling, (Hugo et al. 2016)(Mehta et al. 2018)(Zhan, Rindtorff, and Boutros 2017).

Another evasion mechanism is driven by IFN-γ unresponsiveness by tumor cells. Activated TAA-specific T cells secrete IFN-γ which causes several effects within the tumor microenvironment. IFN-γ binding to its receptor on cancer cells activates the JAK1/JAK2 -STATs signal transduction pathways, culminating in tumor cell apoptosis (Darnell, Kerr, and Stark 1994). INF-γ also induces PD-L1 upregulation by tumor cells, making them more susceptible to anti-PD-L1/PD-1 blockade (Benci et al. 2016)(Ribas 2015). As a result of the potent immune-selective pressure, tumor cells expressing mutated proteins that prevent IFN responsiveness can be selected, by avoiding tumor cell apoptosis. Inactivating mutations in IFN-γ-receptor 1 and 2 (*IFNGR1/2*), *JAK1* and *JAK2* and interferon regulatory factor 1 (*IRF1*) have been found in patients resistant to PD-L1/PD-1 blockade (Zaretsky et al. 2016)(Dunn et al. 2005)(Shin et al. 2017). Moreover, mutations in these pathways also prevent PD-L1 upregulation, resulting in the selection of PD-L1 negative tumors which might be less likely to respond to anti-PD-L1/PD-1 blockade (Zaretsky et al. 2016). Despite the high levels of PD-L1

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expression in lung tumors with *EGFR* mutations and *ALK* rearrangements, the responsiveness to PD-L1/PD-1 blockade is very poor. This is due to low or even absence of CD8 T cell infiltration in these subtypes of lung tumors.

Tumor expression of galectin-3 has been recently associated to resistance to PD-L1/PD-1 blockade in NSCLC patients (Capalbo et al. 2019). Galectin-3 is a pleiotropic molecule which also acts as a binding partner of the immune checkpoint molecule LAG-3. Hence, engagement of LAG-3 by galentin-3 on tumor cells may play an important compensatory mechanism by maintaining T cell immunosuppression during PD-L1/PD-1 blockade therapy. Finally, mutations in *MDM2/MDM4*, *EGFR*, *ALK* and *STK11* genes have been associated to failure of PD-L1/PD-1 blockade therapy in NSCLC patients (Skoulidis et al. 2018). Indeed, mutations in *EGFR* and *MDM2/MDM4* genes have been associated to accelerated tumor growth following the administration of immunotherapies, a phenomenon termed hyperprogressive disease (Kato et al. 2017).

5.2. Tumor cell-extrinsic factors contributing to resistance to PD-L1/PD-1 blockade

Tumor cell-extrinsic mechanisms include non-tumor cells or other systemic influences, such as host microbiota, which can support tumor progression and resistance to PD-L1/PD-1 blockade therapy. For example, several populations of immunosuppressive cell types such as MDSCs, M2-polarised TAMs, Tregs and CD4 Th2 cells strongly inhibit anti-tumor CD8 cytotoxicity and CD4 Th1 cells through the release of cytokines, chemokines, and other soluble mediators (Pitt et al. 2016)(Sharma et al. 2017). Tumors secret chemokines and chemokine receptors which allow the trafficking of these suppressive populations to the tumor (Highfill et al. 2014).

A recent study has shown that TAMs can remove anti-PD-1 antibodies from the cell surface of PD-1^{+} CD8 T cells by capturing them through Fcγ receptors (Arlauckas et al. 2017). Effector T cells themselves can also express alternative immune checkpoint receptors such as LAG-3, TIM-3, CTLA-4 and others as a compensatory mechanisms (Koyama et al. 2016)(Thommen et al. 2015)(Blackburn et al. 2009)(Pauken and Wherry 2015). Indeed, TAA-specific cells co-expressing of both PD-1 and LAG-3 are more impaired in effector functions (Matsuzaki et al. 2010). Hence, several studies are linking PD-1/LAG-3 co-expression in T cells to resistance to anti-PD-L1/PD-1 therapies (Mishra et al. 2016)(R. Y. Huang et al. 2017)(Williams et al. 2017)(Johnson et al. 2018)(Zuazo et al. 2019). A recent study of immune checkpoint molecule expression by multiparametric single-cell analyses of lung tumor TILs has uncovered that LAG-3 expression is associated to worse outcome to PD-1 blockade (Datar et al. 2019). Simultaneous blockade of PD-1 and LAG-3 signaling axes has been

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shown more efficient in restoring anti-tumor T cell responses and overcoming resistance to PD-L1/PD-1 monotherapy (Woo et al. 2012)(Goding et al. 2013)(Matsuzaki et al. 2010)(Zuazo et al. 2019). LAG-3 targeting agents are currently being evaluated in clinical trials.

5.3. Hyperprogressive disease

There is accumulating evidence that PD-L1/PD-1 blockade does accelerate tumor progression and death in a certain group of patients. This serious adverse event has been termed hyperprogressive disease (HPD) or hyperprogression. HPD is accompanied by dramatically reduced survival after the initiation of immunotherapy (Saâda-Bouzid et al. 2017)(Champiat et al. 2017)(Ferrara et al. 2018)(Kato et al. 2017). The highest incidence is found in melanoma and NSCLC patients with a rate of 9% and 21%, respectively (Champiat et al. 2017). Phase III trials evaluating PD-L1/PD-1 blockade agents showed an early cross-over between immunotherapy and chemotherapy arms, with a tendency of a lower survival rate just right after the initiation for the immunotherapy arm. This dramatic drop of the curve strongly suggests the existence of intrinsic resistance to PD-L1/PD-1 blockade treatment and HPD.

Different quantitative approaches have been proposed to identify HPD in a clinical context (Champiat et al. 2017)(Kato et al. 2017)(Saâda-Bouzid et al. 2017)(Ferrara et al. 2018). All of them integrate pre-treatment tumor growth kinetics to identify growth acceleration after the initiation of immunotherapies. This requires radiological evaluation in the patients before their enrolment in immunotherapies. These procedures estimate tumor volume changes in the interval of time between radiological evaluation before and after PD-L1/PD-1 blockade treatment. Tumor volume is estimated using the sum of the largest diameter of target lesions according to Response Evaluation Criteria in Solid Tumors (RECIST criteria) 1.1. The most commonly used algorithms are the Tumor Growth Kinetics (TGK) (Saâda-Bouzid et al. 2017) and the Tumor Growth Rate (TGR) (Champiat et al. 2017). TGR assumes an exponential tumor growth model and the evaluation is carried out in three-dimensions. In contrast, the TGK method uses a lineal tumor growth model, for which two-dimensional data is used. Both models establish a minimum cut-off value equal or superior to 2 to identify HPD. However, these approaches might still underestimate HPD incidence due to several reasons. First, most patients suspected from HPD progress too quickly before the first radiological evaluation. Therefore, no data on tumor growth rates is obtained. Champiat et al. proposed the first computerized tomography (CT) assessment at earlier time points (4-6 weeks compared to current 8-12 weeks) (S. et al. 2018). Second, RECIST 1.1 assessment only considers target lesions without considering new lesions or non-measurable disease. Thus, potential hyperpogressors characterized by the surge of new lesions or growth of non-

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measurable lesions are not identified. In contrast, HPD incidence could be also overestimated when tumor burden is low.

All these difficulties and the lack of cellular/molecular mechanisms explaining HPD makes it a controversial topic. Although several factors have been associated to HPD across different studies, these have not been validated. Older age (>65 years) (Champiat et al. 2017), higher number of metastatic sites (>2) (Ferrara et al. 2018), and *MDM2* amplification and *EGFR* alterations (Kato et al. 2017) have been associated with higher HPD rate. Interestingly, no association has been found between disease stage, ECOG status, previous treatments and blood cell counts. Garassino et al. proposed that M2-polarised macrophages could cause HPD upon anti-PD-1 treatment (Lo Russo et al. 2018). The authors suggested that there was a possible deleterious effect of the Fc region after binding to its receptors presented in M2-macrophages in absence of T cell immunity. Another study in gastric cancer uncovered the association of infiltrating $FoxP3^{high}$ $CD45RA^{-}$ CD4 Tregs with HPD. Anti-PD-1 treatment enhanced Treg proliferation in tumors of hyperprogressors (Kamada et al. 2019). Although there are still few studies on the subject, all of them seem to point towards the implication of different immune cell populations which support T cell dysfunctionality as a driver force of the phenomena.

No predictive biomarker for HPD risk exists so far that would allow an adequate selection of patients. A high-scale study with NSCLC patients treated with PD-L1/PD-1 blockade therapy showed that hyperprogressors had low pre-treatment frequencies of peripheral effector memory CD8 T cells ($CD8^{+}$ $CCR7^{-}CD45RA^{-}$) and a higher frequency of exhausted $CD8^{+}$ $PD1^{+}$ $TIGIT^{+}$ cells (C. G. Kim et al. 2019). Therefore, the uncovering of biomarkers for HPD that could be applied to routine clinical practice is of paramount importance.

6. BIOMARKERS OF RESPONSE TO PD-L1/PD-1 BLOCKADE THERAPY

The low efficacy rates of PD-L1/PD-1 blockade and the existence of deleterious phenomena highlight the need for the identification of accurate predictive biomarkers for proper patient selection (Topalian et al. 2016). Several biomarkers have been proposed so far. The first to be evaluated was PD-L1 tumor expression, being the only one accepted by the FDA so far (Blank et al. 2016). However, its reliability as a truly predictive biomarker is still under debate, because patients with PD-L1 negative tumors can still benefit from the therapy (Grigg and Rizvi 2016). Other potential biomarkers under evaluation include TMB, TIL quantification and detection of interferon gene signature profiles within the tumor microenvironment **(Table 2)**. However, these techniques require biopsies and technologies not usually available in standard clinical practice. The identification of non-invasive biomarkers in peripheral

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blood or serum is also being contemplated, especially for cancers such as NSCLC for which tumor biopsying is a limitation.

6.1. PD-L1 expression in tumors as a biomarker

Quantification of PD-L1 tumor expression by IHC is the only FDA-approved biomarker for patient stratification in NSCLC. It is generally assumed that significant PD-L1 tumor expression confers susceptibility to PD-L1/PD-1 blockade, being the primary target of the therapeutic antibodies. Its predictive value has been assessed in several phase III clinical trials. A correlation between high PD-L1 positivity and increased overall response rate (ORR) and OS was indeed observed (Topalian et al. 2012)(Rosenberg et al. 2016)(Garon et al. 2015)(Reck et al. 2016)(Hellmann et al. 2018)(Borghaei et al. 2015)(Motzer et al. 2015)(Ferris et al. 2016). However, other studies have shown no association, with patients having "PD-L1-negative" tumors whom also benefit from anti-PD-L1/PD-1 agents (Carbone et al. 2015)(Hanna et al. 2018)(Motzer et al. 2015)(Borghaei et al. 2015). Accordingly, nivolumab and atezolizumab were approved for patients independently of the PD-L1 tumor expression status. In contrast, pembrolizumab was approved for patients with PD-L1 positive tumors. These apparently contradictory results have been explained by several reasons: PD-L1 detection assays are not standardized and utilize a variety of different antibodies, IHC platforms and threshold systems (Hansen AR and Siu LL 2015). PD-L1 expression is not homogeneous within the tumor, and biopsy sampling can introduce a strong bias (Ilie et al. 2016). PD-L1 expression is dynamic and varies during cancer evolution and under different treatments. For instance, chemotherapy, radiotherapy and exposure to TIL-derived cytokines induce PD-L1 expression (Wimberly et al. 2014)(Nguyen et al. 2016)(Shaverdian et al. 2017). Moreover, mutations which might be acquired during tumor progression such as JAK-3 activating mutations increase PD-L1 expression in NSCLC (Van Allen et al. 2015). Interestingly, while inducible PD-L1 expression has been associated with better clinical outcomes, constitutive expression triggered by activation of oncogenic signaling pathways correlates with worse outcome (Inoue et al. 2016). Finally, several studies with mice models and patients have showed that PD-L1 expression in tumor infiltrating immune cells is associated with objective responses (Tang et al. 2018)(Lin et al. 2018)(Lau et al. 2017)(Rosenberg et al. 2016)(Herbst et al. 2014). Hence, OAK and POPLAR clinical trials found an association between response to atezolizumab and PD-L1 expression in both immune cells and tumor cells (Rittmeyer et al. 2017)(Fehrenbacher et al. 2016). In addition, a recent study has characterized the immune cell infiltrating populations within baseline NSCLC tumor biopsies. They have uncovered that the predominant myeloid population that expresses PD-L1 within the tumor are $CD68^{+}$ macrophages, correlating with a positive clinical outcomes to PD-1/PD-L1 blockade (Liu et al. 2019). Although

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further validation is required, these emerging studies suggest that PD-L1 expression on macrophages might be responsible for the predictive value of the biomarker.

6.2. Tumor mutational burden (TMB) as a biomarker

TMB is defined as the total number of non-synonymous somatic mutations of the encoding genome. Specifically, it is represented as total number of somatic mutations per DNA megabases in the tumor exome, mut/Mb (Frampton et al. 2013)(Yarchoan et al. 2017). Targeted Next-Generation Sequencing (NGS) allows the profiling of the tumor somatic mutational landscape, usually with a limited number of genes (Yuza et al. 2017). Several platforms exist for large NGS panel analyses including Foundation Medicine comprehensive gene profiling (FM-CGP) and The Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT). Foundation One CDx ™ and MSK-IMPACT have been approved or authorized by the FDA as *in vitro* diagnostic test for profiling TMB for solid tumors in a clinical setting. TMB can also be quantified by both genome-wide analysis (whole genome sequencing (WGS)) and covering the entire coding regions of genes in the genome (whole exome sequencing (WES)), but with a very high cost which makes it less feasible for standard clinical practice.

Tumors with high TMB are generally more responsive to anti-PD-L1/PD-1 therapy (Topalian et al. 2012)(McGranahan et al. 2016). It is thought that these tumors are more immunogenic and induce a higher number or variety of TAA-specific T cells which would be reinvigorated after PD-L1/PD-1 blockade. Nevertheless, there is a high variability in TMB between different tumor types (Lawrence et al. 2013). NSCLC tumors have usually high TMB due to the chronic exposure to tobacco smoke (Pfeifer et al. 2002). Several studies have evaluated TMB as a potential biomarker for PD-L1/PD-1 blockade in NSCLC patients. Rizvi *et al.* showed for the first time an association between high TMB and increased ORR, longer median progression free survival (mPFS) and grater durable clinical responses to pembrolizumab (Rizvi, Hellmann, et al. 2015). Interestingly, mutations in genes involved in DNA mismatch repair (MMR) such as *POLE*, *POLDI* and *MSH2* were identified in three responder patients high TMB (Rizvi, Hellmann, et al. 2015). These mutations likely increase TMB and possibly neoantigen load. Moreover, patients with high TMB and a homogeneous clonal mutational landscape showed benefit to therapy (McGranahan et al. 2016). In contrast, heterogeneous tumors with subclonal mutations could be more resistant to PD-L1/PD-1 blockade therapy due to the possibility of selection of resistant variants. Goodman et al. also confirmed the positive association between high TMB and good outcomes to PD-L1/PD-1 blockade therapy in NSCLC patients (Goodman et al. 2017).

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Several WES studies in phase III clinical trials support the correlation between high TMB and response to PD-L1/PD-1 inhibitors. CheckMate 026 phase III trial included a post-hoc TMB analysis which uncovered that high TMB (>243 mutations) patients treated with nivolumab showed favorable clinical responses. In contrast, patients with medium (100–242 mutations) and low (0–100 mutations) TMB did not benefit from treatment. Although no association was found between PD-L1 tumor expression and TMB, patients with tumors exhibiting high TMB and PD-L1≥50% showed the highest ORR (Carbone et al. 2017). Another exploratory study of data from CheckMate 026 also showed an increased benefit in patients with high TMB (≥ 13 mutation/Mb) treated with nivolumab (Peters et al. 2017). Similar results were obtained in CheckMate 568 phase II trial evaluating the combination of nivolumab and ipilimumab as a first line treatment where TMB was evaluated by Foundation One CDx^{TM} with a cut-off value for TMB of 10 mutation/Mb (Ready et al. 2019). A retrospective analysis of POPLAL phase II trial data highlighted the association of high TMB evaluated by Foundation One CDx^{TM} and increased of ORR, duration of response, and mPFS with atezolizumab (Marcin Kowanetz et al. 2017). Finally, a recently reported exploratory study of NSCLC patients treated with anti-PD-L1/PD-1 agents showed a good correlation between TMB quantification with NSG (MSK-IMPACT) and WES techniques on improved mPFS and ORR with high TMB (Rizvi et al. 2018).

Although pre-treatment TMB has demonstrated to be relatively efficient predicting responses to PD-L1/PD-1 blockade therapy, validation datasets are required. Nevertheless, TMB quantification has also several drawbacks, including the high cost, standardization of TMB quantification assays to be comparable across studies and the requirement of relatively large biopsies from NSCLC patients.

6.3. Quantification of tumor infiltrating lymphocytes (TILs) as a biomarker

Highly immunogenic tumors (“hot”) usually recruit a variety of immune cell populations including CD8 and CD4 effector T cells, Tregs, DCs and macrophages. The degree of CD8 T cell infiltration is usually a good prognostic factor in several tumor types, correlating with better survival (Fridman et al. 2017). PD-L1/PD-1 blockade therapy reinvigorates TIL effector functions and anti-tumor immunity (Riaz et al. 2017)(Tumeh et al. 2014)(Topalian et al. 2015). Activated TILs produce and secrete effector cytotoxic mediators such as IFN-γ and granzymes. However, these mediators also trigger negative feedback mechanisms to dampen inflammation. For example, IFN-γ induces tumor PD-L1 upregulation to inhibit T cell responses and induce apoptosis of TILs (Dong et al. 2002)(Spranger et al. 2013)(Schalper et al. 2017). In agreement with this, a direct correlation between the degree of TIL infiltration and tumor PD-L1 expression occurs in several tumor types such as lung, breast and ovarian cancer (Kitano et al. 2017)(Lin et al. 2017)(Webb et al. 2016). This phenomenon in turn can favor susceptibility to PD-L1/PD-1 blockade. Thus, both TIL infiltration and PD-L1 quantification are

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considered reliable biomarkers of tumor immunogenicity and potentially predictive of anti-PD-L1/PD-1 responses.

In malignant melanoma, CD8 infiltration often correlates with better response to anti-PD-1 treatment (Tumeh et al. 2014)(Nishino et al. 2017). Single-cell RNA sequencing of baseline $CD8^+$ TILs uncovered that responders were enriched in transcripts related to memory cell differentiation and cell survival, while $CD8^+$ TILs from non-responders were enriched in genes related to exhaustion (Sade-Feldman et al. 2018). The impact of TILs in PD-L1/PD-1 blockade efficacy has been poorly reported for NSCLC due to technical difficulties. Even so, TILs seem to have a prognostic value in lung cancer treated with chemotherapy. Patients with highly-infiltrated tumors showed increased OS (Brambilla et al. 2016). Both high CD8 and CD4 T cell infiltration in the tumor stroma and nest are associated with better OS (Al-Shibli et al. 2008)(Geng et al. 2015)(Kawai et al. 2008)(Donnem et al. 2015)(Schalper et al. 2015). In contrast, the degree of tumor infiltration with Tregs correlate with lower PFS (Tao et al. 2012)(Liu et al. 2017)(Kose et al. 2016). All these evidences strongly support the association between the degree of TIL infiltration and anti-tumor responses in NSCLC.

A limited number of studies have evaluated the predictive value of TILs in NSCLC treated with PD-L1/PD-1 therapy. A prospective study of 65 NSCLC patients uncovered that baseline PD-L1 expression in the tumor stroma and T cell (CD3, CD4 and CD8 T cells) infiltration assessed by IHC predicted response to nivolumab (S. et al. 2017). Another study showed that TIL density superior to 5% correlated with increased PFS and ORR (Gataa et al. 2017). Three subtypes of CD8 TIL subsets with differential PD-1 expression levels were identified in NSCLC patients treated with PD-L1/PD-1 blockade (Thommen et al. 2018). Only the subset with highest PD-1 expression developed efficient responses against autologous cancer cells *in vitro*. Moreover, baseline $PD\text{-}1^{high}$ CD8 TILs levels were strongly associated with anti-PD-1 responses *in vivo*. This specific population might be enriched in tumor-specific T cells reinvigorated by PD-L1/PD-1 therapy. Indeed, the study of lung tumor TIL repertoire might be another indicator of tumor immunogenicity and responses to immune checkpoint inhibitors. A small scale study with NSCLC patients using NGS for TCR sequencing uncovered that early clonal T cell expansion within the tumor and in PBMCs was associated with response to anti-PD-1 treatment (Olugbile et al. 2017).

In addition, decreasing levels of $CD4^+$ $FOXP3^+$ $PD\text{-}1^{high}$ Treg population in tumors and peripheral blood of NSCLC patients treated with anti-PD-1 has been associated with improved OS (Zappasodi et al. 2018). High infiltration of LAG-3 expressing TILs was a predictor of lack of responses to anti-PD-1 treatment (Datar et al. 2017). These results were further supported by a recent study using

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multiparametric single-cell analyses where immune checkpoint molecule expression in TILs was evaluated within several NSCLC patient cohorts. Patients with LAG-3 expressing TILs showed worse clinical outcome to PD-1 blockade monotherapy compared to LAG-3 negative, PD-1 and TIM-3 positive TILs tumors (Datar et al. 2019).

6.4. Tumor immune gene signatures as a biomarker

Microarray technology can be applied to provide an assessment of multiple gene expression from the tumor microenvironment from relatively small tumor samples. Several gene signatures with prognostic and predictive value have been found for NSCLC treated with chemotherapy (Tang et al. 2017)(Chen et al. 2007). Identification of specific pre-treatment tumor immune gene signatures can predict clinical response in melanoma patients treated with ipilimumab (Ji et al. 2012). Although limited data are available for NSCLC, an exploratory analysis from the phase II POPLAR trial uncovered that patients with tumors showing baseline T effector IFN-γ gene signatures had increased OS after atezolizumab treatment (L. et al. 2016). Supporting these data, high IFN-γ mRNA expression indicated improved response to nivolumab in a small cohort of NSCLC patients, without differences in OS (Karachaliou et al. 2018).

6.5. Blood cell counts in routine clinical practice

A dominant neutrophil infiltration inversely correlates with the degree of TILs infiltration in NSCLC (Kargl et al. 2017). *In vivo* studies show that increased tumor-associated neutrophilia correlates with poor efficacy to anti-PD-1 therapy efficacy (Akbay et al. 2017). Absolute neutrophil count (ANC), the classical prognostic neutrophil-to-lymphocyte ratio (NLR), and the derived NLR (dNRL; absolute neutrophil count/ white blood cell count-absolute neutrophil count) have been extensively assessed because they are easily to implement in clinical routine. In a cohort of advanced NSCLC patients treated with nivolumab, a baseline ANC≥ 7500/ul correlated with worse PFS and OS (Tanizaki et al. 2018). A pre-treatment NLR≥ 5 significantly correlated with worse PFS and OS in patients treated with nivolumab as a second line therapy (Bagley et al. 2017). NLR changes were monitored during treatment, and NLR values above 5 after 6 weeks of treatment were associated with poor PFS and OS (K.J. et al. 2018). The dNRL seems to be more consistent as a biomarker, as considers monocytes and other granulocyte populations. High dNRL (>3) was associated with worse OS in NSCLC patients treated with anti-PD-1 agents. dNRL greater than 3 together with lactate dehydrogenase (LDH) values greater than the upper limit of normal (ULN) can be integrated into a parameter termed “lung immune prognostic index”. This parameter efficiently identifies 3 groups of patients with good (0 factor),

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intermediate (1 factor), and poor survival (2 factors) under PD-L1/PD-1 blockade therapy (Mezquita et al. 2018). Thus, it might serve to identity patients unlikely to benefit from immunotherapies.

The baseline absolute lymphocyte count (ALC) ≥ 1000/ul was significantly associated with PFS and OS in nivolumab-treated NSCLC patients (Tanizaki et al. 2018). Platelet-to-lymphocyte ratio (PLR) has also been used for NSCLC patients treated with nivolumab. Elevated pre-treatment PLR and 6 weeks-PLR ≥ 169 were associated with longer OS (Akbay et al. 2017)(K.J. et al. 2018). Finally, the prognostic value of circulating monocytes has also been evaluated. A pre-treatment ratio ≥ 3.68 correlated with worse OS in nivolumab-treated NSCLC patients (Hu et al. 2014). Baseline absolute eosinophil count (AEC) ≥ 150/ul was associated with PFS and OS in patients treated with nivolumab (Tanizaki et al. 2018). No data are available on the impact of baseline absolute-monocytic count (AMC) in anti-PD-L1/PD-1 outcome.

Nevertheless, parameters such as ANC, NLR, dNLR and AMC calculated in standard clinical practice do not accurately differentiate the wide ranges of myeloid-derived populations at different activation stages in peripheral blood which may play differential roles in therapy. For example, high levels of classical and non-classical HLA-DR$^+$ monocytes positively correlate with better response to ipilimumab in melanoma, while this association was inverse with high AMC (Romano et al. 2015).The elevated AMC values might have corresponded to increased monocytic MDSC populations which are identified as monocytes in classical analytical data (Prelaj et al. 2019).

6.6. Monitoring the dynamics of specific immune cell populations in peripheral blood

Monitoring of peripheral immune populations is a promising non-invasive method to find biomarkers of response to PD-L1/PD-1 therapy. Several circulating cell types have been put forward as biomarkers, but these studies will require large-scale validation studies (Havel, Chowell, and Chan 2019).

A recent study evaluating peripheral blood samples of NSCLC patients treated with anti-PD-1 agents demonstrated that early expansion of peripheral PD-1$^+$ CD8 T cells was associated with efficacy (Kamphorst, Pillai, et al. 2017). These expanded cell subsets expressed CD28 and other activation and effector markers, suggesting that CD28 co-stimulation could be a requirement for anti-PD-1 therapy efficacy (Kamphorst, Pillai, et al. 2017)(Kamphorst, Wieland, et al. 2017). Another study with two independent NSCLC patient cohorts also correlated the expansion of peripheral PD1$^+$ CD8$^+$ T cells, measured as the fold-change in the percentage of Ki67$^+$ cells after the first week of treatment, with durable clinical benefit to anti-PD-1 treatment (K. H. Kim et al. 2019). Early clonal T cell expansion as evaluated by genome-wide sequencing was positively associated with clinical responses in NSCLC

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under anti-PD-1 treatments (Olugbile et al. 2017). Moreover, TCR clonal expansion and loss of corresponding TAAs could be used for monitoring the response to immunotherapy and acquisition of resistances (Anagnostou et al. 2017)(Olugbile et al. 2017). A high proportion monocytic MDSCs (M-MDSCs) and granulocytic MDSCs (PMN-MDSCs) in NSCLC patients has been correlated with poor responses to chemotherapy (Feng et al. 2012)(Vetsika et al. 2014)(Koinis et al. 2016)(Liu et al. 2010). Few studies have evaluated the role of MDSCs and their predictive value for PD-L1/PD-1 blockade. Thus, a low baseline proportion of circulating M-MDSCs was associated to positive clinical outcomes in melanoma patients treated with ipilimumab (Meyer et al. 2014).

Mass cytometry allows the identification of immune peripheral blood cell populations in a highly specific manner due to its capacity to detect multiple markers. Increased baseline peripheral $CD14^{+}$ and $CD16^{-}$ $HLA\text{-}DR^{high}$ monocytes correlated with high response rates in melanoma patients under anti-PD-1/PD-L1 therapy (Krieg et al. 2018). Another study identified high levels of $CD69^{+}MIP^{-}1\beta^{+}$ NK cells in responders to anti-PD-1 treatment (Subrahmanyam et al. 2018). High PD-1, PD-L1 and PD-L2 expression on PBMC from NSCLC patients ascertained by multi-parametric flow cytometry have been associated with worse OS (Arrieta and Montes-Servín 2017).

6.7. Cytokine quantification in serum/plasma

Cytokines are key effectors and regulators of many cell types including immune effector cells. Their quantification in serum or plasma is straightforward and easily implemented in clinical practice. Therefore, their quantification in cancer patients could most likely uncover biomarkers for response to PD-L1/PD-1 therapy. Indeed, cytokines present in tumor and peripheral blood can provide relevant information about the status of the patients and the outcome of therapies. High baseline levels of IFN-γ, IL-6, and IL-10 correlated with objective responses in melanoma patients treated with nivolumab (Yamazaki et al. 2017). Analysis of NSCLC samples from the Cancer Genome Atlas (TCGA) and from two MD Anderson datasets identified elevated serum IL-6 and indoleamine 2,3-dioxygenase (IDO) in inflammatory tumors (Y. et al. 2015). A parameter based on cytokines termed the “cytoscore” was developed to analyze data from two phase III clinical trials in NSCLC patients treated with nivolumab. The “cytoscore” was generated from baseline selected cytokines quantified in sera from previous phase III trials and was defined as high or low based on the median cytokine cut-off values observed in these studies. NSCLC patients with a high cytoscore had better OS compared with those with low cytoscores (Borghaei et al. 2016). In addition, early changes in serum IL-8 levels after treatment initiation have been associated with response to anti-PD-1 therapy in NSCLC and melanoma patients (Sanmamed et al. 2017).

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Table 2. Biomarkers of clinical outcomes to PD-L1/PD-1 immunotherapy in NSCLC patients

Biomarkers	Method of assessment	Tissue type	Association with favorable clinical outcome	Validated in phase- III clinical trial/References
PD-L1 tumor expression	IHC	Tumor	Positive PD-L1 tumor expression	Yes (Topalian et al. 2012)(Rosenberg et al. 2016)(Garon et al. 2015)(Reck et al. 2016)(Hellmann et al. 2018)(Borghaei et al. 2015)(Motzer et al. 2015)(Ferris et al. 2016)
Tumor mutation burden (TMB)	NGS: Foundation One CDx ™ and MSK-IMPACT WES	Tumor Blood	High mutational rate	Yes (Peters et al. 2017)(Carbone et al. 2017)(M. Kowanetz et al. 2017)
TCR clonality	Predicted neoantigens derived from WES data	Tumor Blood	Tumor homogeneous TCR clonality Systemic clonal expansion	No
TILs	IHC, FC analyses and multiparametric single cell analyses (cytoff)	Tumor	CD3, CD4 and CD8 T cell infiltration TIL density>5% Decrease in CD4 $FOXP3^{+}$ $PD\text{-}1^{high}$ T cells	No
Tumor immune gene signatures	Microarray platform technology	Tumor	Baseline T effector IFN-γ gene signatures	No
Blood cell counts	FC	Blood	Baseline ALC≥1000/ul Baseline ANC≥7500/ul Baseline NLR≥5 6weeks NRL≥5 Baseline dNRL>3 Elevated baseline PLR 6 weeks PLR≥169 Baseline AEC≥150/ul	No
Immune cell population	FC, Multiparametric single cell analyses (cytoff)	Blood	Expansion of peripheral PD-1+ CD8 CD28 T Baseline CD4 T_{HD} >40% and tumor PD-L1 positivity	No
Cytokines in serum	ELISA, protein microarray or enzyme-linked immunospot	Blood	High cytoscore High IL-6 and indoleamine 2,3-dioxygenase (IDO) Serum IL-8 elevation after treatment	No
Liquid biopsy bTMB	NGS	Blood	High mutational rate	No
Microbiome	MiSeq and Ion Torrent	Gut Microbiota	*Akkermansia muciniphila*	No

Abbreviations: IHC, Immunohistochemistry; NGS, Next generation sequencing; WES, Whole exome sequencing; FC, Flow cytometry; ALC, absolute lymphocyte count; ANC, Absolute neutrophil count; NLR,

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neutrophil-to-lymphocyte ratio; dNLR, derived NLR; AEC, absolute eosinophil count; PLR, Platelet-to-lymphocyte ratio; TCR, T cell receptor.

6.8. Liquid biopsy

Liquid biopsy is a non-invasive sampling method of circulating tumor cells (CTC), tumor DNA (ctDNA) and exosomes. These approaches provide information on genetic alterations within the tumor throughout treatments. This method is particularly useful for monitoring mutations associated to acquired resistances to therapies. Liquid biopsy is routinely used to detect the T790M resistance mutation in *EGFR* mutant NSCLC. CtDNA analyses can also be applied to quantify TMB in a non-invasive manner. A retrospective study of ctDNA from POPLAR and OAK clinical trials evaluating PD-L1/PD-1 blockade in NSCLC patients demonstrated that high TMB quantified from ctDNA correlates with clinical benefit to atezolizumab (Gandara et al. 2018).

Soluble PD-L1 (sPD-L1) is under evaluation as a predictive biomarker of responses as well. PD-L1 splice variants lacking the transmembrane or the intracellular domain are secreted and can inhibit T cell activation by a poorly understood mechanism (Kruger et al. 2017). Although it is assumed that increased level of sPD-L1 might be associated with poor prognosis, a recent study has found that increased sPD-L1 levels were present in melanoma patients who responded to anti-PD-1 treatment (Yue et al. 2018). Moreover, the presence of PD-L1 on circulating exosomes during anti-PD-1 treatment has also been evaluated in melanoma patients, correlating with better outcomes (Chen et al. 2018).

6.9. The microbiome

Gut commensal microbiota influences anti-tumor responses (Garrett 2015)(Zitvogel et al. 2016). It seems to strongly influence PD-L1/PD-1 blockade therapy efficacy in mice and in several human cancer types including NSCLC, RCC and urothelial cancer (Matson et al. 2018)(Gopalakrishnan et al. 2018)(Routy et al. 2018). Specific bacterial strains have been identified that could be associated to either response or resistance to PD-L1/PD-1 blockade therapy. Fecal microbiota transplantation from responder patients into mice restored their susceptibility to PD-L1/PD-1 blockade therapy in melanoma and MCA-205 mice models (Sivan et al. 2015)(Routy et al. 2018). A recent study with a cohort of 60 NSCLC patients treated with anti-PD-1 therapy showed that responder microbiota was specially enriched in *Akkermansia muciniphila* (Routy et al. 2018). Nevertheless, the underlying mechanism by which microbiota influences immunosuppression is still under research.

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MATERIALS AND METHODS

7.1. Study design

The study was approved by the Ethical Committee at the Complejo Hospitalario de Navarra. Informed consent was obtained from all subjects and all experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Samples were collected by the Blood and Tissue Bank of Navarre, Health Department of Navarre, Spain. 46 patients diagnosed with non-squamous and 19 with squamous NSCLC were recruited at the Complejo Hospitalario de Navarra. Patients had all progressed to first-line chemotherapy or concurrent chemo-radiotherapy. Eligible patients were 18 years of age or older who agreed to receive PD-L1/PD-1 blockade immunotherapy following the current indications. Tumor PD-L1 expression could be quantified in 50 of these patients before the start of therapies. Measurable disease was not required. The exclusion criteria consisted of concomitant administration of chemotherapy or previous immunotherapy treatment. NSCLC patients had an age of 63.17 ± 8.9 (mean ± SD, $N = 65$). Age-matched healthy donors were recruited from whom written informed consent was also obtained, with an age of 64.5 ± 8.5 (mean ± SD, $N = 40$).

Therapy with nivolumab, pembrolizumab, and atezolizumab was provided following current clinical indications (Herbst et al. 2016)(Horn et al. 2017)(Rittmeyer et al. 2017). 10 ml of peripheral blood samples were obtained prior and during immunotherapy before administration of each cycle. The participation of each patient in the study was concluded when a radiological evaluation confirmed response or progression, with the withdrawal of consent or after death of the patient. Tumor responses were evaluated according to RECIST 1.1 (Eisenhauer et al. 2009) and Immune-Related Response Criteria (Wolchok et al. 2009). Objective responses were confirmed by at least one sequential tumor assessment. Hyperprogression was identified according to the radiological criteria established by Champiat et al. (Champiat et al. 2017).

7.2. Cell culture and growth

7.2.1. Cell lines

Human embryonic kidney (HEK) 293T cells were purchased from the American Type Cell Culture Collection (ATCC) and were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% antibiotics PenStrep (Gibco). The human lung cancer adenocarcinoma A549 cell line was obtained from Prof. Ruben Pío, authenticated by his group and were grown in Roswell Park Memorial Institute medium (PRMI) medium (Gibco) supplemented with 10% FBS (Sigma) and 1% antibiotics PenStrep (Gibco). This cell line harbors

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mutations in *KRAS* and *CDKN2A*, while it is wild type for *EGFR*, *PIK3CA*, *TP53*, *ALK* and *PTEN* genes. The A549-SC3 cell line was obtained from the A549 parental cells by transduction with a pDUAL lentivector (Karwacz et al. 2011) encoding a single-chain version of a membrane-bound anti-OKT3 antibody (SC3 molecule) and blasticidin resistance following standard procedures. CHO cells were obtained from the ATCC and cultured in DMEM-F12 (Gibco) medium supplemented with 10% fetal FBS (Sigma) and 1% antibiotics PenStrep (Gibco). The CHO cell line producing an antibody equivalent to pembrolizumab was generated by transduction with two pDUAL lentivectors expressing the heavy and light chains of the published pembrolizumab sequence (Scapin et al. 2015). Jurkat E-6 cell line was obtained from the ATCC and cultured in RPMI (Gibco) medium supplemented with 10% fetal FBS (Sigma) and 1% antibiotics PenStrep (Gibco). All cells were confirmed to be mycoplasma-free by PCR using as oligonucleotides MYA (GGCGAATGGGTGAGTAACACG) and MYB (CGGATAACGCTTGCGACCTATG), which hybridize specifically with mycoplasma ribosomal genes.

7.2.2. Peripheral blood mononuclear cells (PBMCs)

10 ml of heparinized blood samples were obtained from patients and healthy donors. PBMCs were immediately isolated by FICOL gradients right after the blood extraction. Briefly, fresh blood retrieved from EDTA-collection tubes was diluted 1:2 with PBS. 9 ml of diluted blood were layered on top of 3 ml FICOL solution (GE Healthcare) in 15 ml falcon tubes. Samples were then centrifuged at 800g for 20 min which resulted on a mononuclear cell-ring layer at the interface between erythrocyte sedimentation on the bottom and plasma on the top. Plasma was stored at -80ºC for further analyses. The mononuclear cell-containing ring was collected, washed with PBS and centrifuged at 800g for 10 min. Erythrocyte contamination was eliminated by resuspending the cell pellet in 200 µl of ACK solution (Ammonium-Chloride-Potassium) (Gibco) for 5 min at room temperature (RT). Samples were washed with PBS and centrifuged at 300g for 10 min. PBMCs were then cultured in TexMACS ™ Medium (Miltenyi Biotech) at 37°C in a humidified 5% CO_2 incubator and plated on 6-well cell culture plates. Myeloid cells could adhere to the plastic cell culture plates overnight, and non-adherent T cells were collected, centrifuged, and resuspended for further applications.

7.2.3. Monocyte-derived dendritic cells (DCs)

Monocyte derived DCs were generated from adherent mononuclear cells in the presence of recombinant 50 ng/ml of GM-CSF (PeproTech) and IL-4 (PeproTech) in RPMI medium (Gibco), 10% FBS (Sigma) and antibiotics PenStrep (Gibco) for 7 days as described (Escors et al. 2008).

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7.3. Lentivectors

The pDUAL-SC3-BlastR lentivector was generated by standard DNA recombinant techniques to express single-chain OKT3 (scAb) antibody construct under the control of the SFFV promoter and blasticidin resistance from the human ubiquitin promoter in a pDUAL lentivector construct **(Figure 10A).** The cDNAs encoding the variable heavy and light chains of the anti-CD3 OKT3 antibody fused with a G-S linker were ordered from GeneArt (Arakawa et al. 1996). The linker was introduced by overlap extension PCR with the primers indicated in **Table 3.** Then, the scAb was fused to a human IgG1 constant sequence followed by the transmembrane domain of the human PD-1 gene.

The lentivector pDUAL-PembroIgL-PuroR was obtained by cloning in pDUAL vectors the published sequence of the light chain from the anti-PD-1 antibody pembrolizumab (Scapin et al. 2015) under the control of the SFFV promoter. Puromycin resistance was expressed under the control of the ubiquitin promoter **(Figure 10B)**. The lentivector pDUAL-PembroIgH-BlastR was generated by cloning in pDUAL vectors the published sequence of the heavy chain from the anti-PD-1 antibody pembrolizumab (Scapin et al. 2015) under the control of the SFFV promoter. Blasticidin resistance was expressed under the control of the ubiquitin promoter **(Figure 10B)**.

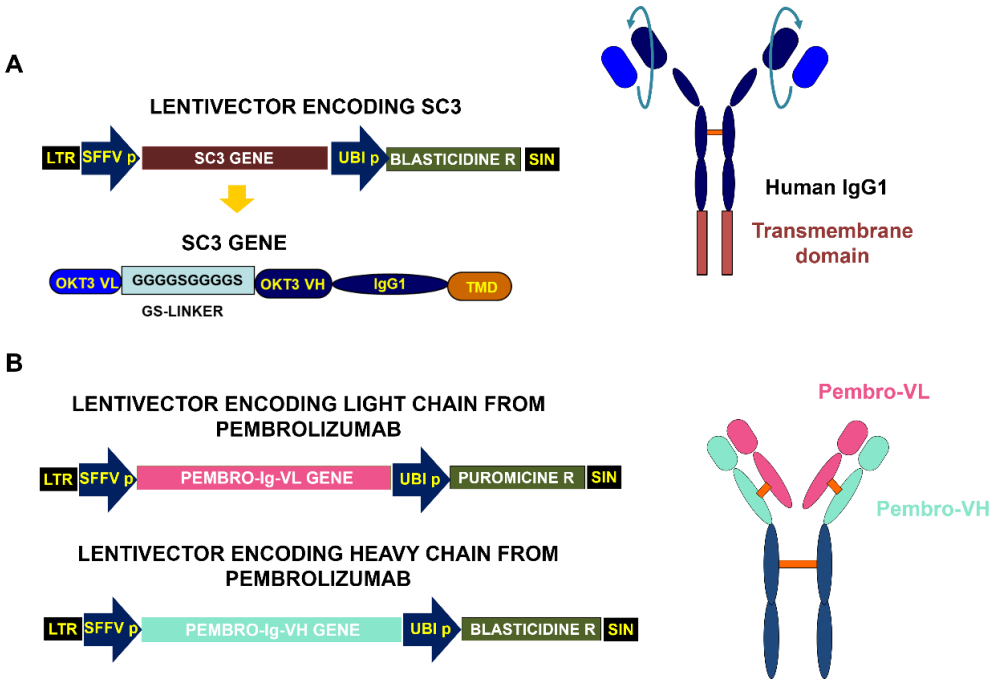

Figure 10. Generation of SC3 single-chain antibody and anti-PD-1 equivalent to pembrolizumab by lectivectors. (A) *Left, lentivector co-expressing an anti-CD3 single-chain antibody gene (SC3) and blasticidin resistance for selection; and the structure of SC3 gene. The cDNAs encoding the variable heavy and light chains of the anti-CD3 OKT3 antibody fused with a G-S linker. Then, the sequence was fused to a human IgG1 constant sequence followed by the transmembrane domain of the human PD-1 gene. SFFVp, spleen focus-forming virus promoter; UBIp, human ubiquitin promoter; LTR, long terminal repeat; SIN, U3-deleted self-inactivating LTR; OKT3 VL, variable light chain of the single-chain OKT3; OKT3 VH, variable heavy chain of the single-chain*

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OKT3; IgG1, immunoglobulin G1; TMD, transmembrane domain. Right, molecular structure of the anti-CD3 SC3 molecule, which is anchored to the cell membrane by a transmembrane domain as indicated. OKT3 VL, variable region of the light chain from the anti-CD3 antibody OKT3; VH, variable region of the heavy chain from the anti-CD3 antibody OKT3. ***(B)*** *Left, lentivectors co-expressing the light chain from the anti-PD-1 antibody pembrolizumab (PEMBRO-IgVL) and puromycin resistance for selection; and lentivectors co-expressing the heavy chain from the anti-PD-1 antibody pembrolizumab (PEMBRO-IgVH) and blasticidine resistance for selection. SFFVp, spleen focus-forming virus promoter; UBIp, human ubiquitin promoter; LTR, long terminal repeat; SIN, U3-deleted self-inactivating LTR. Right, molecular structure of the anti-PD-1 antibody. Pembro-VL, variable region of the light chain from the anti-PD-1 pembrolizumab antibody; Pembro-VH, variable region of the heavy chain from the anti-PD-1 pembrolizumab antibody.*

Table 3. Primers used for the generation of the single-chain OKT3

Targeted gene	Oligo forward	Oligo Reverse
Variable Light OKT3-GS linker	GGCGCGCCgccaccATGGATTTT CAGGTGCAGATTTTCAGCTTC	ccgccagagccacctccgcctgaaccgcctccac ctgaggagacggtgaccgtggtgccGTTTAT TTCCAACTTTGTCCCCGAGCCga acgtgaa
GS-linker-Variable Heavy OKT3	Ggcggttcaggcggaggtggctctggcggtgg cggatcgggcggcggcggctcgcaggtccag ctgcagcagtctggggc	GGATCCTGAGGAGACTGTGAG AGTGGTGCC
Single chain OKT3	GGCGCGCCgccaccATGGATTTT CAGGTGCAGATTTTCAGCTTC	GGATCCTGAGGAGACTGTGAG AGTGGTGCC

7.4. Lentivector production and cell transduction

Lentivector stocks were produced by the three-plasmid co-transfection system into HEK 293T cells, following published procedures (Karwacz et al. 2011). Cells were transduced with the required lentivectors (or combination of lentivectors) for 24-48 hours. To select transduced cells, the appropriate antibiotics at lethal concentrations were added to eliminate non-transduced cells.

7.5. T cell activation assay

Primary human T cells were stimulated by three different procedures. The first procedure was based on CD3/CD28-stimulation by coating 24 well-plates for 2h at 37ºC with 0.1 µg/ml functional grade OKT3 anti-CD3 monoclonal antibody (eBioescience ™ 16-0037-85) and functional grade anti-CD28 monoclonal antibody (CD28.2, eBioescience ™ 16-0289-85) diluted in PBS. Wells were washed and T cells added to coated wells in RPMI medium (Gibco) supplemented with 10% FBS (Sigma) and 1% antibiotics PenStrep (Gibco). T cells were stimulated for 3 days. The second procedure was based on activation by co-culture with A549-SC3 cells grown in 6 well-plaques. Primary human T cells were added to A549-SC3 cultures in a 1:1 ratio for 3 days. The third procedure was based on incubation with DCs loaded with A549 cell protein extracts. A549 cell extracts were obtained by freezing and thawing following by sonication. DCs were incubated with A549 cell extracts overnight and maturated

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with 10 ng/ml of IFN-γ (PeproTech) overnight. Human T cells were then added to the activated antigen-loaded DC cultures in a 1:10 ratio and co-cultured during 7 days as described (Escors et al. 2008).

7.6. Flow cytometry

T cells isolated from fresh blood samples and stimulated T cells were collected from the culture supernatant, centrifuged and resuspended in PBS for surface and intracellular flow cytometry staining procedures. The antibodies indicated in **Table 4** were used in a final volume of 50 µl and surface staining was performed for 10 min in ice. Cells were washed and resuspended in 300 µl PBS and analyzed immediately using a BD FACS Canto II (BD Biosciences).

For intracellular cytokine staining, Golgi-plug Protein Transport Inhibitor (BD Bioesciences) was added to T cell culture 5 hours before surface/intracellular staining procedures, following the recommendations of the manufacturer. For intracellular staining, T cells that had been stained for surface molecules were fixed 20 min in ice using 200 µl Fixation/Permeabilization solution (BS Bioescience) and washed twice with 500 ul Perm/Wash™ Buffer (10x) solution (BD Biosciences). To stain nuclear proteins such as Ki67, a similar protocol was performed using Transcription factor set (BD Biosciences).

Table 4. Antibodies used for flow cytometry analyses

Antibody	Reference	Clone	Dilution	Producer
CD3-APC	130-113-135	clone REA613	1:50	Miltenyi Biotech
CD3-violet fluor 450	75-0036-T100	Clon SK3	1:50	TONBO
CD3-PercP-Cy5	65-0037-T100	Clone OKT3	1:50	TONBO
CD4-FITC	130-080-501	clone M-T466	1:50	Miltenyi Biotech
CD4-APC-Vio770	130-100-455	clone M-T466	1:50	Miltenyi Biotech
CD4-PECy7	4129769,	clone SK3	1:50	BD Biosciences
CD8-APC-Cy7	A15448	clone RFT-8	1:50	Molecular probes by Life technologies
CD8-FITC	344703	clone SDK1	1:50	Biolegend
CD28-PECy7	302926	clone CD28.2	1:50	Biolegend
CD28-FITC	302906	clone CD28.2	1:50	Biolegend
CD27-PE	reference 130-093-185	clone M-T271	1:50	Miltenyi Biotech

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CD27-APC	130-097-922	clone M-T271	1:50	Miltenyi Biotec
PD-1-PE	339905	clone EH12.2H7	1:50	Biolegend
LAG-3-PercP-Cy5.5	369312	Clone 11C3C65	1:50	Biolegend
CD62L-APC	130-099-252	Clone MEL14-H2.100	1:50	Miltenyi Biotec
CD45RA-FITC	130-098-183	Clone T6D11	1:50	Miltenyi Biotec
CD57-PE	322311	clone HCD57	1:50	Biolegend
H2AX-FITC	613403	clone 2F3	1:100	Biolegend
CD14-VF450	75-0149-T100	clone 61D3	1:500	TONBO
IL-2 Alexa Fluor 647	500315	clone MQ1-17H12	1:100	Biolegend
IFN-γ-FITC	502506	clone 4S.B3	1:100	Biolegend
IL-10-APC	130-096-042	Clone JES3-9D7	1:100	Miltenyi Biotech
IL4-PE	130-091-647	Clone 7A3-3	1:100	Miltenyi Biotech
IL-17A-BV421	512321	BL168	1:100	Biolegend
IL-17A-Violet 667	130-120-554	clone CZ8-23G1	1:100	Miltenyi Biotech

7.7. Anti-PD-1 antibody production and purification

Supernatants from CHO-Pembrolizumab cultures were collected and antibodies purified by affinity chromatography following standard procedures. Purified antibodies were quantified by Nanodrop, and their binding activities confirmed by flow cytometry over Jurkat cells and primary T cells.

7.8. PD-1 and LAG-3 blockade assays

PD-1 (clone EH12.2H7, BioLegend) and LAG-3 (clone 17B4, BioLegend) blocking antibodies were added for 3 days to A549-SC3/T cell co-cultures at a final concentration of 5 µl/ml.

7.9. Cytokine arrays

Baseline plasma samples from patients and healthy donors were retrieved from storage at -80ºC and serum-derived cytokine profile analysis was performed using a dot-blot protein array (Abcam). Membranes with 80 cytokine antibodies were blocked with blocking buffer at RT for 30 min. Then,

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membranes were incubated overnight at 4ºC with 1 ml sera diluted 1:4 in blocking buffer. After washing, membranes were incubated with 500 µl biotinylated anti-cytokine antibody mix overnight at 4ºC. After washing, the membranes were incubated with HRP-conjugated streptavidin overnight at 4ºC. Membranes were then exposed to developing solution and revealed by ChemiDoc imaging system. Signals were quantified by ImageLab software (BioRad) and normalized to the positive control signal. Perseus (1.5.6) software was used for statistical analysis.

7.10. ELISA assays

Baseline plasma samples from patients were retrieved from storage at -80ºC. Cytokines fms-related tyrosine kinase 3 ligand (FLT3LG), brain-derived neurotrophic factor (BDNF), macrophage-derived chemokine (MDC) and vascular endothelial growth factor (VEGF) were quantified by ELISAs according to the indications of the manufacturer (human BDNF Elisa Kit ab99978, human MDC Elisa Kit ab100591, human FTL3-Ligand ab100521, VEGF-A Human ELISA Kit BMS277-2). All samples were analyzed in triplicates and cytokine levels quantified with an Epoch detection system (Biotek). Standard curves were obtained for each cytokine and concentrations expressed as pg/ml and µl/ml.

7.11. Data collection and Statistics

T cell percentages were quantified using Flowjo (Lanna et al. 2014)(Lanna et al. 2017). The percentage of CD4/CD8 T_{HD} ($CD27^{-}$ $CD28^{low/neg}$) and non-T_{HD} T cells ($CD28^{+}$ $CD27^{+}$) were quantified prior to therapy (baseline), and before administration of each cycle of therapy within CD4 and CD8 cells. Gates in flow cytometry density plots were established taking $CD27^{+}$ $CD28^{+}$ T cells as a reference. Data was recorded and independently analyzed thrice by two different individuals. Cohen´s kappa coefficient was utilized to test the inter-rater agreement in classification of immunological profiles (κ=0.939).

The mode of action, pharmacokinetics, adverse events and efficacies of the three PD-L1/PD-1 blocking agents are comparable in NSCLC, which act through the interference with the inhibitory interaction between PD-L1 and PD-1 (Herbst et al. 2016)(Horn et al. 2017)(Rittmeyer et al. 2017). Treatments administered to the patients were allocated strictly based on their current indications, and independently of any variable under study. All data was pre-specified to be pooled to enhance statistical power, and thereby reducing type I errors from testing the hypotheses after *ad hoc* subgrouping into specific PD-L1/PD-1 blockers. The number of patients assured statistical power for Fisher´s exact test of 0.95 and superior for Student t and Mann-Whitney tests (G*Power calculator)(Faul et al. 2009), taking into account that the expected proportion of responders is around 25% to 35% without stratification (Herbst et al. 2016)(Horn et al. 2017)(Rittmeyer et al. 2017). Two pre-specified subgroup analyses in the study were contemplated. The first, baseline T cell values; the

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second, post-first cycle T cell changes from baseline. The study protocol contemplated the correlation of these values with responses using Fisher´s exact test, paired Student t tests/repeated measures ANOVA (if normally distributed) or U of Mann-Whitney/Kruskal-Wallis (if not normally distributed, or data with intrinsic high variability). Two-tailed tests were applied with the indicated exceptions (see below).

The percentage of T cell subsets in untreated cancer patients was normally distributed (Kolmogorov-Smirnov normality test), but not in age-matched healthy donors. Hence, to compare T cell values between two independent cancer patient groups, two-tailed unpaired Student t tests were used, while comparisons between healthy subjects and cancer patients were carried out with the U of Mann-Whitney. Percentages of T cell populations in treated patients were not normally distributed, so response groups were compared with either Mann-Whitney (comparisons between two independent groups) or Kruskal-Wallis for multi-comparison tests if required. Two-tailed paired t tests were carried out to compare changes in the proportion of $CD28^+$ CD8 T cells between baseline and post-therapy paired groups, and to compare Ki67 expression in T cell subsets activated with A549-SC3 cells subjected to PD-1 or LAG-3 blockade. For comparison of paired samples with anti-PD1-/anti-LAG-3 combinations, two-way ANOVA tests with a random criterium (subjects) were used. Fisher´s exact test was used to assess the association of the baseline values of T_{HD} cells with clinical responses. The same tests were performed to assess associations between G1/G2 groups with well-established prognostic variables as indicated in the text.

Progression free survival (PFS) was defined as the time from the starting date of therapy to the date of disease progression or the date of death by any cause, whichever occurred first. PFS was censored on the date of the last tumor assessment demonstrating absence of progressive disease in progression-free and alive patients. PFS rates at 12 and 28-weeks was estimated as the proportion of patients who were free-of-disease progression and alive at 12 and 28 weeks after the initiation of immunotherapies. Patients who dropped out for worsening of disease and did not have a 28-week tumor assessment were considered as having progressive disease. Overall survival (OS) was defined as the time from the starting date of therapy to the date of death due to the disease or unrelated causes. Response rate (ORR) was the proportion of patients who achieved best overall response of complete or partial responses.

PFS and OS were represented by Kaplan-Meier plots and long-rank tests utilized to compare cohorts. Hazard ratios were estimated by Cox regression models. Receiver operating characteristic (ROC)

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analysis was performed with baseline T_{HD} numbers and response/no response as a binary output. Statistical tests were performed with GraphPad Prism 5 and SPSS statistical packages.

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RESULTS

8. IDENTIFICATION OF SYSTEMIC PREDICTIVE BIOMARKERS FOR CLINICAL RESPONSES TO PD-L1/PD-1 BLOCKADE THERAPY

8.1. Baseline CD4 T_{HD} cell profile separates NSCLC patients into two groups with differential clinical outcomes to anti-PD-L1/PD-1 immunotherapy

Changes in specific peripheral T cell populations during anti-PD-L1/PD-1 immunotherapy treatment have been previously correlated with objective clinical responses in NSCLC patients (Kamphorst, Pillai, et al. 2017)(K. H. Kim et al. 2019). However, none of those studies identified immunological profiles before treatment initiation which could reliably predict clinical outcomes for PD-L1/PD-1 blockade therapies.

To identify whether there was a correlation between specific systemic T cell subsets and responses to anti-PD-L1/PD-1 immunotherapy in NSCLC patients, a prospective study was carried out in a cohort of 65 patients who were enrolled to be treated with PD-L1/PD-1 inhibitors following their current clinical indications. The baseline characteristics of the cohort under study are shown in **Table 5**. These patients had all progressed to conventional cytotoxic therapies and received immune checkpoint inhibitors (13.8% pembrolizumab, 46.2% nivolumab and 40% atezolizumab) as part of their second- and third-line treatments. 80% of the patients presented an ECOG of 0-1, 69.2% with at least three affected organs and 24.6% with liver metastases **(Table 5)**.

As a first approach, the percentages of CD4 T cell differentiation subsets according to CD27/CD28 expression profiles were quantified within total CD4 T cells in patients before the start of immunotherapies (baseline), which have been previously shown to discriminate T cells in distinct differentiation stage (Lanna et al. 2014)(Lanna et al. 2017). Accordingly, human T cells are classified according to CD27/CD28 profiles into poorly differentiated ($CD27^{+}$ $CD28^{+}$), intermediately differentiated ($CD27^{neg}$ $CD28^{+}$) and highly differentiated ($CD27^{neg}$ $CD28^{low/neg}$, or T_{HD}) subsets. T cell quantification was performed from fresh peripheral blood samples and compared to healthy age-matched donors (N=40) (63.17±8.9 *vs* 64.5±8.5 years, mean±standard deviation, (SD)). As freezing and storage significantly altered surface expression markers in T cells, T cell subsets were directly analyzed from fresh blood samples.

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Table 5. Baseline patient characteristics

Variable	All patients (N=65) (%)
Sex	
Female	17 (26,2)
Male	48 (73,8)
Age	
<60	17 (26,2)
≥60	48 (73,8)
Histology	
Squamous	19 (29,2)
Non- Squamous	46 (70,8)
Inmunotherapy treatment	
Pembrolizumab	9 (13,8)
Nivolumab	30 (46,2)
Atezolizumab	26 (40)
PDL1 status	
0%	23 (35,4)
1-4%	5 (7,6)
5-49%	12 (18,5)
≥ 50%	10 (15,4)
Undetermined	15 (23,1)
Mutation status	
No	63 (97)
EGFR	1 (1,5)
ROS1	1 (1,5)
Smoking status	
Smoker	59 (90,8)
Non-smoker	6 (9,2)
Treatment line	
2nd	46 (70,8)
3th	16 (24,6)
4th or higher	3 (4,6)
Previous systemic therapies (previous 3 months)	
Platinium-based therapy	20 (30,8)
Non-platinium based therapy	23 (35,4)
No	22 (33,8)
ECOG	
0-1	52 (80)
2-4	13 (20)
GRImScore	
0-1	36 (55,4)
2-3	15 (23,1)
Undetermined	14 (21,5)
Liver metastases	
No	49 (75,4)
Yes	16 (24,6)
Number of sites involved	
≤2	20 (30,8)
≥3	45 (69,2)
CD4 THD Profiling	
G1 profile	31 (47,7)
G2 profile	34 (52,3)
Responses	
Partial response	15 (23,1)
Progression disease	41 (63,1)
Stable disease	9 (13,8)

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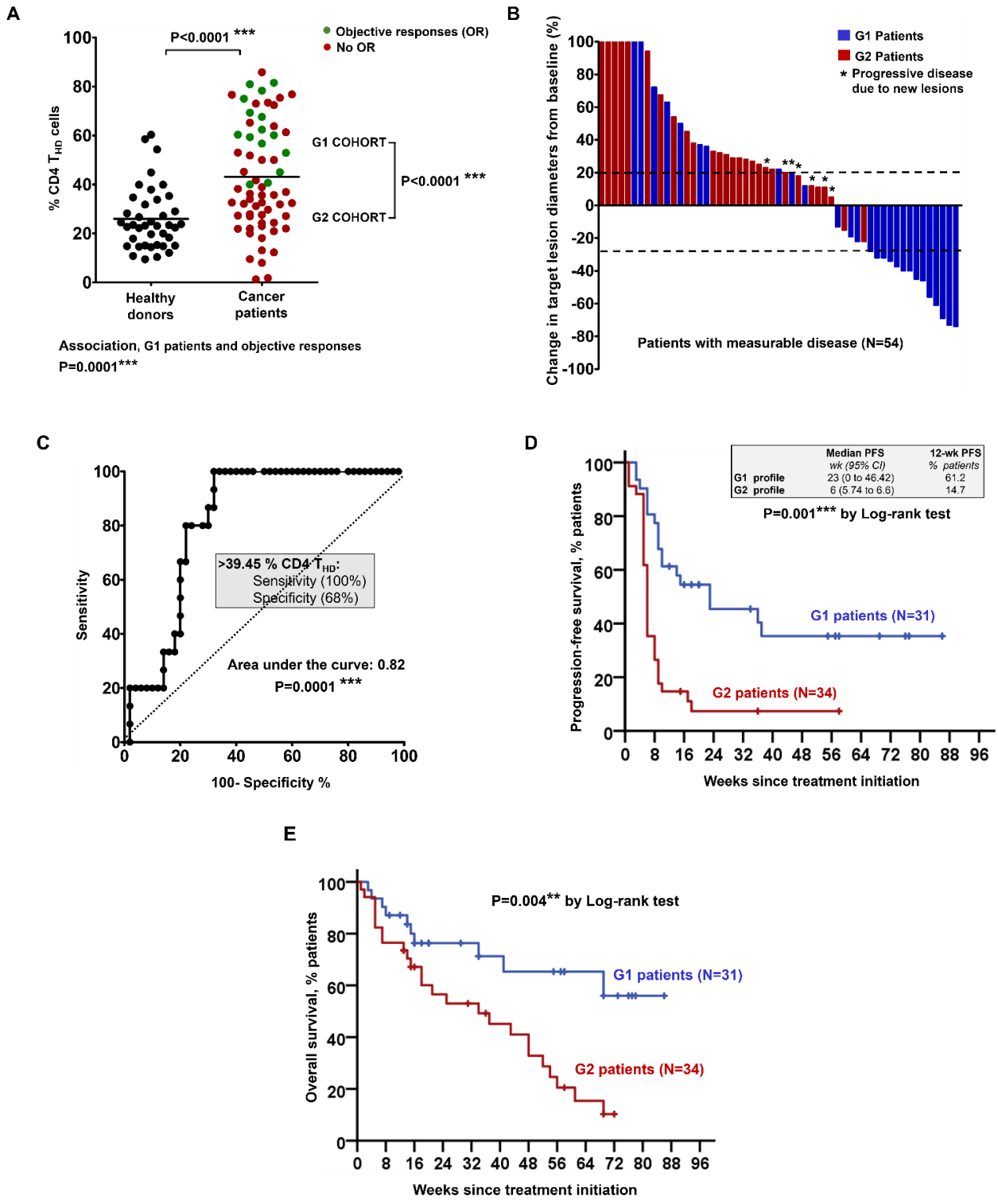

Figure 11. Baseline profiling of CD4 T cell differentiation subsets stratifies clinical responses to PD-L1/PD-1 blockade. (A) *Percentage of circulating highly differentiated CD4 T cells (CD4 T_{HD}) within CD4 cells in age-matched healthy donors (N=40) or NSCLC patients (N=65) before undergoing immunotherapies. G1 and G2, groups of patients classified according to high CD4 T_{HD} cells (G1, >40% CD4 T_{HD} cells) and low CD4 T_{HD} cells (G2, <40% CD4 T_{HD} cells). Relevant statistical comparisons are shown by the test of Mann-Whitney. In green, objective responders (OR). In red, no OR. Below the graph, correlation of objective responses to G1 and G2 groups by the Fisher´s exact test.* ***(B)*** *Waterfall plot of change in lesion size in patients with measurable disease (N=54) classified as having a G1 (blue) or G2 (red) CD4 T_{HD} cell baseline profile. Dotted line represents the limit to define significant progression (increase>20%, upper line) or significant regression (decrease >30%,*

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lower line). Patients starting therapy with a G1 and G2 CD4 T_{HD} profile had an overall response rate (ORR) of 48.4%, and 0% respectively. ***(C)*** *ROC curve and optimal cut-off value of CD4 T_{HD} baseline percentage for predicting objective clinical responses to immunotherapies.* ***(D)*** *Kaplan-Meier plot for progression free survival (PFS) in patients treated with immunotherapies stratified only by G1 (blue) and G2 (red) CD4 T_{HD} cell profiles. In the gray square, median mPFS and 12-weeks PFS are indicated for G1 and G2 cohorts. Statistical comparison is shown by the long-rank test.* ***(E)*** *Same as (D) for overall survival (OS). *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences.*

Overall, cancer patients showed a significantly higher baseline percentage of CD4 T_{HD} cells (43.12±22.19 %, N=65) than healthy controls (25.97±12.69 %, N=40) (P<0.0001) **(Figure 11A)**. Furthermore, patients were separated in two groups by an approximate cut-off value of 40% CD4 T_{HD} cells **(Figure 11A);** we denominated "G1 cohort" to patients with more than 40% CD4 T_{HD} cells (62.04±13.22 %, N=31), and "G2 cohort" to patients with less than 40% (25.22±10.08 %, N=34). Differences between G1 and G2 cohorts were also highly significant (P<0.0001) **(Figure 11A)**. Accordingly, the G1 cohort showed very significantly lower numbers of $CD28^+$ $CD27^+$ CD4 T (CD4 non-T_{HD}) cells compared to the G2 cohort, in whom this subset represented the majority.

All the patients were evaluated for response based on RECIST 1.1 criteria. The overall response rate (ORR) in the cohort was 23.1% consistent with the published efficacies for these agents (Herbst et al. 2016)(Rittmeyer et al. 2017)(Horn et al. 2017). Interestingly, in our study objective responders were found only within the G1 cohort (P=0.0001) which included all patients that showed significant tumor regression **(Figure 11A-B)**. Accordingly, ROC analysis demonstrated a highly significant association of CD4 T_{HD} baseline percentage with objective clinical responses (AUC 0.82, 95% C.I., 0.72–0.92; P=0.0001), and confirmed the cut-off value of >40% to identify objective responders with 100% sensitivity and 68% specificity **(Figure 11C)**. In contrast, clinical outcomes of patients with G2 profiles were significantly associated with tumor progression (p=0.0001), with an ORR of 0% **(Figure 11A-B)**. In agreement with these results, the G1 patient cohort had a significantly longer progression-free survival (PFS) compared to the G2 cohort. The median PFS (mPFS) of G2 patients was only 6 weeks (95% C.I., 5.4-6.6) compared to 23 weeks for G1 patients (95% C.I., 0-46.42; P<0.0001) **(Figure 11D)**. 86% of G2 profile patients experienced progression or death by week 12. A comparison of G2 *versus* G1 baseline profiles showed hazard ratios for disease progression or death that favored the latter [3.15 (95% C.I., 1.71-5.78); P < 0.0001]. In addition, the G1 cohort also exhibited a significant benefit in overall survival (OS) compared to the G2 cohort. Median OS (mOS) for the G2 cohort was 34 weeks (95% C.I., 8.7-59.3) while the corresponding for G1 patients was not reached yet at the present time (P=0.004) **(Figure 11E).**

In contrast to CD4 T_{HD} cells, the relative percentage of circulating baseline CD8 T_{HD} cells within the CD8 population did not significantly differ from age-matched healthy donors **(Figure 12A)**. Moreover,

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ROC analysis demonstrated the lack of significant discriminating value of CD8 T_{HD} cell baseline percentage for objective clinical responses (AUC 0.63, 95% C.I., 0.48–0.79; P=0.12) **(Figure 12B).**

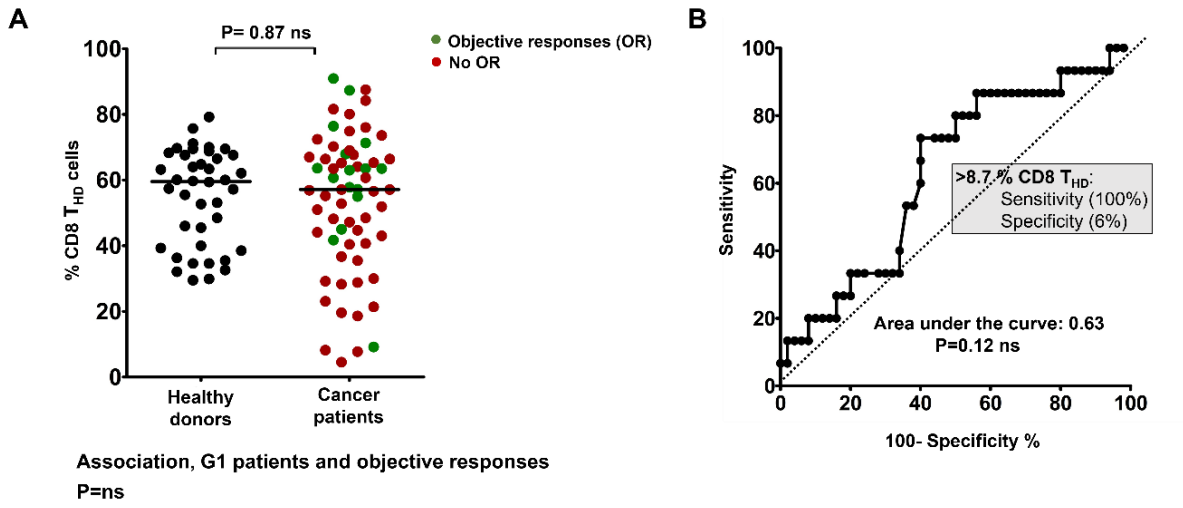

Figure 12. Baseline profiling of CD8 T cell differentiation subsets according to CD27/CD28 expression does not stratify clinical responses to PD-L1/PD-1 blockade. (A) *Percentage of circulating CD8 T_{HD} within CD8 T cells in age-matched healthy donors (N=40) or NSCLC patients (N=65) before undergoing immunotherapies. In green, objective responders (OR). In red, no OR. Relevant statistical comparisons are shown by the test of Mann-Whitney. Below the graph, correlation of objective responses to G1 and G2 groups by the Fisher´s exact test. Ns, no significant differences (P<0.05).* ***(B)*** *ROC curve of CD8 T_{HD} cell quantification for predicting objective clinical responses.*

Hyperprogressors represent a subgroup of patients that exhibit fast worsening of clinical conditions following immunotherapy, with fatal consequences. The identification of hyperprogressors is a critical issue in clinical oncology, as the causes of hyperprogressive disease are currently unclear. Therefore, hyperprogressors were identified within our cohort of patients following the radiological criteria established by Champiat et al., which applies an exponential tumor growth model to estimate tumor growth rates (TGR) before and after the start of immunotherapy. In this model, the TGR estimated during previous treatments is compared to the TGR calculated after the first follow-up CT scan evaluation. A threshold of TGR increase equal or superior to 2 is frequently considered to identify hyperprogressors (Champiat et al. 2017).

TGR could not be evaluated in 44.7% of the patients due to unmeasurable tumor burden by RECIST criteria, or unavailable CT scan evaluation from previous treatments. Ten patients were identified as radiological hyperprogressors, representing the 15.4% of our cohort **(Figure 13A)**, similar to other studies in NSCLC (S. et al. 2018). All of them experienced early progression of disease with a mPFS of 6 weeks (95% C.I., 4.9-7.1) compared to 8.1 weeks for the rest of progressors (95% C.I., 5.1-11.2; P=0.01) **(Figure 13B)**. Hazard ratios for disease progression or death favored the latter over hyperprogressors [2.61 (95% C.I., 1.17-5.82); P = 0.002]. Moreover, 90% of identified

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hyperprogressors had a G2 baseline profile. We found a significant association between G2 profiles and incidence of hyperprogression (P=0.01) **(Figure 13A).** However, no association was found between hyperprogression and other variables including age (P=0.246), gender (P=0.89), performance status (ECOG score) (P=0.154), immunotherapy drug (P=0.235), tumor histology (P=0.589), PD-L1 tumor expression (P=0.48), tumor load (P=0.583), number of liver metastases (P=0.677) or GRIm score (P=0.965).

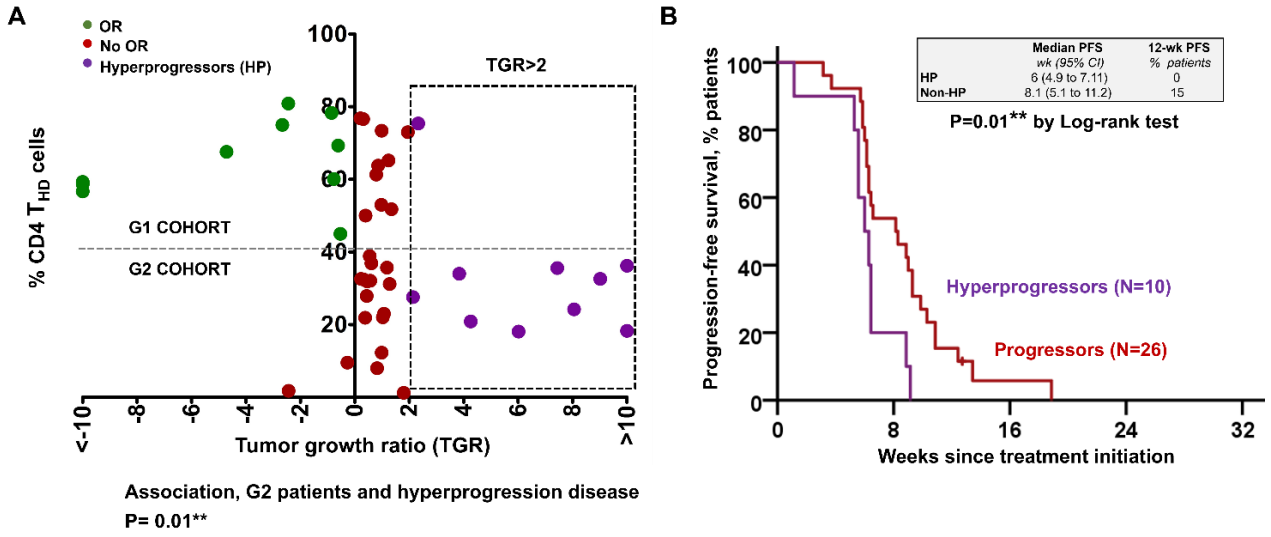

Figure 13. Baseline G2 T cell profile is associated with risk of hyperprogression. ***(A)*** *Scatter plot of baseline percentage of circulating CD4 T_{HD} cells as a function of tumor growth rate (TGR) in patients with measurable TGR (N=47). Dotted line shows the 40% CD4 T_{HD} cut-off value separating G1 and G2 cohorts. The dotted square includes patients with TGR>2 identified as hyperprogressors by radiological criteria in our cohort of patients. In green, objective responders (OR). In red, no OR. In purple, hyperprogressors (HP). Below the graph, correlation of hyperprogressive disease to G2 profiles by the Fisher´s exact test.* ***(B)*** *Kaplan-Meier plot for PFS in patients treated with immunotherapies with measurable TGR stratified as non-hyperprogressive progressors (non-HP) (red) and hyperprogressors (HP) (purple). Statistical comparison is shown by the long-rank test. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences.*

These results showed that the baseline relative percentage of circulating CD4 T_{HD} cells separates NSCLC patients before initiation of immunotherapies in two groups with distinct clinical outcomes to PD-L1/PD-1 blockade therapy. A cut-off value of >40% CD4 T_{HD} identified a group of patients containing the objective responders. In contrast, <40% CD4 T_{HD} values were strongly linked to disease progression and risk of developing hyperprogressive disease. Although independent validation studies are required, it represents a promising non-invasive blood-based predictive biomarker of clinical responses to PD-L1/PD-1 blockade therapy in advanced NSCLC patients.

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8.2. CD4 T_{HD} cell profiling has no significant prognostic value in NSCLC patients

To assess whether CD4 T_{HD} profiling had prognostic instead of a predictive value, the time elapsed from diagnosis to the start of immunotherapies was compared between G1 and G2 patient cohorts. Longer recruitment times would be associated to good prognosis, and this approach has been previously used for immunotherapies in a clinical context (Le et al. 2015). No significant differences were observed between G1 and G2 cohorts, indicating that G1/G2 classification did not have prognostic value **(Figure 14)**. This was supported by no association between G1/G2 patient cohorts with several well-stablished prognostic variables including ECOG score (P=0.33) **(Table 6),** liver metastases (P=0.15) **(Table 7)**, tumor load (P=0.18) **(Table 8)** or with the Gustave-Roussy immune score (GRIm) (P=0.54) **(Table 9)** which englobes three classical prognostic variables; serum LDH levels, serum albumin concentration and NLR ratio (Bigot et al. 2017). The hazard ratio for progression or death of G2 patients maintained its statistical significance by multivariate analyses (HR 9.739; 95% C.I., 2.501 to 37.929) when adjusted for tumor histology, age, gender, smoking habit, liver metastases, number of organs affected, PD-L1 tumor expression, NLR, serum LDH and albumin. Hence, we concluded that CD4 T_{HD} profiling has no significant prognostic value.

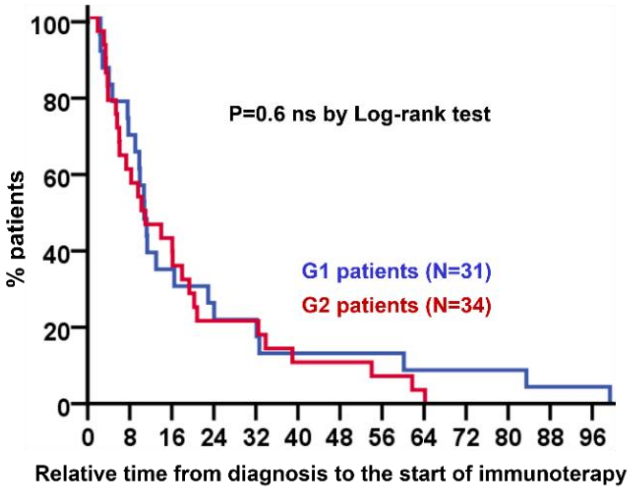

Figure 14. Baseline CD4 T_{HD} profiling does not have significant prognostic value. *Kaplan–Meier plot of relative time elapsed from diagnosis to the start of immunotherapy for G1 (blue) and G2 (red) patient cohorts, as indicated. Statistical comparison is shown by the long-rank test. Ns, no significant differences (P>0.05).*

Table 6. Association of CD4 T cell profiles with ECOG score

	CD4 T_{HD} profiles		Clinical responses	
ECOG score	**G1**	**G2**	**Objective responders**	**Progressors**
0-1	22	31	10	43
2-4	9	3	5	7
Association, P	0.05 ns		0.13 ns	

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Table 7. Association of CD4 T cell profiles with liver metastases

	CD4 T_{HD} profiles		Clinical responses	
Liver metastases	**G1**	**G2**	**Objective responders**	**Progressors**
Yes	5	11	3	13
No	26	23	12	37
Association, P	0.16 ns		0.74 ns	

Table 8. Association of CD4 T cell profiles with tumor load

	CD4 T_{HD} profiles		Clinical responses	
Affected organs	**G1**	**G2**	**Objective responders**	**Progressors**
</= 2	12	8	8	12
>/= 3	19	26	7	38
Association, P	0.28 ns		0.05ns	

Table 9. Association of CD4 T cell profiles with GRIm score

	CD4 T_{HD} profiles		Clinical responses	
GRImScore	**G1**	**G2**	**Objective responders**	**Progressors**
0-1	18	18	10	26
2-3	6	9	1	14
Association, P	0.55 ns		0.14 ns	

8.3. Combination of CD4 T_{HD} profiling and tumor PD-L1 positivity accurately identifies objective responders

Even though a baseline G1 CD4 T_{HD} cell profile was associated to objective responses to immunotherapy in our cohort of patients, ORR within G1 patients was still about 50%. PD-L1 tumor expression is the only approved biomarker available in clinical practice, and high PD-L1 scoring is associated with benefit from PD-L1/PD-1 blockade therapies in NSCLC patients (Borghaei et al. 2015). Therefore, we wondered whether there was an association between PD-L1 tumor expression and clinical responses in G1 and G2 patient cohorts for whom PD-L1 tumor expression values were available (N=50). Interestingly, the ORR in G1 patients with PD-L1 tumor positivity >5% increased up to 65%, with a 12-week PFS of 67% **(Figure 15A)**. Moreover, the mPFS was significantly longer compared to the remaining patients, which also included G1 patients with PD-L1<5% and all G2 patients regardless of their PD-L1 status (P=0.001) **(Figure 15A)**. The same benefit was observed

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when the stratification was extended to include patients with unknown PD-L1 tumor status in our cohort **(Figure 15B)**. In addition, G1 patients with the highest PD-L1 scores (PDL1>50%) showed an ORR of 75% and a 12-week PFS of 88% compared to the G1 cohort alone and G1 patients with PD-L1≤5% **(Figure 11D, 15A and 15C)**. Moreover, mPFS was significantly longer compared to the remaining patients (P=0.003) **(Figure 15C)**. Again, the same benefit was observed when patients with unknown PD-L1 tumor status were included in our cohort **(Figure 15D)**. In conclusion, within the G1 cohort tumor PD-L1 positivity identifies objective responders to anti-PD-L1/PD-1 immunotherapy more accurately.

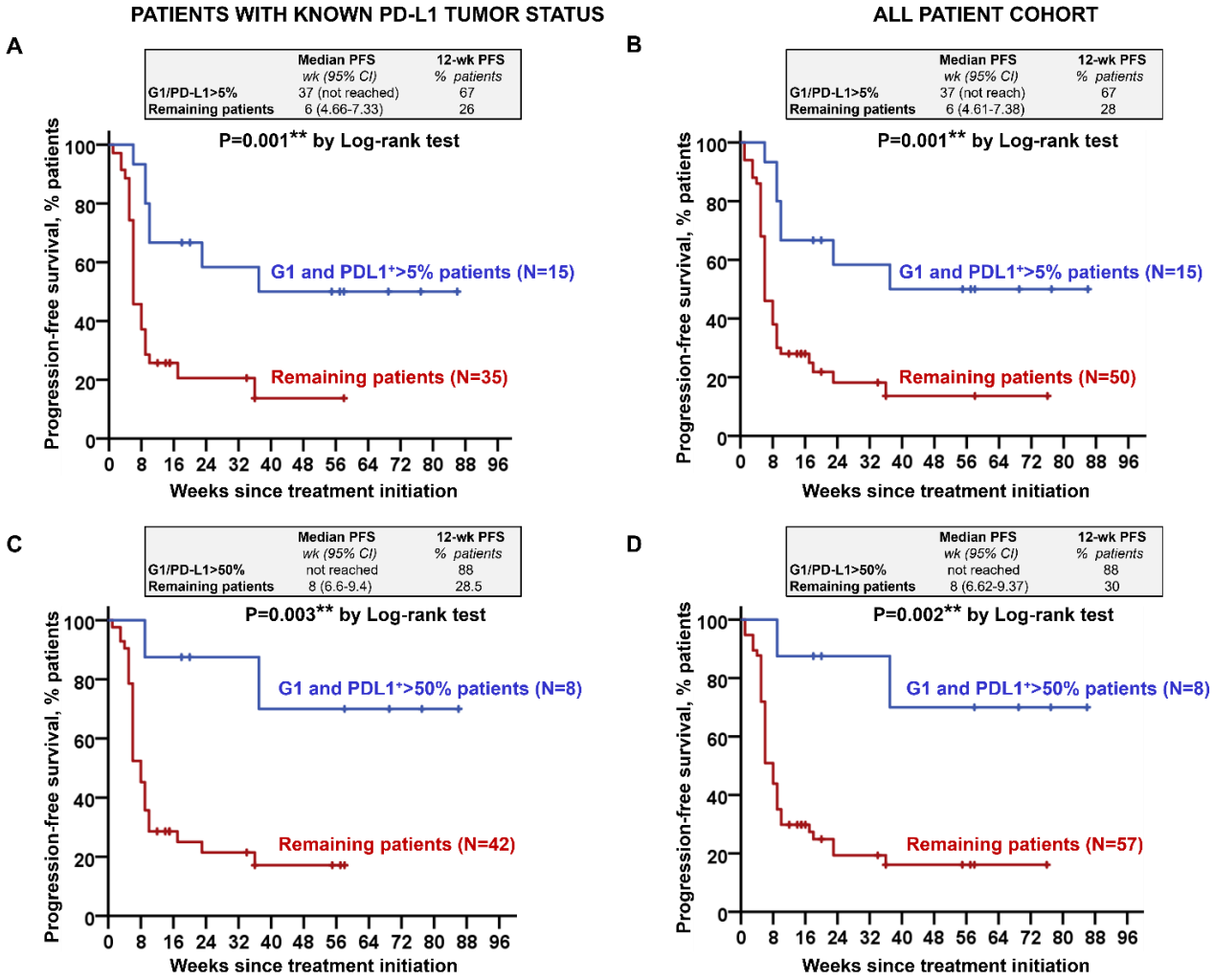

Figure 15. Objective responders to anti-PD-L1/PD-1 immunotherapy are found within G1 patients with PD-L1+ tumors. ***(A)*** *Kaplan-Meier plot of PFS in patients undergoing immune checkpoint inhibitor therapies stratified by G1/PD-L1> 5% tumors (blue) and remaining patients including G1 patients with PD-L1 low or negative tumors, and G2 patients with either PD-L1+ or PD-L1 negative tumors (red). In the gray square, mPFS and 12-week PFS values are shown. Long-rank test was used to test for statistical significance.* ***(B)*** *Same as in (A) but including all patients in the study cohort. Remaining patients (red) also included patients with unknown PD-L1 tumor status.* ***(C)*** *Same as (A) but in patients stratified by G1/PD-L1>50% tumors (blue) and remaining patients including G1 patients with PD-L1 low or negative tumors, and G2 patients with either PD-L1+ or PD-L1 negative tumors (red).* ***(D)*** *Same as in (C) but including all patients in the study cohort.*

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*Remaining patients (red) also included patients with unknown PD-L1 tumor status. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences.*

8.4. Baseline systemic cytokine profiles as potential discriminating factors between responders and progressors in our discovery cohort

Even though tumor PD-L1 positivity in combination with baseline CD4 T_{HD} cell profile identified most of the objective responders, determination of tumor PD-L1 expression in lung cancer patients is often inaccessible in clinical practice. In our cohort, we could not assess PD-L1 tumor expression in 23% of our patients. To look for other systemic markers that could be used in a simple way, systemic cytokine and chemokine profiles were analyzed in baseline sera from a selection of responders, progressors and healthy donors by a cytokine array panel of 80 immune-related cytokines and chemokines. A comparison between three independent responders and progressors uncovered four potentially differential cytokines including Fms-related tyrosine kinase 3 ligand (FLT3LG), brain-derived neurotrophic factor (BDNF), macrophage-derived chemokine (MDC) and vascular endothelial growth factor (VEGF). While FLT3LG (P=0.016) and VEGF (P=0.038) were significantly increased in baseline sera from responders, BDNF (P=0.024) and MDC (P=0.045) were increased in progressors **(Figure 16A-C).**

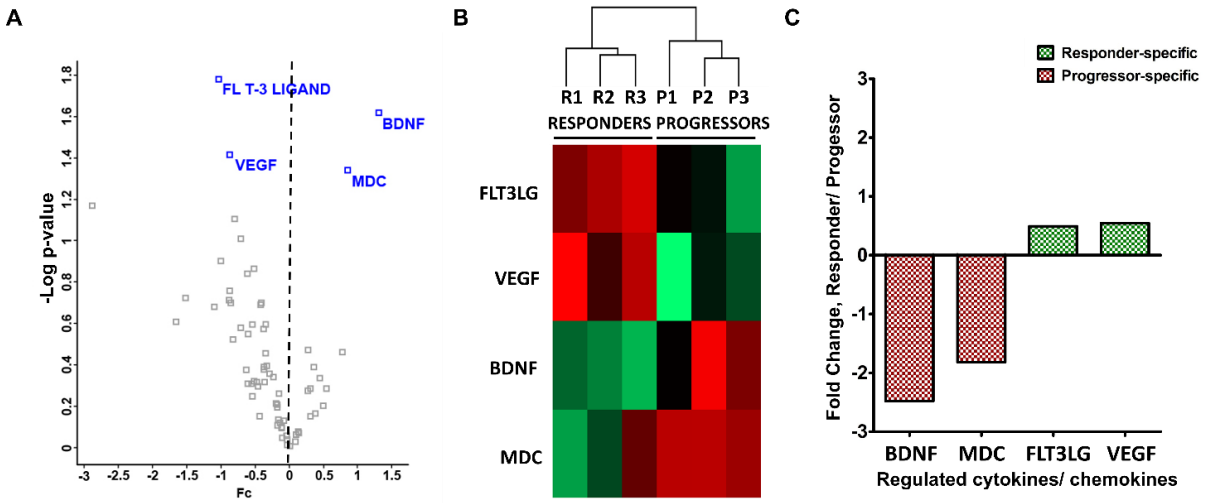

Figure 16. Differential systemic cytokine/chemokine profiles identified in baseline sera. (A) *Volcano plot representing the fold-change of identified cytokine/chemokine expression between 3 responders and 3 progressors with associated p-values. In blue, cytokines exhibiting significant changes* (-log p-value>1.2). *The vertical dotted line separates increased and decreased cytokines.* ***(B)*** *Heat map representation of the relative expression levels of the differential cytokines between responders and progressors, as indicated. In red, increased levels. In green, decreased levels.* ***(C)*** *Column graphs representing the fold change of the indicated cytokines differentially expressed in responders versus progressors. In green, changes specific for responders and in red for progressors.*

Results were validated by ELISA from sera of the entire patient cohort. Only FLT3LG and BDNF differentiated responders from progressors **(Figure 17A)**. In contrast to results in the cytokine array,

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VEGF tended to be increased in progressors compared to responders. We then hypothesized that G1 responders could be identified from progressors by quantification of FTL3LG and BDNF serum values. To that end, two validation studies were performed using independent lung cancer cohorts from Universidad Clínica de Navarra **(Figure 17B)** and the Gustave-Roussy Hospital **(Figure 17C)**. These results could not be validated using samples from these institutions **(Figure 17B-C).** In conclusion, although FLT3LG and BDNF are not useful as predictive biomarkers of PD-L1/PD-1 blockade responses, they might still play a role influencing systemic T cell immunity in lung cancer.

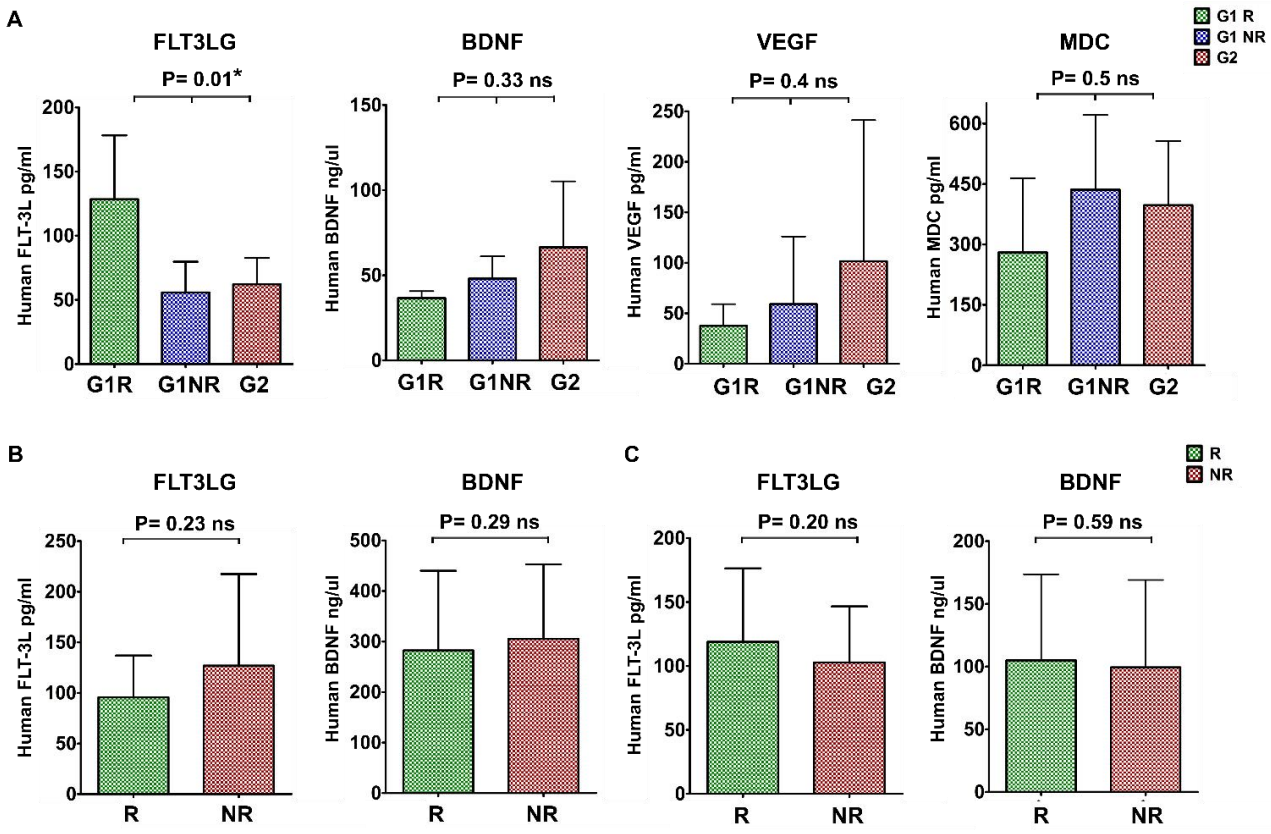

Figure 17. Validation studies of FTL3LG and BDNF serum values in independent patient cohorts. (A) *Column graphs representing the concentrations of FLT3LG (pg/ml), BDNF (ng/ml), VEGF (pg/ml), and MDC (pg/ml) in our patient cohort. Error bars correspond to standard deviations, from 28 biological replicates (patients). In green, objective responders (OR). In blue, G1 no responders. In red, G2 patients. Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis. Ns, no significant differences (P<0.05).* ***(B)*** *Same as in (A) but representing the concentration of FLT3LG (pg/ml) and BDNF (ng/ml) within the cohort of patients from Clínica Universidad de Navarra (N=45).* ***(C)*** *Same as in (B) but in the cohort of patients from the Gustave-Roussy Cancer Center (N=40). *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences. Ns, no significant differences (P<0.05).*

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9. FUNCTIONAL SYSTEMIC T CELL IMMUNITY IS REQUIRED FOR EFFICACY OF PD-L1/PD-1 BLOCKADE THERAPY IN NSCLC PATIENTS PROGRESSING FROM CONVENTIONAL THERAPIES

9.1. Functionality of systemic CD4 immunity before the start of immunotherapy discriminates clinical responses to PD-L1/PD-1 blockade therapy

We hypothesized that the relative percentage of CD4 T_{HD} cells was a biomarker for functional differences in systemic CD4 immunity between the two patient cohorts before the start of immunotherapies. To find out if this was the case, we first evaluated PD-1 expression in non-stimulated CD4 T cells directly after blood sampling. However, no differences were observed between G1 and G2 patient cohorts, or even with healthy age-matched donors **(Figure 18)**. We then tested if there were differences in PD-1 upregulation after *ex vivo* stimulation with lung cancer cells. As we did lack viable samples of autologous tumor cells from our patients, we decided to set up an *in vitro* assay that could replicate tumor antigen recognition by T cells. To this end, we engineered a T cell-stimulator cell line by expressing a membrane-bound anti-CD3 single-chain antibody in the surface of A549 human lung adenocarcinoma cells (A549-SC3 cells) using lentivectors. This genetically modified cell line stimulated T cells in co-cultures with the same affinity and specificity (given by the anti-CD3 single chain) while preserving other inhibitory interactions such as PD-L1/PD-1 or MHC II-LAG-3 **(Figure 19A-B).** This ensured the same standard assay for cancer cell-T cell recognition for each patient.

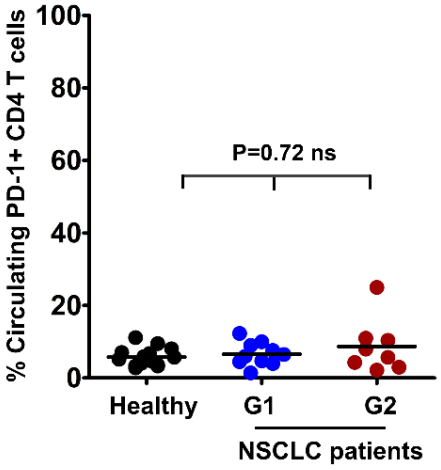

Figure 18. Circulating non-stimulated CD4 T cells from healthy donors and lung cancer patients express low levels of PD-1. *Scatter plot graph with the percentage of PD-1$^+$ CD4 circulating T cells from healthy donors (black), G1 (blue) and G2 (red) lung cancer patient cohorts. Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis. Ns, no significant differences (P>0.05).*

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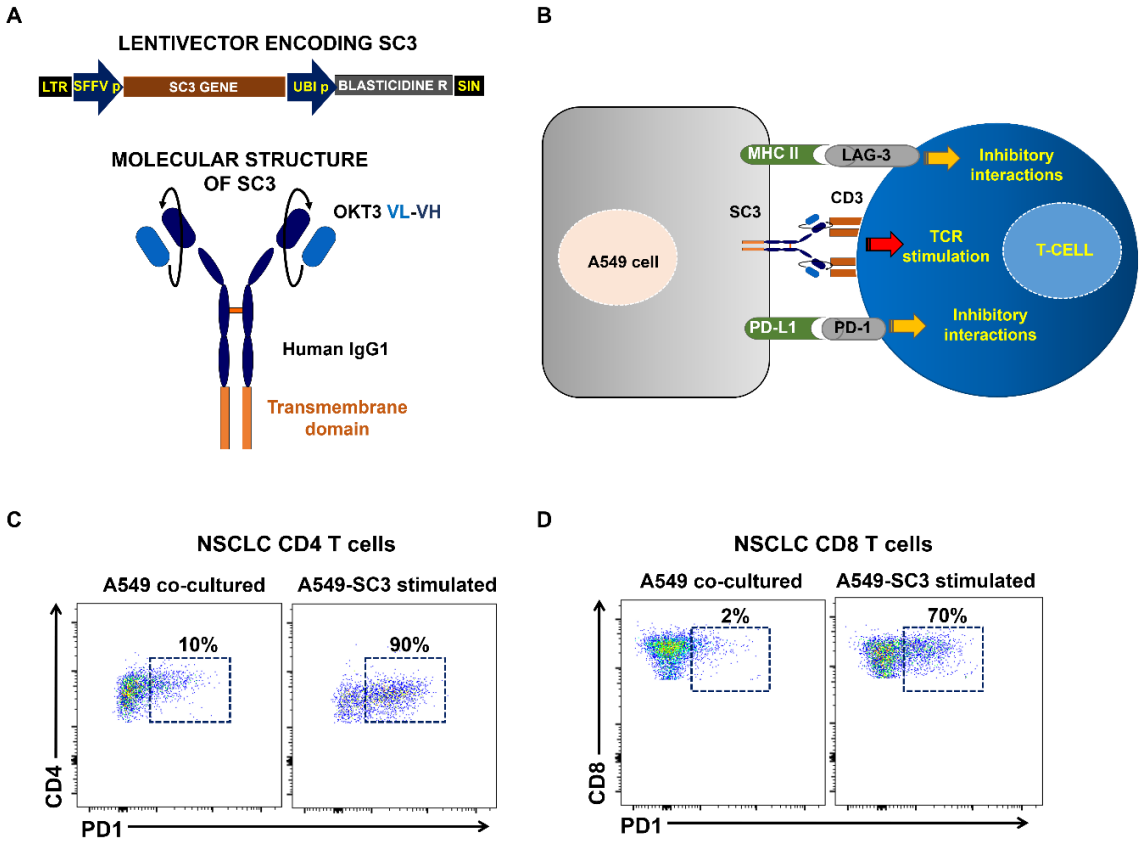

Figure 19. Ex vivo human lung adenocarcinoma T cell recognition system. (A) *Top, lentivector co-expressing an anti-CD3 single-chain antibody gene (SC3) and blasticidin resistance for selection. SFFVp, spleen focus-forming virus promoter; UBIp, human ubiquitin promoter; LTR, long terminal repeat; SIN, U3-deleted self-inactivating LTR. Bottom predicted molecular structure of the anti-CD3 SC3 molecule, which is anchored to the cell membrane by a transmembrane domain as indicated. OKT3 VL, variable region of the light chain from the anti-CD3 antibody OKT3; VH, variable region of the heavy chain from the anti-CD3 antibody OKT3.* ***(B)*** *Scheme of the cell-to-cell interactions mediated by the lentivector-modified A549 cell and T cells including SC3/CD3, PD-L1/PD-1, and MHC-II/LAG-3 interactions as indicated.* ***(C and D)*** *Representative flow cytometry density plots with the upregulation of PD-1 expression in CD4(C) and CD8 T cells (D) from NSCLC patients following co-incubation with A549-SC3 cell as indicated (right graph), or with unmodified A549 control (left graph). Dotted squares enclose PD-1$^+$ T cells which percentages are shown within the graphs.*

CD4 T cells from NSCLC patients significantly upregulated PD-1 compared to cells from age-matched healthy donors after incubation with A549-SC3 cells (P<0.001) **(Figure 19C and 20A)**. However, no differences were found between T cells obtained from patients of G1 and G2 cohorts. PD-1 and LAG-3 co-expression has been previously suggested to identify dysfunctional tumor-infiltrating lymphocytes in NSCLC (He et al. 2017). Interestingly, G2 donors presented a significantly higher percentage of CD4 T cells co-expressing both markers than G1 donors after stimulation of T cells with A549-SC3 cells **(Figure 20B)**. To test if there were also differences in proliferation, the percentage of Ki67$^+$ cells was compared after stimulation with A549-SC3 cells. Accordingly, CD4 T cells from G2 patients were remarkably impaired in proliferation after *ex vivo* activation with A549-SC3 cells compared to T cells from G1 patients **(Figure 20C and 20D)**. As we had observed that G1 and G2

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patient cohorts differed in baseline percentages of CD4 T_{HD} cells **(Figure 11A)**, we tested if this subset was responsive to activation by A549-SC3 cells. Interestingly, CD4 T_{HD} cells strongly proliferated in all patients, although they constituted a minority in the G2 patient cohort **(Figure 20D)**.

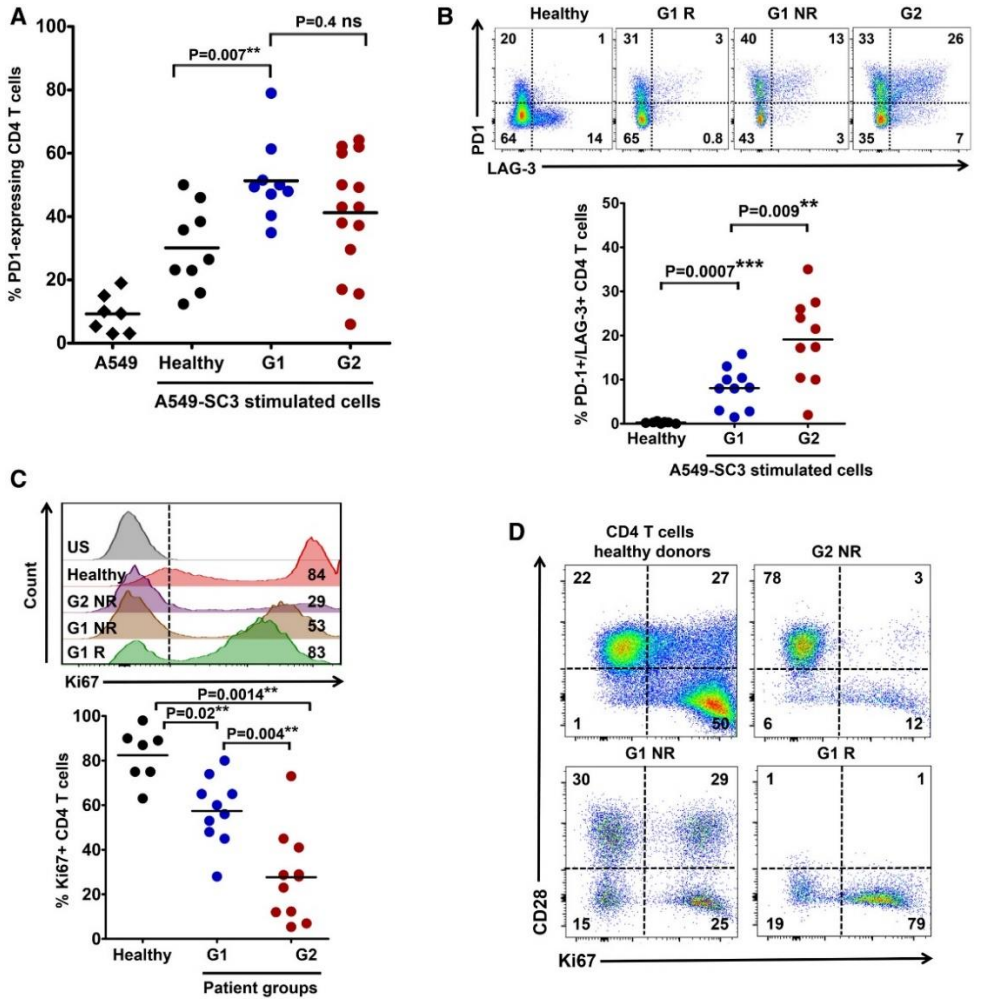

Figure 20. Functional differences in systemic CD4 immunity between the G1 and G2 patient cohorts. (A) *The scatter plot shows PD-1 expression after co-culture of CD4 T cells from healthy donors (n=9) or NSCLC patients (n=14), as indicated, with A549-SC3 lung cancer cells. Relevant statistical comparisons with the test of Mann–Whitney are indicated.* ***(B)*** *Upper graphs, flow cytometry density plots of PD-1 and LAG-3 co-expression in CD4 T cells from healthy donors, a G1 responder (G1R), a G1 non-responder (G1NR), and a G2 non-responder as indicated, following stimulation with A549-SC3 cells. Percentages of PD-1, LAG-3 and PD-1/LAG-3 expressing cells are indicated within each quadrant. Us, unstained control. Below, same as in the upper graphs but as a scatter plot of the percentage of CD4 T cells that simultaneously co-express PD-1 and LAG-3 in healthy donors (n=10), G1 (n=10) and G2 (n=10) patients. Relevant statistical comparisons are shown with the test of Mann–Whitney.* ***(C)*** *Upper flow cytometry histograms of Ki67 expression in CD4 T cells from the representative subjects as indicated on the left, after stimulation with A549-SC3 cells. Vertical dotted line indicates the cut-off value of positive versus negative Ki67 expression. The percentage of Ki67-expressing CD4 T cells is shown within the histograms. Below, same data represented as a scatter plot from a sample of healthy, G1 and G2 donors as indicated, with relevant statistical comparisons with the test of Mann–Whitney (n=7–10).* ***(D)*** *Flow cytometry density plots of proliferation marker Ki67 expression together with CD28 expression in CD4 T cells stimulated by A549-SC3 cells from healthy donor and the indicated patient groups. Percentages of cells within each quadrant are shown. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences. Ns, no significant differences (P>0.05).*

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The strong proliferative capacities of CD4 T_{HD} cells indicated that these were not exhausted, anergic or senescent subsets, but probably highly differentiated memory subsets. To test this, their baseline phenotype according to CD62L/CD45RA surface expression was assessed in a sample of patients. The majority of CD4 T_{HD} cells were central-memory ($CD45RA^{negative}$ $CD62L^{+}$) and effector-memory ($CD45RA^{negative}$ $CD62L^{negative}$) cells, without differences between G1 and G2 cohorts **(Figure 21A)**. Increased genotoxic damage is strongly associated to T cell senescence and can be evaluated by H2AX expression (Lanna et al. 2014). Interestingly, NSCLC CD4 T cells exhibited extensive genotoxic damage in both T_{HD} and non-T_{HD} subsets without differences between G1 and G2 patient cohorts, unlike T cells from age-matched healthy donors **(Figure 21B)**. Therefore, genotoxic damage did not identify senescent T cells in patients that had been treated with conventional therapies. Then, the expression of the replicative senescence marker CD57 was used to identify *bona fide* senescent T cells, which accounted to 30% of T_{HD} cells in healthy age-matched donors, and about 15% in NSCLC patients **(Figure 21C)**. Our results strongly suggested that circulating CD4 T_{HD} cells in our cohort of NSCLC patients mostly corresponded to non-senescent, non-exhausted memory subsets.

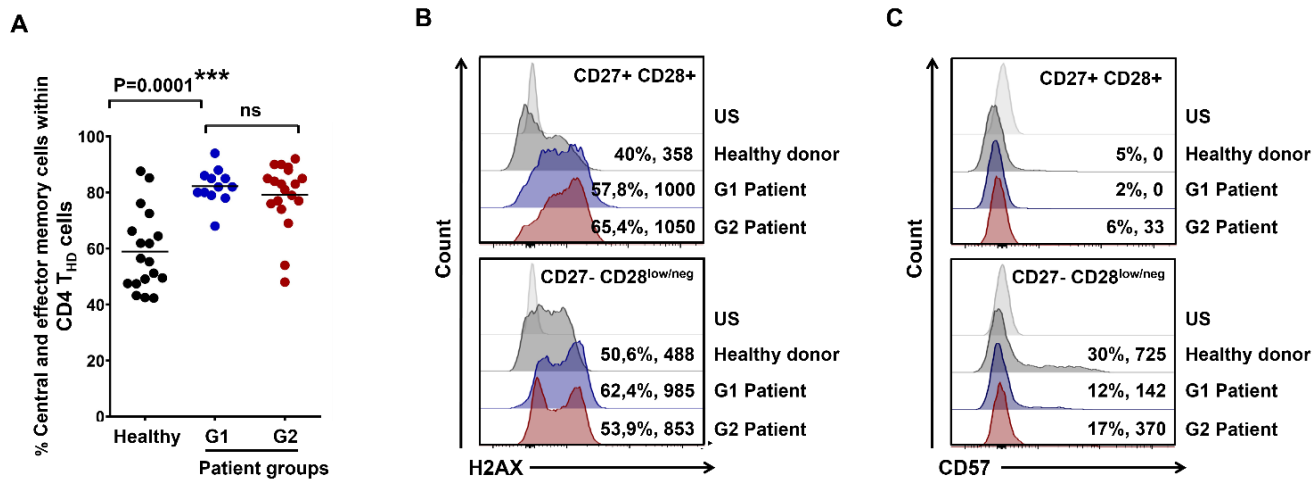

Figure 21. CD4 T_{HD} cells in NSCLC patients are mainly non-senescent memory subsets. (A) *Scatter plot graph of percentage of memory phenotypes in baseline CD4 T_{HD} cells according to CD62L-CD45RA expression (% $CD45RA^{negative}$ $CD62L^{+}$ central-memory + % $CD45RA^{negative}$ $CD42L^{negative}$ effector memory) in a sample of healthy donors (N=18), G1 (N=12) and G2 (N=19) patients. Relevant statistical comparisons are shown by one-way ANOVA followed by Tukey´s pairwise tests.* ***(B and C)*** *Expression of (B) the genotoxic damage marker H2AX and (C) replicative senescent marker CD57 by flow cytometry in CD4 T cell subsets from an aged-matched healthy donor, and NSCLC G1 and G2 patients as indicated. Percentage of positivity and mean fluorescent intensities are indicated for each population. Top, histogram analysis within $CD27^{+}$ $CD28^{+}$ (non-THD) CD4 T cells, and bottom, $CD27^{negative}$ $CD28^{low/negative}$ (T_{HD}) counterparts as indicated. Us, unstained control. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences. Ns, no significant differences (P>0.05).*

CD4 T cells of G2 patients strongly co-upregulated PD-1/LAG-3 after stimulation with A549-SC3 and exhibited impaired proliferative capacities. We wondered if lack of clinical responses in G2 patients

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could be explained by resistance to single blockade of PD-1, which could be likely caused by the co-expression of both immune checkpoints. Hence, proliferation of baseline CD4 T cells activated with A549-SC3 in the presence of an anti-PD-1 antibody equivalent to pembrolizumab was assessed (Scapin et al. 2015) **(Figure 22A)**. As expected, PD-1 blockade increased proliferation of non-T_{HD} and T_{HD} CD4 T cells in patients from the G1 cohort. In contrast, their G2 counterparts were largely refractory. To find out if CD4 T cells from G2 patients remained unresponsive to PD-1 blockade *in vivo*, cells were obtained from patients after at least three cycles of therapy and tested for their proliferative capacities. Systemic CD4 T cells from G2 patients remained poorly proliferative during immunotherapy **(Figure 22B).** In contrast, T cells from G1 patients, particularly from objective responders, maintained or even increased proliferative capacities during treatment **(Figure 22B)**.

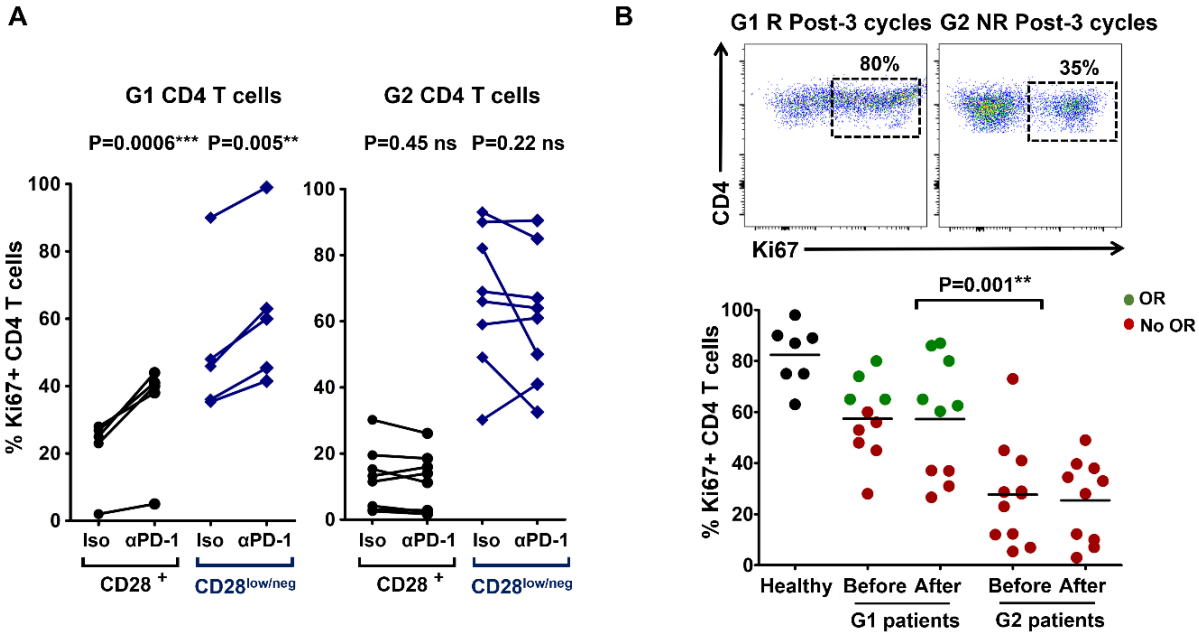

Figure 22. T cells of patients from the G2 cohort are refractory to PD-1 blockade ex vivo and in vivo. (A) *Proliferation of CD4 T cells stimulated by A549-SC3 cells from the indicated patient groups in the presence of an isotype control antibody or an anti-PD-1 antibody with the equivalent sequence to pembrolizumab. The effects on CD4 T cells from a G1 and a G2 patients are shown, divided into CD28^{+} or CD28 $^{low/negative}$ subsets as indicated. Relevant statistical comparisons are shown with paired Student's t-test.* ***(B)*** *Top, flow cytometry density plots of Ki67 expression in CD4 T cells from representative G1 or G2 patients after three cycles of therapy, activated by incubation with A549-SC3 cells. Below, same as above but as a dot-plot graph (n=7–10). A comparison between proliferating CD4 T cells before and after therapy is shown in unpaired patient samples from G1 and G2 patients. G1 R, G1 objective responder patient. G2 NR, G2 patient with no objective responses. In green, objective responders (OR). In red, no OR. Statistical comparisons were performed with the test of Mann–Whitney. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences. Ns, no significant differences (P>0.05).*

All together, these results indicated that pre-treatment functional systemic CD4 immunity observed in G1 cohort patients is required to achieve clinical responses to PD-L1/PD-1 blockade immunotherapy. In contrast, CD4 T cell dysfunctionality observed in G2 patients, which was reflected as high co-

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expression of PD-1/LAG-3 immune-checkpoint molecules and strongly impaired proliferative capacities after stimulation, resulted in a resistance to *ex vivo* and *in vivo* PD-L1/PD-1 blockade therapy.

9.2. Absence of lung cancer-specific CD4 T cells or systemic T cell exhaustion is not responsible for the lack of objective clinical responses to PD-L1/PD-1 blockade therapy

Then we thought that G2 patients could be refractory to anti-PD-1 immunotherapy by not having systemic cancer-specific CD4 T cells. To this end, we quantified CD4 T cells reactive to lung adenocarcinoma antigens using IFN-γ-activated autologous monocyte-derived DCs as antigen presenting cells, as described (Escors et al. 2008). DCs were loaded with A549 cell lysate, as these cells contain numerous common lung adenocarcinoma antigens (Madoz-Gúrpide et al. 2008). We used this approach as we lacked enough biopsy material to obtain tumor antigens or tumor-infiltrating T cells from patients. CD4 T cells reactive to A549 cell antigens were identified by IFN-γ upregulation. Interestingly, lung cancer specific CD4 T cells were present at varying proportions before the start of immunotherapy in both G1 and G2 patients **(Figure 23A)**. Indeed, percentages of lung cancer specific CD4 T cells did not differ significantly between G1 (responders and non-responders) and G2 patients. These T cells consisted of both T_{HD} and non-T_{HD} subsets, without significant differences in relative percentages between G1 and G2 cohorts in our cohort **(Figure 23B)**. These results suggested that poor responses in G2 patients were not caused by lack of tumor specific CD4 T cells but rather by having dysfunctional T cells.

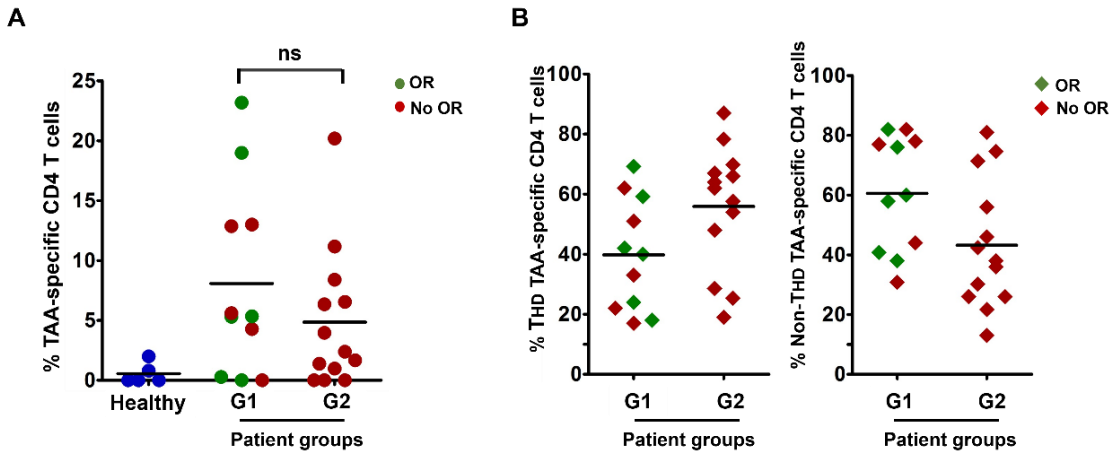

Figure 23. Lung cancer antigen specific CD4 T cells in NSCLC patients. (A) *Scatter plot graph with the percentage of lung cancer-specific systemic CD4 T cells quantified by an autologous DC-based antigen presentation assay in a sample of healthy donors (n = 5), G1 (n = 11) and G2 (n = 13) patients, as indicated. Objective responders (OR) are shown in green. In red, patients with no OR. Relevant statistical comparisons are shown within the graphs with the test of Mann–Whitney. Ns, no significant differences (P<0.05).* ***(B)*** *The scatter plot graph on the left represents the percentage of CD4 T_{HD} cells within lung cancer specific CD4 T cells in a sample of patients from the indicated G1 and G2 groups. On the right, same as left but representing the*

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percentage of non-T_{HD} ($CD28^+$) CD4 T cells within lung cancer specific CD4 T cells. Objective responders (OR) are shown in green. In red, patients with no OR.

To get further insight into the dysfunctional status of systemic CD4 T cells in G2 patients, we evaluated PD-1 and LAG-3 surface expression directly after blood sampling, as constitutive high-level co-expression of these markers is a frequent characteristic of T cell exhaustion. Low PD-1/LAG-3 co-expression levels were observed in circulating non-stimulated CD4 T cells from both patient groups in either non-T_{HD} or T_{HD} subsets **(Figure 24)**. Moreover, no differences were found between age-matched healthy donors and G1/G2 patient cohorts **(Figure 24).** Nevertheless, the defining hallmark of T cell exhaustion is the loss of cytokine production following stimulation, particularly multi-cytokine expression (Crawford et al. 2014). Interestingly, CD4 T cells from both G1 and G2 patient cohorts were as proficient in IFN-γ, IL-4, IL-10 and IL-2 expression as T cells from healthy donors independently of their CD28 expression **(Figure 25A)**. Indeed, CD4 cells (total, non-T_{HD} and T_{HD} subsets) in both G1 and G2 patient cohorts were significantly skewed towards IL-17 responses compared to age-matched healthy donors **(Figure 25A)**. Importantly, only a minority of CD4 T cells from either G1 or G2 patient groups were single-cytokine producers **(Figure 25B)** while most of the non-T_{HD} CD4 T cells were very proficient in multiple cytokine production with a preference for IL-17-expressing subsets **(Figure 25C-D)**. These results indicated that the majority of CD4 T cells from G2 patients were not exhausted according to our current understanding (Hashimoto et al. 2018). Indeed, they responded to stimulation by producing cytokines although with strong co-upregulation of PD-1/LAG-3 associated with markedly diminished proliferative capacities.

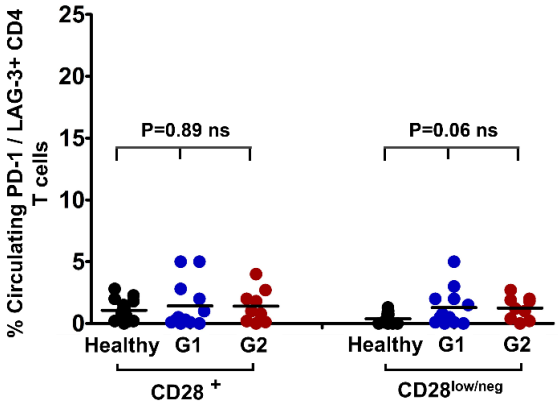

Figure 24. Circulating unstimulated CD4 T cells from healthy donors and lung cancer patients co-express low levels of PD-1 and LAG-3 molecules. *Scatter plot graph with the percentage of $PD\text{-}1^+/LAG\text{-}3^+$ co-expressing CD4 circulating T cells from healthy donors (black), G1 (blue) and G2 (red) patients divided into $CD28^+$ or $CD28^{low/negative}$ subsets. Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis. Ns, no significant differences ($P<0.05$).*

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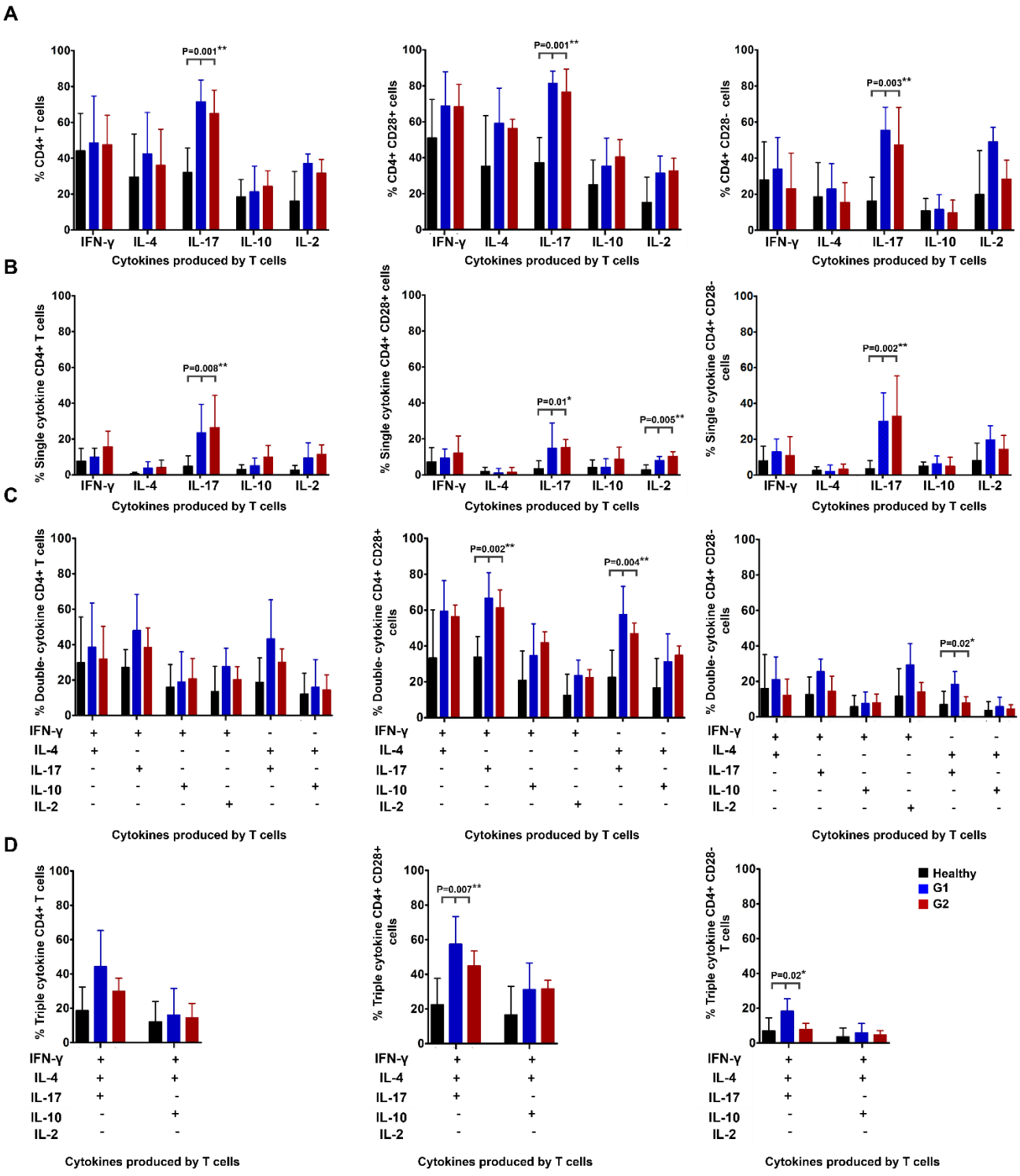

Figure 25. Systemic circulating CD4 T cells in NSCLC patients are proficient in cytokine production with an overall Th17 profile. (A)*Column graphs representing the percentage of CD4 T cells from NSCLC patients (G1 in blue, G2 in red) or age-matched healthy donors (in black) as represented in the graph, expressing the indicated cytokines after T cell stimulation with anti-CD3/anti-CD28 antibodies. Data on total CD4 (left graph), CD28^{+} subsets (center graph) and CD28- subsets (right graph) are shown. Error bars correspond to standard deviations, and bars represent means from 9 independent biological replicates (healthy donors) and 6 independent replicates (patients). Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis.* ***(B)(C)(D)*** *Same as in (A) but representing CD4 T cells expressing only one cytokine (B), two (C) or three cytokines simultaneously (D). Error bars correspond to standard deviations, and bars represent*

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*means from 6 independent biological replicates (patients). Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences.*

9.3. Systemic CD8 immunity recovers in objective responder patients from the G1 cohort undergoing PD-L1/PD-1 immunotherapy

Interestingly, CD8 T cells from both G1 and G2 patient cohorts obtained before the start of immunotherapies failed to proliferate after stimulation by A549-SC3 cells **(Figure 26A)**. To test if anti-PD-1 therapy could recover CD8 dysfunctionality *in vivo*, the proliferative capacities of CD8 T cells from G1 and G2 patients obtained after at least 3 cycles of treatment were evaluated *in vivo* by stimulation with A549-SC3 cells. CD8 T cells from G1 responders had recovered significant proliferative capacities, while only limited enhancements were observed in G2 patients **(Figure 26B)**. Similarly to CD4 cells, systemic CD8 T cells specific for lung adenocarcinoma antigens were quantified in G1 and G2 patients, and found to be comparable **(Figure 26C)** and distributed within non-T_{HD} and T_{HD} subsets **(Figure 26D).**

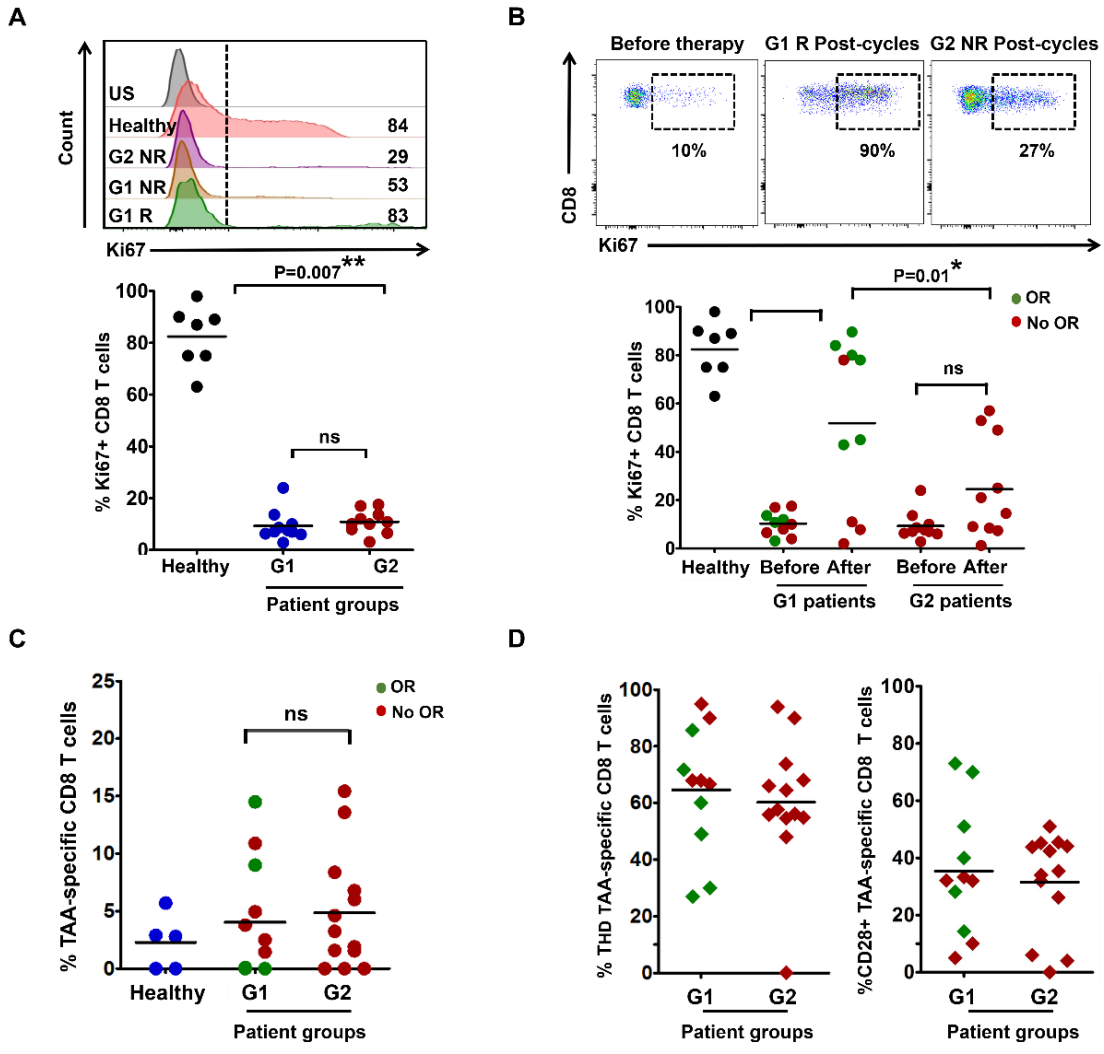

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Figure 26. CD8 dysfunctionality recovers in G1 patients undergoing anti-PD-L1/PD-1 immunotherapy. (A) *Upper flow cytometry histograms, expression of the proliferation marker Ki67 in CD8 T cells from the indicated patients or healthy donor before the start of immunotherapy, stimulated ex vivo by A549-SC3 cells. Numbers indicate mean fluorescence intensities. G1R and G1NR, responder and non-responder G1 patient, respectively; G2NR, non-responder G2 patient. US, unstained control. Below, same as above but as a dot plot graph with percentage of proliferating Ki67$^+$CD8 T cells from the indicated groups (n=7–10). Relevant statistical comparisons are shown with the test of Mann–Whitney.* ***(B)*** *Upper flow cytometry density plots, expression of Ki67 in ex vivo-stimulated CD8T cells from the indicated patients before and after the start of immunotherapies. NR, non-responder patient; R, responder patient. Below, dot-plots of the percentage of Ki67$^+$ proliferating CD8 T cells after ex vivo activation by A549-SC3 cells. CD8 T cells were obtained from samples of G1 or G2 patients before immunotherapy and after three cycles of anti-PD-1 therapy (n=7–10). Relevant statistical comparisons are shown with the test of Mann–Whitney. Green, objective responders (OR) and red, no ORs.* ***(C)*** *Dot-plot of lung cancer antigen-specific CD8 T cells obtained before the start of immunotherapies and stimulated with A549-loaded autologous DCs in healthy donors (n = 5), G1 (n = 11) and G2 (n = 13) patients, as indicated. Relevant statistical comparisons are indicated by the test of Kruskal–Wallis. Ns, no significant differences (P>0.05).* ***(D)*** *Left dot-plot, percentage of CD28-negative CD8 T cells within TAA-specific CD8 subsets in G1 (n = 11) and G2 (n = 13) patients, as indicated. Right dot-plot, same as left but with CD28-positive subsets. Green, objective responders (OR). Red, no OR. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences.*

To find out whether CD8 T cells in G1 patients in contrast to those from G2 patients were more susceptible to PD-1 blockade *ex vivo*, baseline samples of CD8 T cells from G1 and G2 patients were activated with A549-SC3 cells in the presence of an anti-PD-1 antibody (molecularly equivalent to pembrolizumab) or an isotype control. In agreement with the *in vivo* results, *ex vivo* PD-1 blockade improved significantly the proliferation of CD8 T cells from G1 patients, and especially non-T_{HD} subsets **(Figure 27A**). Recent studies have demonstrated the requirement of CD28 co-stimulation for effective responses to anti-PD-L1/PD-1 therapy. Particularly, *in vivo* expansion of CD28$^+$ CD8 T cells in murine models correlate with anti-PD-1 efficacy (Kamphorst, Wieland, et al. 2017). To confirm this observation in our cohort of patients, the changes in the relative abundance of CD8$^+$ CD28$^+$ T cells were compared in G1 and G2 patients from baseline to post-anti-PD-L1/PD-1 therapy. Accordingly, the CD28$^+$ CD8 T cell compartment significantly expanded (P<0.001) only in G1 patients **(Figure 27B)**.

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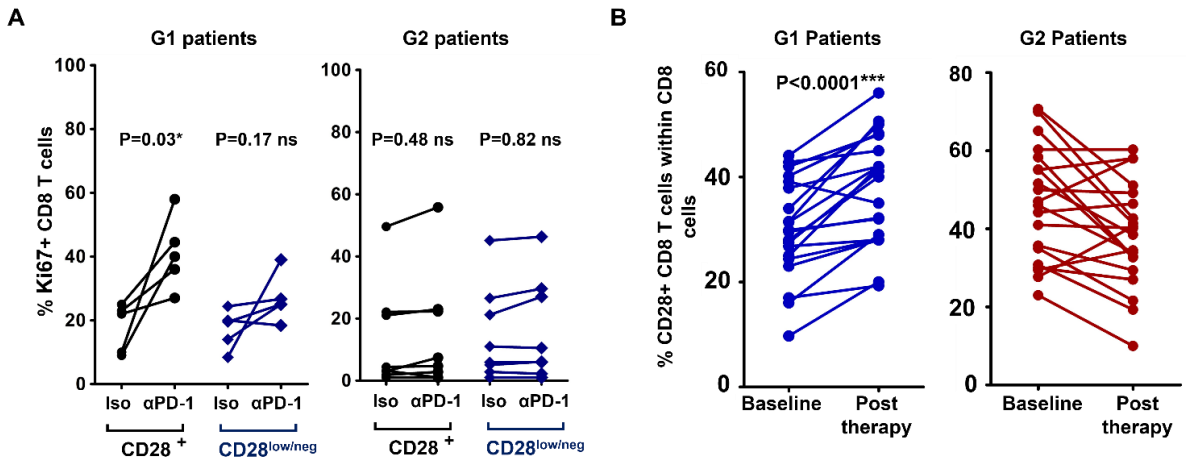

Figure 27. CD28$^+$ CD8 T cell subset significantly expanded ex vivo and in vivo after anti-PD-L1/PD-1 therapy in objective responders. ***(A)*** *Proliferation of CD8 T cells stimulated by A549-SC3 cells from the indicated patient groups in the presence of an isotype control antibody or an anti-PD-1 antibody with the equivalent primary sequence to pembrolizumab. The effects on CD8 T cells from a G1 and a G2 patients are shown, divided into CD28$^+$ or CD28 $^{low/negative}$ subsets as indicated. Relevant statistical comparisons are shown with paired Student's t-test.* ***(B)*** *Change in percentage of CD28$^+$ CD8 T cells from baseline to post-therapy in G1 patients (left) or in G2 patients (right). Statistical comparisons were carried out with paired Student's t-test. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences. Ns, no significant differences (P>0.05).*

We wondered whether proliferative dysfunctionality and resistance to anti-PD-1 monotherapy observed in baseline CD8 T cells from G2 patients correlated with high PD-1/LAG-3 co-upregulation after activation, as we had found for CD4 T cells. PD-1/LAG-3 co-expression was tested *ex vivo* after stimulation with A549-SC3 cells, and G2 patients presented a significantly higher proportion of PD-1/LAG-3 co-expressing CD8 T cells in both T_{HD} and non-T_{HD} subsets compared to G1 counterparts **(Figure 28A)**. Indeed, PD-1/LAG-3 co-upregulation in CD8 T cells was associated with resistance to *ex vivo* and *in vivo* anti-PD-L1/PD-1 blockade therapy. Moreover, as for CD4 T cells, CD8 T cells from both G1 and G2 patient cohorts were as proficient in IFN-γ and IL-2 cytokine production after stimulation as T cells from healthy donors independently of their CD28 expression, indicating that they were neither exhausted nor anergic **(Figure 28B-C)**. Hence, CD8 T cells from G2 patients responded to stimulation by producing cytokines although with strong co-upregulation of PD-1/LAG-3 associated with resistance to anti-PD-L1/PD-1 monotherapy.

All together, these results indicated that baseline CD8 proliferative dysfunctionality observed in both patient groups was only recovered by PD-L1/PD-1 blockade therapy in G1 patients if their baseline CD4 T cell immunity was functional in proliferation.

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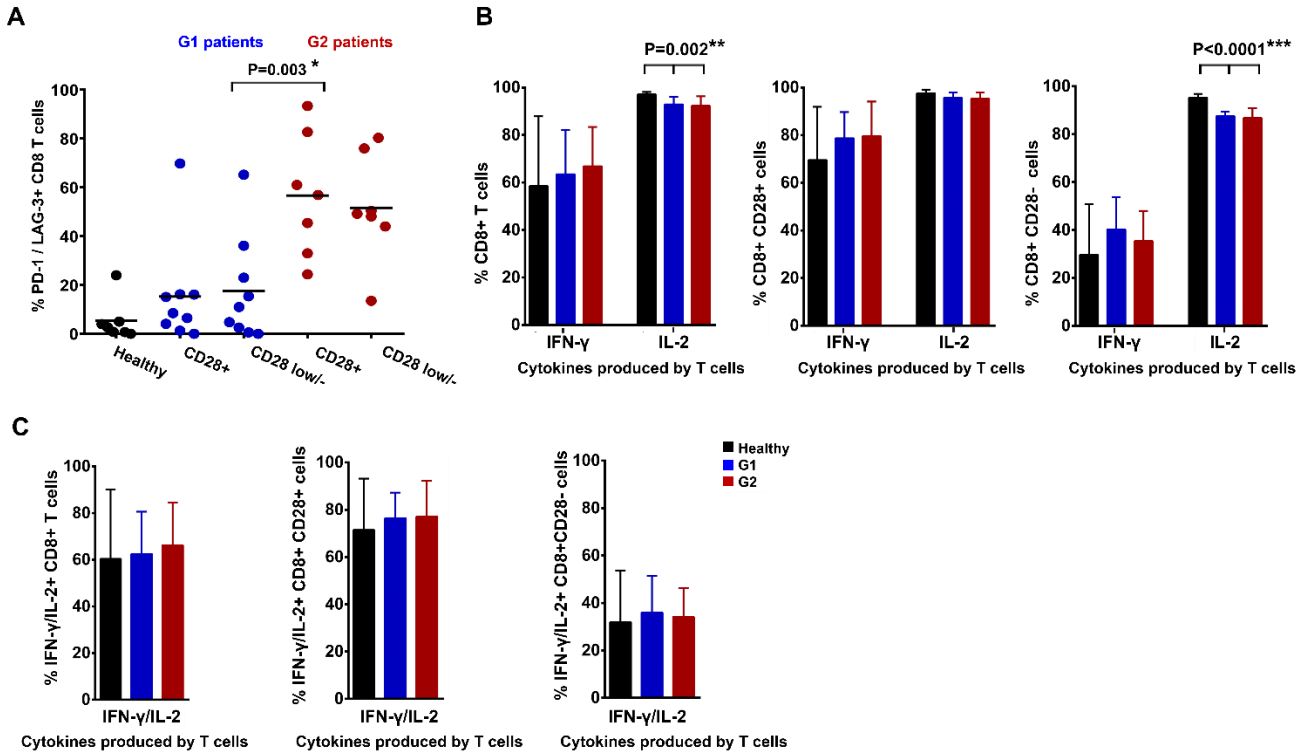

Figure 28. Systemic CD8 T cells from G2 patients are proficient in IFN-γ and IL-2 cytokine production after stimulation with strong co-upregulation of PD-1/LAG-3. (A) *Scatter plots of the percentage of PD-1/LAG-3 co-expressing CD8 T cells within CD8 cells after activation by A549-SC3 cells in a sample of G1 (n=9) and G2 (n=7) patients, in CD28$^+$ and CD28- populations as indicated in the figure. Relevant statistical comparisons are shown with the test of Kruskal-Wallis.* ***(B)*** *Column graphs representing the percentage of CD8 T cells from G1 (in blue) and G2 (in red) NSCLC patients or age-matched healthy donors (in black) as shown in the graph, expressing the indicated cytokines after T cell stimulation with anti-CD3/anti-CD28 antibodies. Data on total CD8 (left graph), CD28$^+$ subsets (center graph) and CD28- subsets (right graph) are shown. Error bars correspond to standard deviations, and bars represent means from 9 independent biological replicates (healthy donors) and 6 independent replicates (patients). Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis.* ***(C)*** *Same as in (B) but representing CD8 T cells expressing IFN-γ and IL-2 cytokines simultaneously. Error bars correspond to standard deviations, and bars represent means from 6 independent biological replicates (patients). Relevant statistical comparisons are shown by the test of Kruskal–Wallis. *, **, *** in the figures indicate significant (P<0.05), very significant (P<0.01) and highly significant (P<0.001) statistical differences.*

9.4. Proliferative dysfunctionality of CD4 and CD8 T cells from G2 patients is partially reversible *ex vivo* after PD-1/LAG-3 dual blockade

Overall, our data indicated that PD-1/LAG-3 co-upregulation was contributing to proliferative dysfunctionality in both CD4 and CD8 populations and possibly to resistance to anti-PD-L1/PD-1 blockade monotherapy. We hypothesized that blocking both signalling axes might restore T cell dysfunctionality, and potentially the resistance to anti-PD-L1/PD-1 therapy observed in G2 patients. To test if this was the case, baseline samples of CD4 and CD8 T cells from G2 patients were co-incubated *ex vivo* with A549-SC3 cells in the presence of an isotype antibody control, anti-PD-1, anti-LAG-3 or anti-PD-1/anti-LAG-3 antibodies simultaneously. We confirmed that each antibody was

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specifically blocking PD-1, LAG-3 or both in our assays by epitope masking using flow cytometry **(Figure 29A).** Only co-blockade of PD-1 and LAG-3 in both CD4 **(Figure 29B)** and CD8 T cells **(Figure 29C)** from G2 patients significantly increased proliferation independently of CD28 expression. These results confirmed that PD-1/LAG-3 co-upregulation contributed to keeping systemic CD4 and CD8 T cells from G2 patients in a proliferative dysfunctional state following stimulation, and that this T cell dysfunctionality can be reverted by co-blockade of both immune checkpoints.

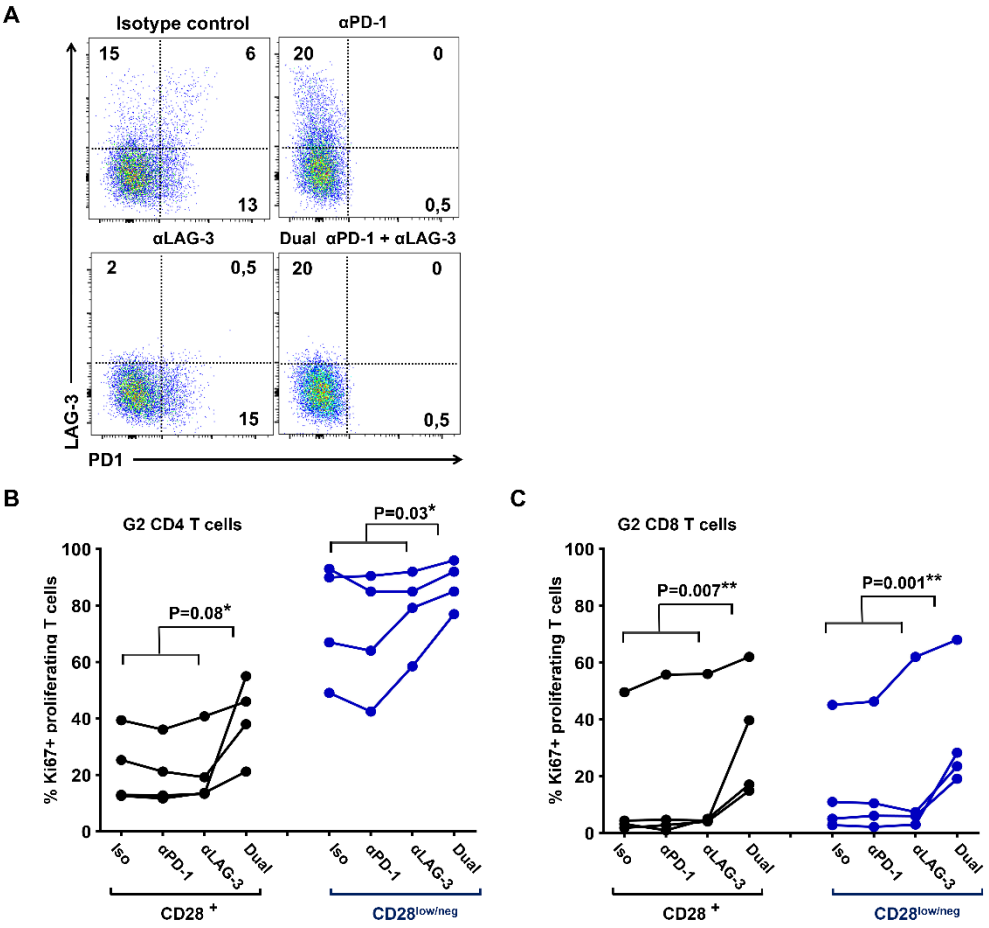

Figure 29. PD-1/LAG-3 co-blockade recovers proliferative capacities of CD4 and CD8 T cells from G2 patients. (A) *Flow cytometry density plots of PD-1, LAG-3 and PD-1/LAG-3 co-expression in T cells from a selected NSCLC patient following stimulation with A549-SC3 cells in the presence of irrelevant isotype, anti-PD-1, anti-LAG-3 and the anti-PD-1/anti-LAG-3 combination (dual). Percentage of expressing cells is indicated within each quadrant.* ***(B)(C)*** *Dot-plot representing the percentage of proliferating (B) CD4 T cells and (C) CD8 T cells from a sample of G2 patients before starting immunotherapy, activated ex vivo by A549-SC3 cells in the presence of the indicated antibodies. Data from CD28^{+} and CD28 $^{low/negative}$ subsets are shown as indicated. Appropriate statistical comparisons are shown within the graph with two-way paired ANOVA, followed by Tukey´s a posteriori pairwise tests.*

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DISCUSSION AND FUTURE PERSPECTIVES

In the last decade, PD-L1/PD-1 blockade immunotherapy in advanced NSCLC patients has significantly improved duration of responses and long-term survival with limited adverse effects compared to conventional cytotoxic treatments. Unlike the latter, PD-L1/PD-1 inhibitors target both tumor and the host immune system by interfering with immunosuppressive interactions which prevent immune system recognition of cancer cells and their efficient elimination. However, the complex and often individual-dependent crosstalk between tumor and the immune system results in heterogeneous clinical responses among patients and cancer types. In the case of NSCLC patients progressing from cytotoxic agents, there is still a high percentage of patients intrinsically refractory to anti-PD-L1/PD-1 antibodies with a high risk of developing HPD. In addition, the high costs of these treatments reinforce the urgent need for patient selection. To overcome all these limitations, extensive efforts are being put into the identification of predictive biomarkers for an adequate patient selection, coupled to better understanding of the mechanisms of resistance. Tumor PD-L1 expression is the only approved predictive biomarker but its utility is frequently under question. So far not a single factor has been associated with objective responses or progression. This is not surprising due to the wide range of tumor-intrinsic and extrinsic mechanisms which might be influencing clinical responses to PD-L1/PD-1 blockade immunotherapy (Sharma et al. 2017).

Most of the research is centered on the immunological status of the tumor microenvironment as a major driving force for the efficacy of PD-L1/PD-1 blockade. Investigation into tumor mutational and neoantigen burden, TAA-specific TIL repertoire, presence of effector T cell responses and immune suppressive mechanisms are pursued to identify tumors susceptible to anti-PD-L1/PD-1 treatments. However, such studies require expensive techniques which are not currently present in clinical practice, while large biopsy materials can be difficult to obtain for many tumor types. Moreover, it is often ignored the fact that PD-L1/PD-1 inhibitors are systemically administrated and consequently they have an important impact on systemic immunity. Indeed, several studies have demonstrated that these therapies cause systemic changes in immune cell populations which can be correlated with clinical efficacy (Kamphorst, Pillai, et al. 2017)(Krieg et al. 2018)(K. H. Kim et al. 2019). Therefore, systemic responses unleashed by immunotherapy probably have a significant anti-tumor effect. A growing number of studies are analyzing baseline immunological characteristics and their impact on immunotherapy efficacy. However, results are most of the times difficult to interpret possibly due to the high complexity and variety of systemic immune variables. Here, we analyzed systemic T cell profiles before PD-L1/PD-1 treatment initiation in a cohort of advanced NSCLC patients progressing

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from cytotoxic treatments. In contrast to other similar studies, the analyses were carried out from fresh blood samples because we observed that freezing PBMCs led to altered expression patterns of immune cell surface markers. Hence, sample manipulation had a significant impact on our results, which limited our study to prospective data. We found that baseline relative percentages of CD4 T_{HD} cells in our cohort stratified patients into two groups with differential clinical outcomes. ROC analysis provided a cut-off value of > 40% CD4 T_{HD} to predict responses to PD-L1/PD-1 blockade. Indeed, patients with baseline CD4 T_{HD} > 40% (G1 cohort) contained all the objective responses, while no objective responders and hyperprogressors were found in patients with baseline CD4 T_{HD} < 40% (G2 cohort) within our cohort study. In addition, when PD-L1 high tumor positivity was combined with CD4 T_{HD} values, G1 patients with high PD-L1-tumor expression exhibited response rates of 75%. Apart from providing a potential predictive biomarker, our results strongly suggested that pre-treatment systemic CD4 T cell immunity might be playing a crucial role in the efficacy of PD-L1/PD-1 blockade in lung cancer patients. Nevertheless, it has to be stressed that our cohort under study was restricted to patients heavily pre-treated before starting immunotherapies and not to immunotherapies as first line treatment.

Although several blood-based potential predictive biomarkers of responses to PD-L1/PD-1 blockade therapy are constantly emerging, most of them are either difficult to validate and implement in clinical routine or have rather a prognostic value (Krieg et al. 2018)(Takahashi et al. 2016). Therefore, the baseline quantification of CD4 T_{HD} cells from routine small blood samples represents a promising non-invasive biomarker. This biomarker could be used to identify patients with primary resistance to PD-L1/PD-1 blockade and high risk of developing hyperprogressive disease. On the other hand, potential responders in combination to tumor PD-L1 expression can be accurately identified. Hence, its strong predictive value demonstrated by the ROC analysis together with the low-cost and easy applicability in clinical practice makes it a very promising potential predictive biomarker. However, independent validation studies will be required with proper protocol standardization to guarantee its reliability.

Our data already suggested that baseline systemic CD4 immunity could have a significant impact on PD-L1/PD-1 blockade therapy efficacy. $CD27^{-}$ $CD28^{low/negative}$ CD4 T cells are usually described as terminally differentiated replicative senescent T cells. Interestingly, in our study CD4 T_{HD} cells from patients corresponded to central and effector memory subsets with highly proliferative capacities which did not express classical markers for replicative senescence. This opens the question of whether the reduced expression of CD27 and CD28 is a true hallmark of T cell senescence, at least in the context of cancer. Moreover, this subset probably represents a pre-existing repertoire of antigen-

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specific memory T cells with potential anti-tumor capacities. However, we did not find major baseline differences in the proportion of these T cells between responders and non-responders at least in our cohort. Therefore, the experimental evidence pointed to the baseline intrinsic functionality of CD4 immunity as the most influential factor in our study.

In agreement with our hypothesis, we found that proliferative functionality of systemic CD4 immunity was the baseline differential factor between objective responders and non-responders to PD-L1/PD-1 blockade therapy. Patients from the G1 cohort thus had a high degree of functionality in baseline systemic CD4 T cells, while G2 patients exhibited a strong baseline CD4 T cell dysfunctionality. Such dysfunctionality was reflected as strongly impaired proliferation capacities after stimulation, high co-expression of LAG-3/PD-1, and resistance to *ex vivo* and *in vivo* PD-1 monoblockade. Surprisingly, it was not a consequence of tumor-induced exhaustion or anergy as systemic $CD28^+$ CD4 T cells in G2 or in G1 patients were not truly exhausted or *bona fide* anergic T cells. No constitutive high-level co-expression of PD-1 and LAG-3 was observed unless stimulated, and T cells were proficient in multi-cytokine expression following stimulation but skewed toward Th17-expressing phenotypes compared to healthy donors. All these characteristics were indicators of systemic CD4 T cell proliferative dysfunctionality in G2 patients. Thus, the lower proportion of systemic CD4 T_{HD} observed in G2 patients might be a direct consequence of the intrinsic CD4 proliferative dysfunctionality which might prevent the expansion of memory subsets after antigen re-encounter.

PD-L1/PD-1 therapy aims to recover CD8 cytotoxic responses (Ahmadzadeh et al. 2009). To our surprise, all lung cancer patients in our cohort showed dysfunctional CD8 immunity as assessed by the same techniques described above for CD4 T cells. Nevertheless, the proliferative capacities of CD8 T cells were recovered during immunotherapy but only in patients with functional proliferative CD4 immunity. This was reflected by an expansion of $CD28^+$ CD8 cells in agreement with previous published data in murine models (Kamphorst, Wieland, et al. 2017). The requirement of functional systemic immunity has been previously demonstrated in murine models for the efficacy of other immunotherapy approaches (Spitzer et al. 2017) as well as the importance of CD4 T cells for anti-PD-1 immunotherapy (Markowitz et al. 2018). These evidences together with our data in human patients strongly support the need for proficient systemic CD4 immunity to achieve efficacious clinical responses. Hence, CD4 T cells might be coordinating the reversion of CD8 T dysfunctionality by PD-L1/PD-1 blockade therapy. In addition, CD8 dysfunctionality in G2 patients was again correlated with a higher proportion of CD8 T cells co-expressing PD-1/LAG-3. An increasing number of studies are linking PD-1/LAG-3 co-expression in T cells to resistance to anti-PD-L1/PD-1 therapies (Mishra et

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al. 2016)(R. Y. Huang et al. 2017)(Williams et al. 2017)(Johnson et al. 2018). Hence, both CD4 and CD8 proliferative dysfunctionality in G2 patients were reversible *ex vivo* by PD-1/LAG-3 co-blockade, confirming that PD-1/LAG-3 co-expression was a contributor to T cell dysfunctionality in G2 patient cohort. These results provide a strong rationale for the combination of PD-L1/PD-1 and LAG-3 blockade therapies in patients exhibiting baseline CD4 T cell dysfunctionality.

Nevertheless, the restoration of CD8 proliferative capacities in G1 patients was not linked to PD-1/LAG-3 co-expression, as these cells also showed high expression levels of these two immune checkpoints. This result suggests that other factors affect CD8 immunity in lung cancer patients. Indeed, accumulation of DNA damage on systemic T cells, most likely due to previous cytotoxic treatments, was a factor shared by all patients compared to healthy individuals which might have an impact. Moreover, prevalent Th17 phenotypes observed in lung cancer patients may keep a systemic immunosuppressive environment through the promotion of PD-L1^{+} suppressive immune populations (Akbay et al. 2017). Moreover, not all patients from the G1 cohort responded to therapy supporting the existence of other compensatory mechanisms preventing the development of efficient anti-tumor responses most likely within the tumor microenvironment. That was supported by the fact that further stratification of patients according to high PD-L1 tumor positivity increased ORR in the selected population.

It has been previously demonstrated that the post-treatment systemic expansion of a specific subset of CD28^{+} CD8 effector T cells also present in the CD8 TIL population correlate with clinical responses to anti-PD-1 agents (Kamphorst, Wieland, et al. 2017). Moreover, a recent study uncovered that tumor-specific TILs after anti-PD-1 treatment did not express the TCRs identified in the pre-treated tumor, suggesting that these cells are recruited *de novo* from the periphery (Yost et al. 2019). Indeed, the authors confirmed that a proportion of novel intratumor T cell clones post-therapy were already present in the pre-treatment peripheral blood. These evidences in combination with our data suggest that, first, the recovery of CD8 T cell responses after therapy may be orchestrated by CD4 T cells in the periphery where CD28 co-stimulation by professional APCs is predominant **(Figure 30)**; and second, restored peripheral CD8 populations may come from pre-existing tumor-specific T cell pools that are newly recruited to the tumor after systemic expansion **(Figure 30)**. Indeed, we have confirmed that patients can have a large systemic lung cancer-specific T cell pool. Nevertheless, PD-L1/PD-1 blockade therapy has a direct impact within the tumor environment. In this thesis we have set up a cancer cell culture-based model that resembles antigen recognition by T cells in a cancer-cell context, which has largely facilitated the study of systemic T cell responses. In our system, CD8 responses also get

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reinvigorated after *in vitro* PD-1 blockade suggesting that PD-L1/PD-1 blockade also plays a fundamental role within the tumor microenvironment. This is supported by recent evidence showing an association of baseline CD4 memory TILs with response to PD-L1/PD-1 blockade (Datar et al. 2017). Based on several emerging studies claiming that the epigenetic reprogramming experienced by TILs is irreversible (Pauken et al. 2016), it might be the case that PD-L1/PD-1 inhibitors cannot revert the pre-existing exhausted TILs. Other studies disagree with this hypothesis and propose that exhaustion protects TILs from overstimulation and cell death (Scott et al. 2019). Therefore, PD-L1/PD-1 blockade would induce activation induced cell-death of the exhausted TIL populations after reinvigoration. Nevertheless, emerging studies are showing that PD-L1/PD-1 blockade differentially targets exhausted TIL populations depending on the severity of the exhaustion (Jadhav et al. 2019)(Miller et al. 2019)(Siddiqui et al. 2019). While these agents might not revert terminally differentiated exhausted TIL populations, they seem to exclusively target stem-cell memory-like progenitor subsets and induce their differentiation towards effector pools. Although the role of PD-L1/PD-1 blockade within the TIL populations is still controversial, the work presented here demonstrates the key contribution of systemic T immunity for clinical responses to PD-L1/PD-1 in lung cancer patients.

Many of the above-mentioned questions are being under investigation in our group, as a follow-up from the results of the present PhD. First, the identification of the molecular pathways regulated by simultaneous activation of PD-1/LAG-3 axes driving T cell proliferative dysfunctionality. An elegant work recently carried out in our group has found that constitutive activation of both PD-1 and LAG-3 signaling axes on Jurkat cells induces a unique transcriptional and epigenetic reprogramming of T cells affecting the cell cycle amongst other functional pathways. These observations will be further extended to T cells from lung cancer patients. The capacities of PD-L1/PD-1/LAG-3 blockade to revert these transcriptional/epigenetic signatures will be tested. A second question that we are addressing as a continuation is the underlying cause for strong PD-1/LAG-3 co-expression in G2 patients following T cell activation. As we did not identify major differences in the clinical characteristics of both patient cohorts, we speculated that underlying distinct tumor characteristics may influence systemic T cell immunity. Indeed, it has been demonstrated in murine lung cancer models that the presence of IL-17 can induce the upregulation of PD-1 and Tim-3 in CD8 T cells preventing them from activation (Akbay et al. 2017). IL-17 together with other soluble cytokines produced by tumors could be influencing the systemic immunity through a variety of mechanisms. Sequencing of tumor biopsies from both patient cohorts will provide us with insight on potential tumor-induced mechanisms. In addition, here we found that FTL3LG and BDNF were differentially upregulated in the baseline sera

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of responders and non-responders within our discovery cohort, respectively. Although this data could not be validated using two independent patient cohorts from different institutions, these cytokines might be playing an important role influencing systemic T cell immunity of lung cancer patients. Nevertheless, the different sample handling, processing and storage protocols implemented in each institution might have affected the quality of the patient sera, and consequently, introduce strong bias. Additional validation studies with more homogeneous patient sera samples might provide more reliable results.

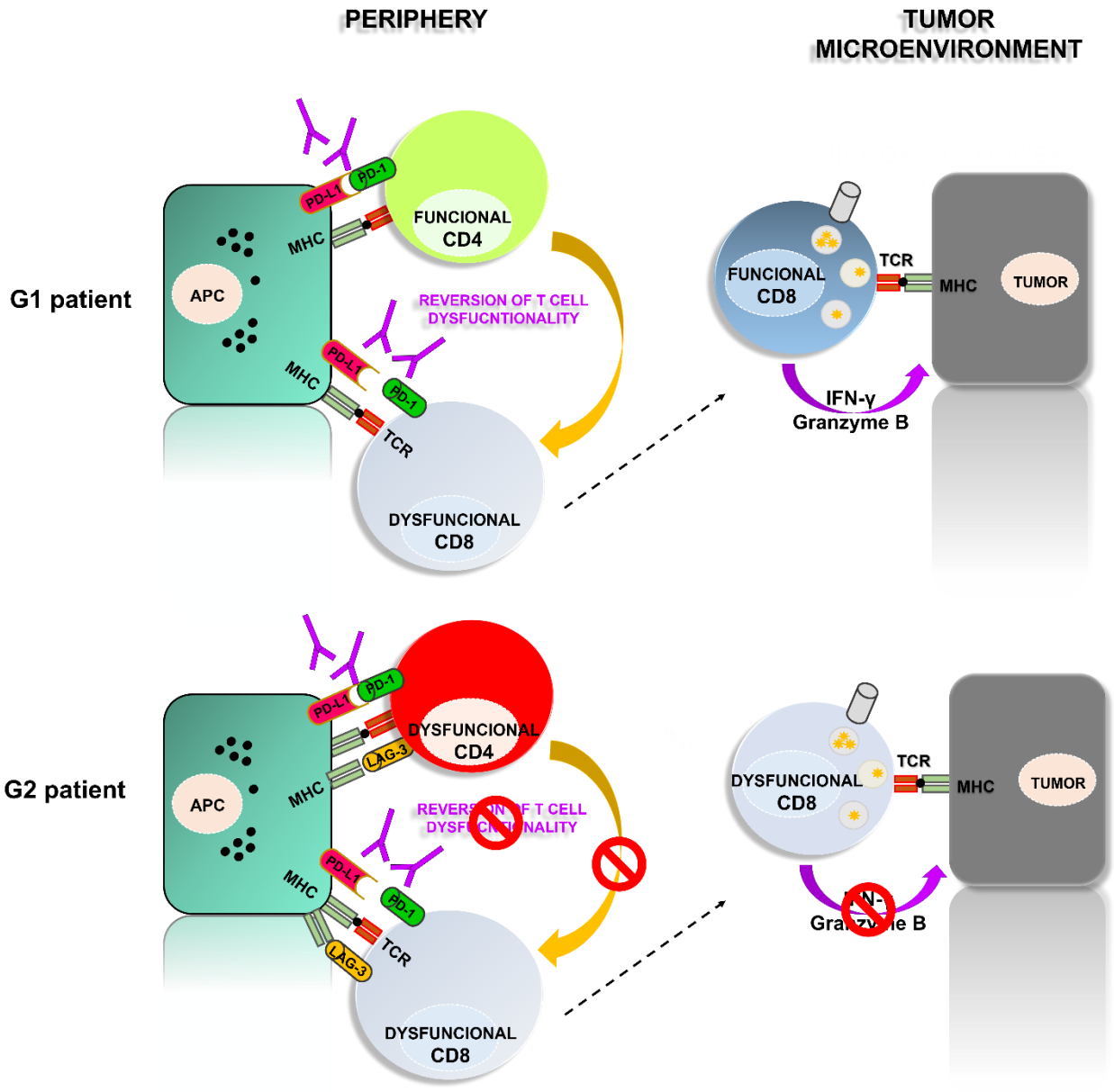

Figure 30. Working model. *Proliferative functionality of systemic CD4 immunity is required for clinical responses to PD-L1/PD-1 blockade therapy. CD8 T cell dysfunctionality can be recovered by PD-L1/PD-1 inhibitors when systemic CD4 responses are functional. The recovery of CD8 T cell responses after therapy may be orchestrated by CD4 T in the periphery where CD28 co-stimulation by professional APCs is present. The reinvigorated CD8 populations may harbor pre-existing tumor-specific T cell pools that are newly recruited*

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to the tumor site after systemic expansion where they undergo cytotoxic anti-tumor responses after antigen encountering.

PD-L1/PD-1 blockade therapies will soon be preferentially applied as first line therapies in lung cancer. Indeed, pembrolizumab is given to NSCLC patients with tumor PD-L1 expression >50, and the rest of the PD-L1/PD-1 blockers are already under evaluation as first-line therapies (Reck et al. 2016)(Carbone et al. 2017)(H. et al. 2017). Therefore, we are currently evaluating CD4 systemic functionality in the context of first-line immunotherapies. Immunological profiling of treatment-naive NSCLC patients is ongoing to answer this question. However, we anticipate that proficiency of T cell immunity in treatment-naive patients strongly differs from heavily pre-treated patients. Indeed, PD-L1/PD-1 monotherapies are more efficacious as first-line therapies, supporting our hypothesis. The role that PD-1/LAG-3 co-expression in T cells from these patients may play in efficacy is currently under investigation in our group.

Finally, the wide-range of immunosuppressor and escape mechanisms present in cancer patients such as the presence of myeloid suppressor populations, the lack of tumor-specific T cell subsets or the genetic modifications by tumor cells among others, constitute a persistent barrier for immunotherapies. Hence, the identification of escape mechanisms compromising T cell functionality is providing new insights for combination of immune checkpoint blockade with other therapies that may show synergy. Indeed, many clinical trials are investigating the potential synergic effects of immune modulators, oncolytic viruses, vaccines, stimulatory antibodies, chemotherapy, radiotherapy, targeted therapies, angiogenic therapies among others, in combination with immune checkpoint inhibitors. All these therapeutic strategies are moving the near future of oncology towards personalized immunotherapies based on immune and molecular/genetic profiling.

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CONCLUSIONS

1- Profiling of CD4 T cell subsets from routine blood sampling before treatment initiation can identify potential responders to PD-L1/PD-1 blockade therapy, especially in combination with PD-L1 tumor expression.

2- Proliferative functionality of systemic CD4 immunity is required for clinical responses to PD-L1/PD-1 blockade therapy. CD8 T cell dysfunctionality is recovered by PD-L1/PD-1 inhibitors when systemic baseline CD4 responses are functional.

3- In our cohort, CD4 and CD8 proliferative dysfunctionality correlates with co-expression of PD-1 and LAG-3, which can confer resistance to PD-L1/PD-1 monoblockade.

4- Our study provides the rational for clinical evaluation of PD-1/LAG-3 dual-blockade strategies in patients with baseline CD4 dysfunctionality.

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CONCLUSIONES

1-La caracterización de las subpoblaciones de células T CD4 a través de muestras de sangre de analíticas rutinarias antes de comenzar el tratamiento puede identificar a potenciales respondedores a las terapias anti-PD-L1/PD-1, especialmente en combinación con la expresión de PD-L1 tumoral.

2- Poseer una inmunidad proliferativa funcional CD4 sistémica es un requisito para obtener respuestas clínicas a la inmunoterapia anti-PD-L1/PD-1. La disfuncionalidad CD8 sistémica es reversible a través de inhibidores de PD-L1/PD-1 cuando las respuestas sistémicas CD4 son funcionales.

3-En nuestra cohorte, la disfuncionalidad CD4 y CD8 sistémica se correlaciona con la co-expresión de PD-1/LAG-3, que confiere resistencia al bloqueo único con inhibidores de PD-L1/PD-1.

4-Este trabajo proporciona evidencia experimental que apoya la combinación de las terapias bloqueadoras de PD-L1/PD-1 y LAG-3 en pacientes que manifiestan una inmunidad CD4 basal disfuncional.

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LIST OF FIGURES AND TABLES

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TABLES

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ANNEXES

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Research Article

SOURCE DATA | TRANSPARENT PROCESS | OPEN ACCESS

EMBO Molecular Medicine

Functional systemic CD4 immunity is required for clinical responses to PD-L1/PD-1 blockade therapy

Miren Zuazo[1,†], Hugo Arasanz[1,†], Gonzalo Fernández-Hinojal[2,†], Maria Jesus García-Granda[1,†], María Gato[1,†], Ana Bocanegra[1], Maite Martínez[2], Berta Hernández[2], Lucía Teijeira[2], Idoia Morilla[2], Maria Jose Lecumberri[2], Angela Fernández de Lascoiti[2], Ruth Vera[2,*], Grazyna Kochan[1,**] & David Escors[1,3,***]

Abstract

The majority of lung cancer patients progressing from conventional therapies are refractory to PD-L1/PD-1 blockade monotherapy. Here, we show that baseline systemic CD4 immunity is a differential factor for clinical responses. Patients with functional systemic CD4 T cells included all objective responders and could be identified before the start of therapy by having a high proportion of memory CD4 T cells. In these patients, CD4 T cells possessed significant proliferative capacities, low co-expression of PD-1/LAG-3 and were responsive to PD-1 blockade *ex vivo* and *in vivo*. In contrast, patients with dysfunctional systemic CD4 immunity did not respond even though they had lung cancer-specific T cells. Although proficient in cytokine production, CD4 T cells in these patients proliferated very poorly, strongly co-upregulated PD-1/LAG-3, and were largely refractory to PD-1 monoblockade. CD8 immunity only recovered in patients with functional CD4 immunity. T-cell proliferative dysfunctionality could be reverted by PD-1/LAG-3 co-blockade. Patients with functional CD4 immunity and PD-L1 tumor positivity exhibited response rates of 70%, highlighting the contribution of CD4 immunity for efficacious PD-L1/PD-1 blockade therapy.

Keywords B7-H1; biomarker; immunotherapy; lung cancer; PD-1/PD-L1
Subject Categories Cancer; Immunology
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Introduction

PD-L1/PD-1 blockade is demonstrating remarkable clinical outcomes since its first clinical application in human therapy (Brahmer *et al*, 2012; Topalian *et al*, 2012). These therapies interfere with immunosuppressive PD-L1/PD-1 interactions by systemic administration of blocking antibodies. PD-L1 is overexpressed by many tumor types and generally correlates with progression and resistance to pro-apoptotic stimuli (Azuma *et al*, 2008; Gato-Canas *et al*, 2017; Juneja *et al*, 2017). PD-1 is expressed in antigen-experienced T cells and interferes with T-cell activation when engaged with PD-L1 (Chemnitz *et al*, 2004; Karwacz *et al*, 2011). The majority of advanced non-small-cell lung cancer (NSCLC) patients progressing from conventional cytotoxic therapies who receive PD-L1/PD-1 blockade therapy do not respond. The causes for these distinct clinical outcomes are a subject for intense research (Topalian *et al*, 2016). Emerging studies indicate that PD-L1/PD-1 blockade therapy does not only affect the tumor microenvironment, but also alters the systemic dynamics of immune cell populations (Hui *et al*, 2017; Kamphorst *et al*, 2017a,b; Krieg *et al*, 2018). Some of these changes do correlate with responses and could be used for real-time monitoring of therapeutic efficacy. For example, PD-1$^+$ CD8 T cells expand systemically after PD-1 blockade therapy in lung cancer patients (Kamphorst *et al*, 2017a). As CD8 T cells are the main direct effectors of responses through cytotoxicity over cancer cells, these changes are thought to be the consequence of efficacious anti-tumor immunity. Indeed, CD8 T-cell infiltration of tumors correlates with good outcomes (Daud *et al*, 2016). However, the role of CD4 immunity in patients undergoing PD-L1/PD-1 blockade therapy remains poorly understood although extensive pre-clinical data link CD4 responses to anti-tumor immunity. Hence, CD4 T cells recognizing tumor neoepitopes contribute significantly to the efficacy of several types of immunotherapies in murine models and in cancer patients (Kreiter *et al*, 2015; Knocke *et al*, 2016; Sahin *et al*, 2017).

Human T cells undergo a natural differentiation process following the initial antigen recognition, characterized by the progressive loss of CD27 and CD28 surface expression, and acquisition of

1 Immunomodulation Group, Biomedical Research Center of Navarre-Navarrabiomed, Fundación Miguel Servet, IdISNA, Pamplona, Spain
2 Department of Oncology, Hospital Complex of Navarre, IdISNA, Pamplona, Spain
3 Division of Infection and Immunity, University College London, London, UK
*Corresponding author. Tel: +34 848 422162; E-mail: ruth.vera.garcia@navarra.es
**Corresponding author. Tel: +34 848 425742; E-mail: grazyna.kochan@navarra.es
***Corresponding author. Tel: +34 848 425742; E-mails: d.escors@ucl.ac.uk; descorsm@navarra.es
† These authors contributed equally to this work

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memory and effector functions (Lanna *et al*, 2014, 2017). Hence, human T cells can be classified according to their CD27/CD28 expression profiles into poorly differentiated ($CD27^{+}$ $CD28^{+}$), intermediately differentiated ($CD27^{negative}$ $CD28^{+}$), and highly differentiated ($CD27^{negative}$ $CD28^{low/negative}$, T_{HD}) subsets (Lanna *et al*, 2014). Highly differentiated T cells in humans are composed of memory, effector, and senescent T cells, all of which could modulate anticancer immunity in patients and alter susceptibility to immune checkpoint inhibitors. To understand the impact of systemic CD4 and CD8 T-cell immunity before the start of immunotherapies, we carried out a discovery study in a cohort sample of 51 NSCLC patients undergoing PD-1/PD-L1 immune checkpoint blockade therapy after progression to platinum-based chemotherapy. Our results indicate that baseline functional systemic CD4 immunity is required for objective clinical responses to PD-L1/PD-1 blockade therapies.

Results

The baseline percentage of systemic CD4 T_{HD} cells within CD4 cells separates NSCLC patients into two groups with distinct clinical outcomes

To study whether there was a correlation between specific systemic T-cell subsets and responses to anti-PD-L1/PD-1 immunotherapy in NSCLC patients, a prospective study was carried out in a cohort of 51 patients treated with PD-L1/PD-1 inhibitors (Table EV1). These patients had all progressed to conventional cytotoxic therapies and received immunotherapies as part of their treatments. 78.4% presented an ECOG of 0–1, 70.6% with at least three affected organs, and 25.5% with liver metastases (Table EV1).

First, the percentages of CD4 T-cell differentiation subsets according to CD27/CD28 expression profiles were quantified within total CD4 cells in patients before the start of immunotherapies (baseline) from fresh peripheral blood samples and compared to healthy age-matched donors. Overall, cancer patients showed a significantly higher baseline percentage of CD4 T_{HD} cells than healthy controls ($P < 0.001$; Fig 1A). Furthermore, patients were separated into two groups by an approximate cut-off value of 40% CD4 T_{HD} cells (Fig 1A); we thus denominated "G1 cohort" to patients with more than 40% T_{HD} cells ($63.25 \pm 13.5\%$, $N = 23$) and "G2 cohort" to patients with less than 40% ($27.05 \pm 10.6\%$, $N = 28$). Differences between G1 and G2 cohorts were also highly significant (Fig 1A).

Objective responders were found only within the G1 cohort ($P = 0.0001$), which included all patients that showed significant tumor regression (Fig 1A and B). Accordingly, ROC analysis demonstrated a highly significant association of the CD4 T_{HD} cell baseline percentage with objective responses ($P = 0.0003$) and confirmed the cut-off value of > 40% to identify objective responders with 100% specificity and 70% sensitivity (Fig 1C).

A validation dataset from 32 patients was performed by parallel independent double-blind sample handling, staining, data collection, and analyses (Fig EV1). While in the discovery cohort T cells were directly analyzed from peripheral blood samples within the same day, validation samples were processed very differently. Briefly, an overnight depletion step of myeloid cells by adherence to plastic was included before T-cell analyses from non-adherent cells. Hence, relative percentages of CD4 T_{HD} cells varied between the discovery and validation cohorts. Even so, there was a significant agreement between the two datasets on patient classification as demonstrated by Cohen's kappa coefficient ($\kappa = 0.932$). The highly significant association between G1 patients and objective responses in the validation set was confirmed ($P = 0.0006$), albeit with a cut-off value of 20% in the validation dataset which was corroborated by ROC analysis (Fig EV1).

In agreement with these results, the G1 patient cohort had a significantly longer progression-free survival (PFS) compared to the G2 cohort. The median PFS (mPFS) of G2 patients was only 6.1 weeks (95% C.I., 5.7–6.6) compared to 23.7 weeks for G1 patients (95% C.I., 0–51.7; $P = 0.001$; Fig 1D). A comparison of G2 versus G1 baseline profiles showed hazard ratios for disease progression or death that favored the latter [3.1 (1.5–6.4; 95% C.I.) $P = 0.002$].

To assess whether CD4 T-cell profiling had prognostic value, the time elapsed from diagnosis to the start of immunotherapies was compared between G1 and G2 patient cohorts, as described (Le *et al*, 2015). No significant differences were observed, indicating that G1/G2 classification did not have prognostic value (Fig EV2). This was supported by no association between G1/G2 patient cohorts and baseline ECOG score ($P = 0.6$), with liver metastases ($P = 0.88$), with tumor load ($P = 0.19$), or with the Gustave-Roussy immune score (GRIm; $P = 0.14$, Table EV2; Bigot *et al*, 2017). The hazard ratio for progression or death of G2 patients maintained its statistical significance by multivariate analyses (HR 9.739; 95% CI 2.501–37.929) when adjusted for tumor histology, age, gender, smoking habit, liver metastases, number of organs affected, PD-L1 tumor expression, NLR, serum LDH, and albumin.

Functionality of systemic CD4 immunity defines clinical outcomes and susceptibility to PD-L1/PD-1 blockade

We hypothesized that the relative percentage of CD4 T_{HD} cells was a biomarker for functional differences in systemic CD4 immunity between the two cohorts before the start of immunotherapy. To find out whether this was the case, we first evaluated PD-1 expression in unstimulated CD4 T cells. However, no differences were observed between G1 and G2 patient cohorts or even with healthy age-matched donors (not shown). We then tested whether there were differences in PD-1 upregulation after *ex vivo* stimulation with lung cancer cells. To this end, we engineered a T-cell stimulator cell line by expressing a membrane-bound anti-CD3 single-chain antibody in A549 human lung adenocarcinoma cells (A549-SC3 cells). This cell line stimulated T cells in co-cultures with the same affinity and specificity while preserving other inhibitory interactions such as PD-L1/PD-1 or MHC II-LAG-3 (Fig EV3A and B). This ensured the same standard assay for cancer cell T-cell recognition for each patient (Fig EV3B–D). CD4 T cells from NSCLC patients significantly upregulated PD-1 compared to cells from age-matched healthy donors after incubation with A549-SC3 cells ($P < 0.001$; Figs EV3C and 2A). However, no differences were found between G1 and G2 patient cohorts. Coexpression of PD-1 and LAG-3 has been suggested to identify dysfunctional tumor-infiltrating lymphocytes in NSCLC (He *et al*, 2017). Interestingly, G2 donors presented a significantly higher percentage of CD4 T cells co-expressing both markers than G1 donors after stimulation (Fig 2B). To test whether there were

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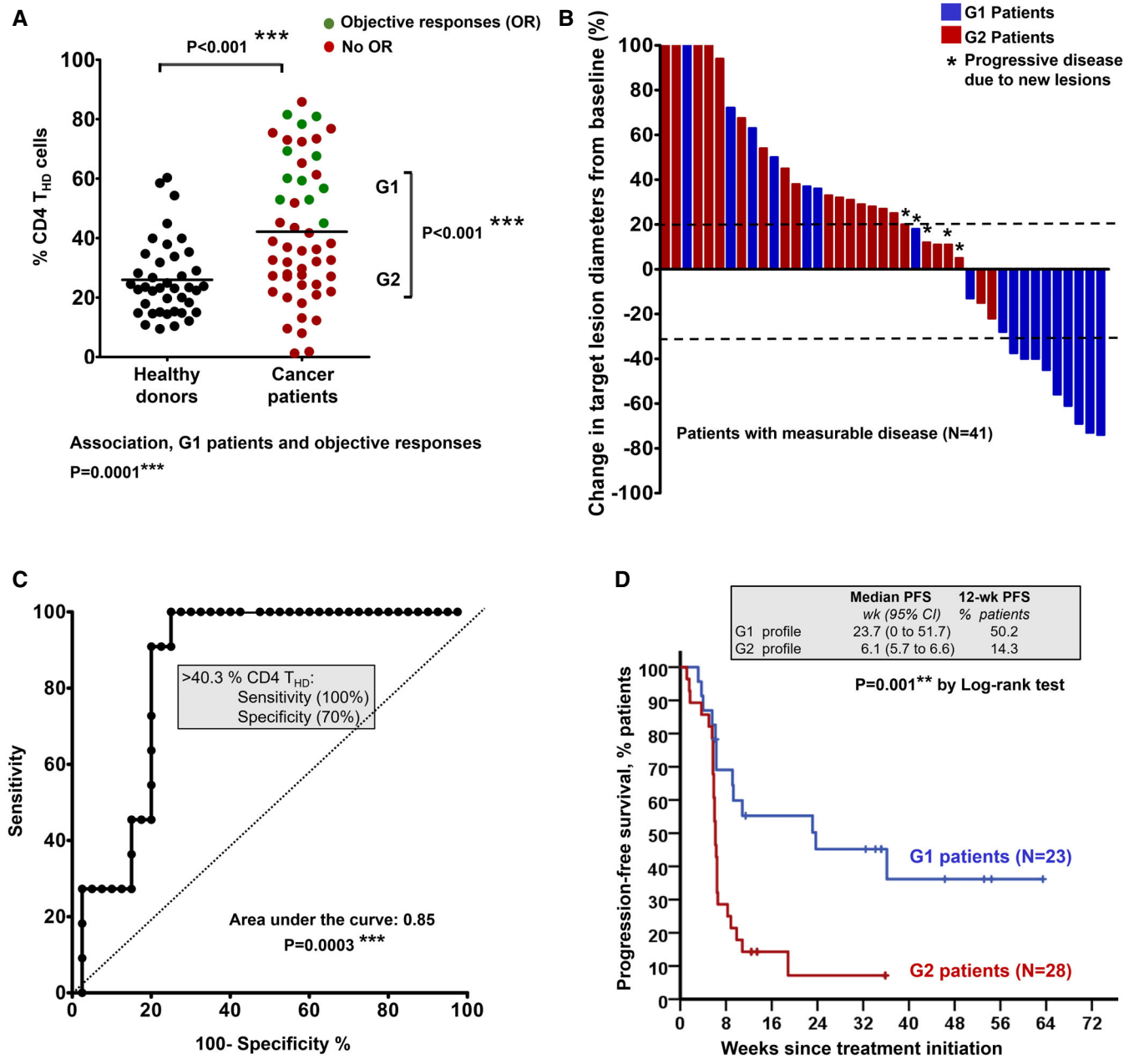

Figure 1. Baseline profiling of CD4 T-cell differentiation subsets stratifies clinical responses to PD-L1/PD-1 blockade.

A Percentage of circulating highly differentiated CD4 T cells within CD4 cells in age-matched healthy donors (N = 40) or NSCLC patients (N = 51) or NSCLC patients before undergoing immunotherapies. G1 and G2, groups of patients classified according to high T_{HD} cells (G1, > 40% CD4 T_{HD} cells) and low T_{HD} cells (G2, < 40% CD4 T_{HD} cells). Relevant statistical comparisons are shown by the test of Mann–Whitney. In green, objective responders (OR). In red, no OR. Below the graph, correlation of objective responses to G1 and G2 groups by Fisher's exact test.

B Waterfall plot of change in lesion size in patients with measurable disease classified as having a G1 (blue) or G2 (red) profile. Dotted lines represent the limit to define significant progression (upper line) or significant regression (lower line).

C ROC analysis of baseline CD4 T_{HD} quantification as a function of objective clinical responses.

D Kaplan–Meier plot for PFS in patients treated with immunotherapies stratified only by G1 (green) and G2 (red) CD4 T-cell profiles. Patients starting therapy with a G2 profile had an overall response rate (ORR) of 0 and 82% of them experienced progression or death by week 9. ORR was 44.8% for G1 patients, and the 12-week PFS was 50.2%.

Source data are available online for this figure.

also differences in proliferation, the percentage of Ki67$^+$ cells was compared (Fig 2C and D). Accordingly, CD4 T cells from G2 patients were remarkably impaired in proliferation after *ex vivo* activation with A549-SC3 cells compared to T cells from G1 patients. As we had observed that G1 and G2 patient cohorts differed in baseline percentages of CD4 T_{HD} cells (Fig 1A), we tested whether this subset

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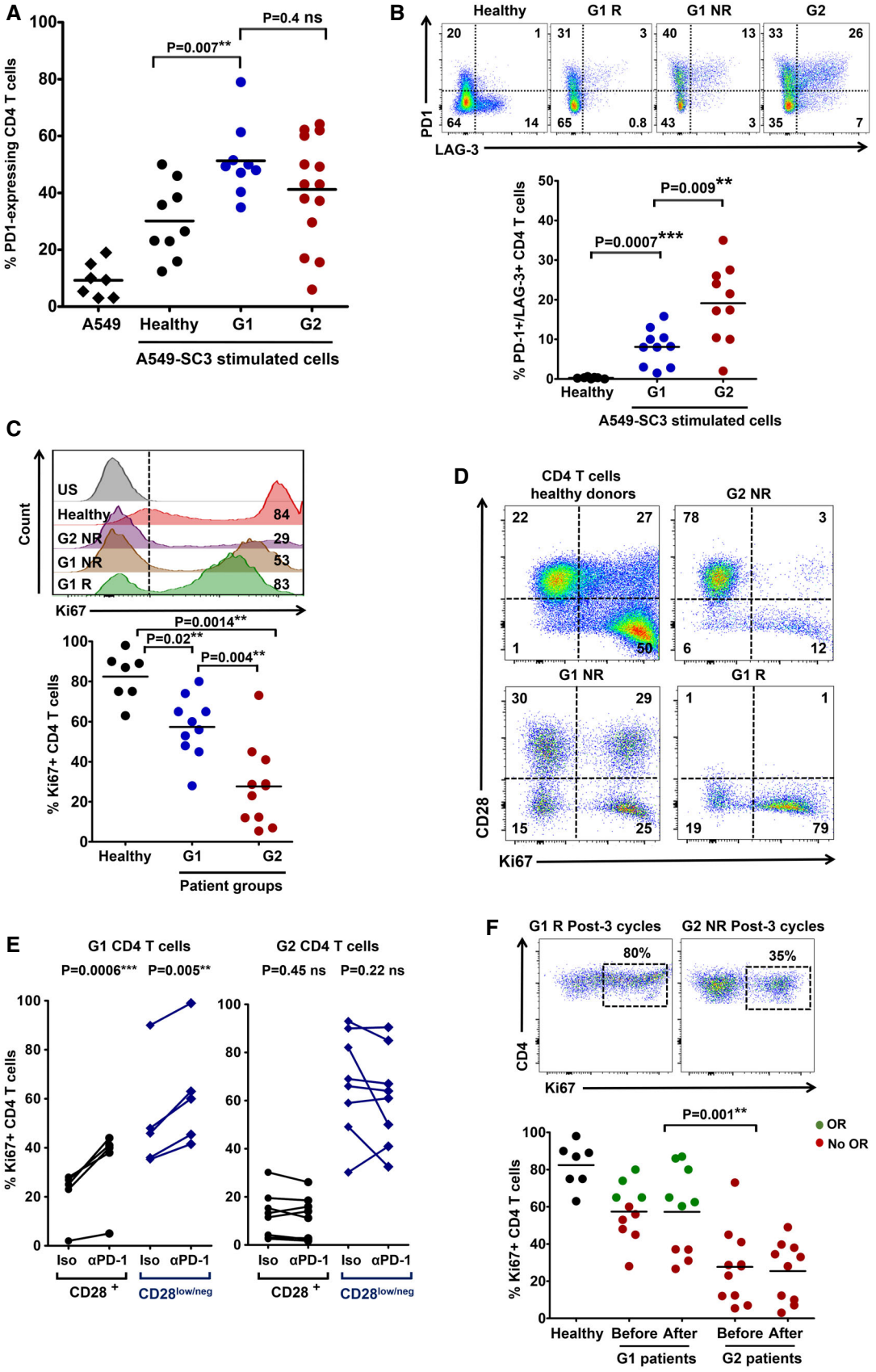

Figure 2.

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Figure 2. Differential systemic CD4 immunity and responses to PD-1/PD-L1 blockade in NSCLC patients.

A The scatter plot shows PD-1 expression after co-culture of CD4 T cells from healthy donors (n = 9) or NSCLC patients (n = 14), as indicated, with A459-SC3 lung cancer cells. Relevant statistical comparisons with the test of Mann–Whitney are indicated.

B Upper graphs, flow cytometry density plots of PD-1 and LAG-3 co-expression in CD4 T cells from healthy donors, a G1 responder (G1 R), a G1 non-responder (G1 NR), and a G2 non-responder as indicated, following stimulation with A549-SC3 cells. Percentage of expressing cells are indicated within each quadrant. Below, same as in the upper graphs but as a scatter plot of the percentage of CD4 T cells that simultaneously co-express PD-1 and LAG-3 that simultaneously co-express PD-1 and LAG-3 in G1 healthy donors (n = 10), G1 (n = 10) and G2 (n = 10) patients. Relevant statistical comparisons are shown with the test of Mann–Whitney.

C Upper flow cytometry histograms of Ki67 expression in CD4 T cells from the representative subjects as indicated on the right, after stimulation with A549-SC3 cells. Vertical dotted line indicates the cut-off value of positive versus negative Ki67 expression. The percentage of Ki67-expressing CD4 T cells is shown within the histograms. Below, same data represented as a scatter plot from a sample of G1 and G2 donors as indicated, with relevant statistical comparisons with the test of Mann–Whitney (n = 7–10).

D Proliferation of CD4 T cells stimulated by A549-SC3 cells from the indicated patient groups. CD28 expression is shown together with the proliferation marker Ki67. Percentages of cells within each quadrant are shown.

E Same as in (D) but in the presence of an isotype control antibody or an anti-PD-1 antibody with the equivalent sequence to pembrolizumab. The effects on CD4 T cells from a G1 and a G2 patient are shown, divided into CD28 high or low/negative subsets as indicated. Relevant statistical comparisons are shown with paired Student's t-test.

F Top, flow cytometry density plots of Ki67 expression in CD4 T cells from representative G1 or G2 patients after three cycles of therapy, activated by incubation with A549-SC3 cells. Below, same as above but as a dot-plot graph (n = 7–10). A comparison between proliferating CD4 T cells before and after therapy is shown in unpaired patient samples. G1 R, G1 objective responder patient. G2 NR, G2 patient with no objective responses; green, objective responders (OR) and red, no OR; Iso, treatment with an isotype antibody control; and α-PD-1, treatment with anti-PD-1 antibody. Statistical comparisons were performed with the test of Mann–Whitney.

was responsive to activation by A549-SC3 cells (Fig 2D). Interestingly, CD4 T_{HD} cells strongly proliferated in all patients, although they constituted a minority in the G2 patient cohort.

The strong proliferative capacities of CD4 T_{HD} cells indicated that these were not exhausted, anergic, or senescent subsets, but probably highly differentiated memory subsets. To test this, their baseline phenotype according to CD62L/CD45RA surface expression was assessed in a sample of patients (Fig EV4A). The majority of CD4 T_{HD} cells were central-memory ($CD45RA^{negative}$ $CD62L^{+}$) and effector-memory ($CD45RA^{negative}$ $CD62L^{negative}$) cells, without differences between G1 and G2 cohorts. Increased genotoxic damage is strongly associated with T-cell senescence and can be evaluated by H2AX expression (Lanna *et al*, 2017). Interestingly, NSCLC CD4 T cells exhibited extensive genotoxic damage in both T_{HD} and non-T_{HD} subsets without differences between G1 and G2 patient cohorts, unlike T cells from age-matched healthy donors (Fig EV4B). Therefore, genotoxic damage did not identify senescent T cells in patients that had been treated with conventional therapies. Then, the expression of the replicative senescence marker CD57 was used to identify *bona fide* senescent T cells, which accounted to 30% of T_{HD} cells in healthy age-matched donors, and about 10% in NSCLC patients (Fig EV4C). Our results strongly suggested that circulating CD4 T_{HD} cells in our cohort of NSCLC patients mostly corresponded to non-senescent, non-exhausted memory subsets.

CD4 T cells of G2 patients strongly co-upregulated PD-1/LAG-3 after stimulation. We wondered if lack of clinical responses in G2 patients could be explained by resistance to single blockade of PD-1. Hence, proliferation of CD4 T cells activated with A549-SC3 in the presence of an anti-PD-1 antibody equivalent to pembrolizumab was assessed (Scapin *et al*, 2015; Fig 2E). As expected, PD-1 blockade increased proliferation of T_{HD} and non-T_{HD} CD4 T cells in patients from the G1 cohort. In contrast, their G2 counterparts were largely refractory. To find out whether CD4 T cells from G2 patients remained unresponsive to PD-1 blockade *in vivo*, cells were obtained from patients after at least three cycles of therapy and tested for their proliferative capacities (Fig 2F). Systemic CD4 T cells from G2 patients remained poorly proliferative during immunotherapy.

Absence of cancer-specific CD4 T cells or systemic T-cell exhaustion is not behind the lack of objective clinical responses to PD-L1/PD-1 blockade therapies

Then, we thought that G2 patients could be refractory to anti-PD-1 immunotherapy by not having systemic cancer-specific CD4 T cells. To this end, we quantified CD4 T cells reactive to lung adenocarcinoma antigens using IFN-γ-activated autologous monocyte-derived DCs as antigen presenting cells, as described (Escors *et al*, 2008). DCs were loaded with A549 cell lysate, as these cells contain numerous common lung adenocarcinoma antigens (Madoz-Gurpide *et al*, 2008). We used this approach as we lacked sufficient biopsy material to get tumor antigens or tumor-infiltrating T cells. CD4 T cells reactive to A549 cell antigens were identified by IFN-γ upregulation. Interestingly, lung cancer-specific CD4 T cells were present at varying proportions before the start of immunotherapy in both G1 and G2 patients (Fig 3A). Indeed, although the average percentages of circulating lung cancer-specific CD4 T cells were low, these did not differ significantly between G1 (responders and non-responders) and G2 patients. These T cells consisted of both T_{HD} and non-T_{HD} subsets, without significant differences in relative percentages between G1 and G2 cohorts (Fig 3B). These results suggested that poor responses in G2 patients were not caused by lack of tumor-specific CD4 T cells but rather by having dysfunctional T cells.

To further study the dysfunctional status of systemic CD4 T cells in G2 patients, we evaluated PD-1 and LAG-3 surface expression directly after blood sampling, as constitutive high-level expression of these markers is a frequent characteristic of T-cell exhaustion. However, no differences were found between age-matched healthy donors and G1/G2 patient cohorts in either T_{HD} or non-T_{HD} subsets (not shown). Nevertheless, the defining hallmark of T-cell exhaustion is the loss of cytokine production following stimulation, particularly multi-cytokine expression (Crawford *et al*, 2014). Interestingly, CD4 T cells from both G1 and G2 patient cohorts were as proficient in IFN-γ, IL-4, IL-10, and IL-2 expression as T cells from healthy donors independently of their CD28 expression (Fig 4A) or whether these were T cells from G1 responders or non-responders (Appendix Fig S1). Indeed, CD4 cells (total, T_{HD}, and non-T_{HD} subsets) in both G1 and G2 patient cohorts were significantly skewed

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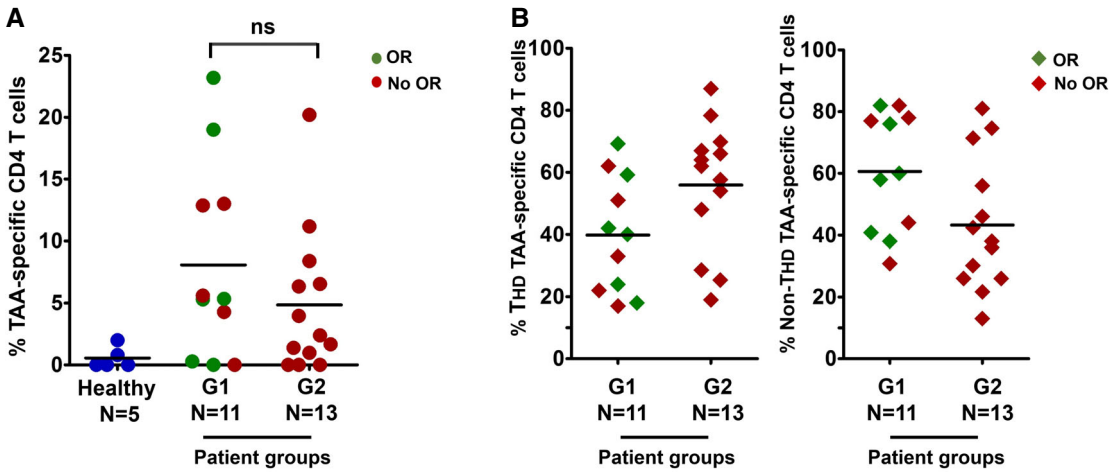

Figure 3. Lung cancer antigen-specific CD4 T cells in NSCLC patients.

A Scatter plot graph with the percentage of lung cancer-specific systemic CD4 T cells quantified by an autologous DC-based antigen presentation assay (see Materials and Methods), in a sample of G1 and G2 patients as indicated. Objective responses (OR) are shown in green. In red, patients with no OR.

B The scatter plot graph on the left represents the percentage of CD4 T_{HD} cells within lung cancer-specific CD4 T cells in a sample of patients from the indicated G1/G2 groups. On the right, same as left but representing the percentage of $CD28^+$ CD4 T cells within lung cancer-specific CD4 T cells. Objective responders (OR) are shown in green. In red, patients with no OR.

Data information: Relevant statistical comparisons are shown within the graphs with the test of Mann–Whitney. *N*, number of biological replicates (independent patients); Ns, no significant differences ($P < 0.05$).

toward IL-17 responses compared to age-matched healthy donors (Fig 4A). Importantly, only a minority of CD4 T cells from either G1 or G2 patient groups were single-cytokine producers (Fig 4B) while most of the non-T_{HD} CD4 T cells were very proficient in multiple cytokine production with a preference for IL-17-expressing subsets (Fig 4C and D). These results indicated that CD4 T cells from G2 patients were not exhausted according to our current understanding (Hashimoto *et al*, 2018). Indeed, they responded to stimulation by producing cytokines although with strong co-upregulation of PD-1/LAG-3 associated with markedly diminished proliferative capacities.

Systemic CD8 immunity recovers in G1 responder patients following immunotherapy

In contrast to CD4 T_{HD} cells, the relative percentage of CD8 T_{HD} cells within the CD8 population did not significantly differ from age-matched healthy donors, nor could be used to identify objective responders (Fig EV5A and B). Interestingly, CD8 cells from both G1 and G2 patient cohorts obtained before the start of immunotherapies did fail to proliferate after stimulation by A549-SC3 cells (Fig 5A). To test whether anti-PD-1 therapy could recover CD8 dysfunctionality *in vivo*, the proliferative capacities of CD8 T cells from G1 and G2 patients obtained after at least three cycles of treatment were evaluated by stimulation with A549-SC3 cells. CD8 T cells from G1 responders had recovered significant proliferative capacities, while only limited enhancements were observed in G2 patients (Fig 5B). Similarly to CD4 cells, systemic CD8 T cells specific for lung adenocarcinoma antigens were quantified in G1 and G2 patients and found to be comparable (Fig EV5C) and distributed within non-T_{HD} and T_{HD} subsets (Fig EV5D).

To find out whether CD8 T cells in G1 patients were especially susceptible to PD-1 blockade *ex vivo*, baseline samples of CD8 T cells from G1 and G2 patients were activated with A549-SC3 cells in the presence of an anti-PD-1 antibody or an isotype control. In agreement with the *in vivo* results, *ex vivo* PD-1 blockade improved significantly the proliferation of CD8 T cells from G1 patients and specially non-T_{HD} ($CD28^+$) subsets (Fig 5C). *In vivo* expansion of $CD28^+$ CD8 T cells in murine models correlate with anti-PD-1 efficacy (Kamphorst *et al*, 2017b). To confirm this observation in our cohort of patients, the changes in the relative abundance of CD8 $CD28^+$ T cells were compared in G1 and G2 patients from baseline to post-anti-PD-1 therapy (Fig 5D). Accordingly, the $CD28^+$ CD8 T-cell compartment significantly expanded ($P < 0.001$) only in G1 patients.

Proliferative dysfunctionality of CD4 and CD8 T cells from G2 patients is reversible after PD-1/LAG-3 dual blockade

As we found that CD4 proliferative dysfunctionality in G2 patients correlated with high PD-1/LAG-3 co-upregulation after activation, we tested if this was also the case for CD8 T cells. PD-1/LAG-3 co-expression was tested *ex vivo* after stimulation with A549-SC3 cells, and G2 patients presented a significantly higher proportion of PD-1/LAG-3 co-expressing CD8 T cells compared to G1 counterparts (Fig 6A). Overall, our data indicated that PD-1/LAG-3 co-upregulation was contributing to proliferative dysfunctionality. To test whether this was the case, baseline samples of CD4 and CD8 T cells from G2 patients were co-incubated *ex vivo* with A549-SC3 cells in the presence of an isotype antibody control, anti-PD-1, anti-LAG-3, or anti-PD-1/anti-LAG-3 antibodies. We confirmed that each antibody was specifically blocking PD-1, LAG-3, or both in our assays by epitope masking using flow cytometry (not shown). Only co-blockade of PD-1 and LAG-3 in both CD4 (Fig 6B) and CD8 T cells (Fig 6C) from G2 patients significantly increased proliferation independently of CD28 expression. These results

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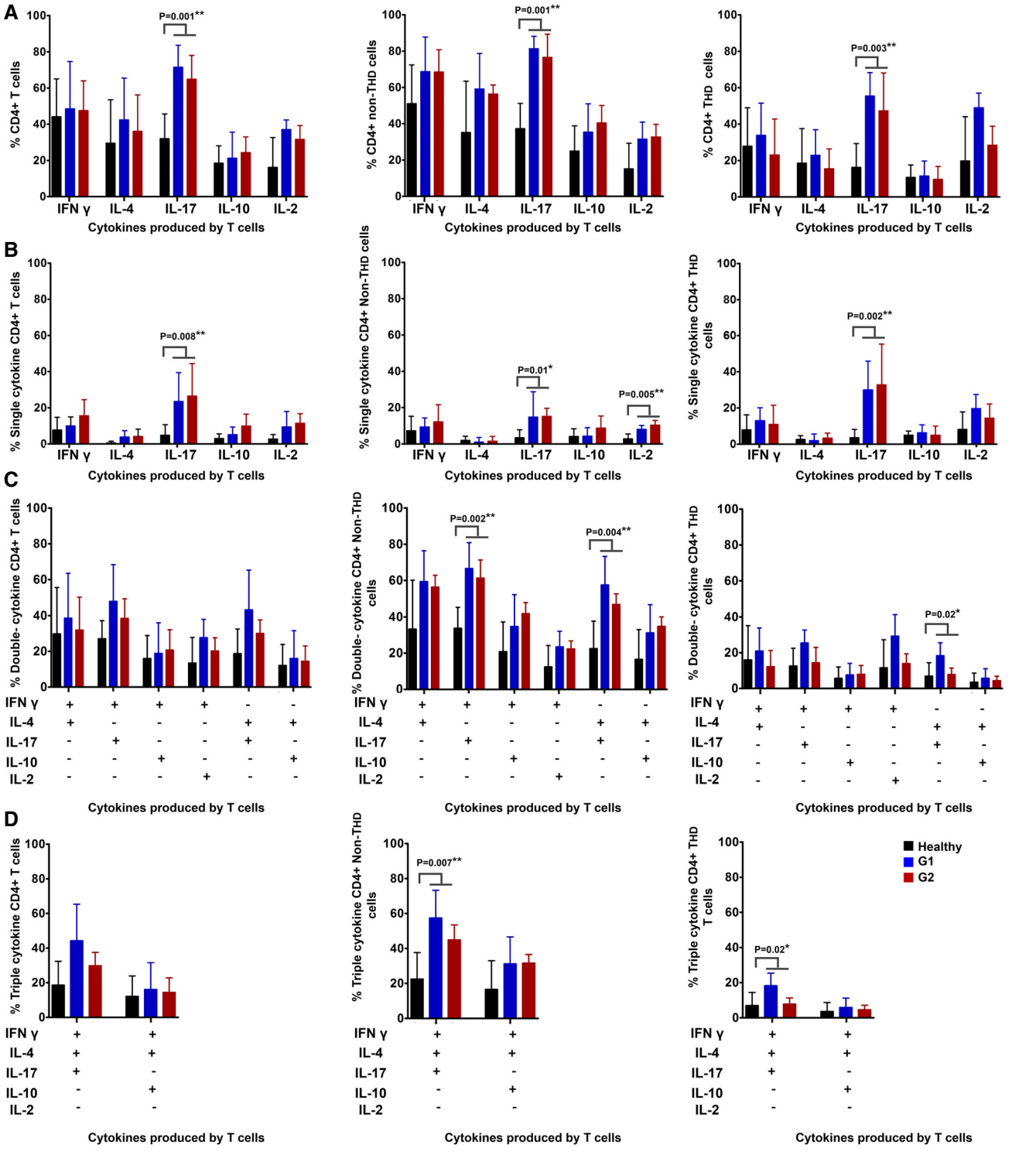

Figure 4. Systemic circulating CD4 T cells in NSCLC patients are proficient in cytokine production with an overall Th17 profile.

A Column graphs representing the percentage of CD4 T cells from NSCLC patients or age-matched healthy donors as represented in the graph, expressing the indicated cytokines after T-cell stimulation with anti-CD3/anti-CD28 antibodies. Data on total CD4 (left graph), $CD28^{+}$ subsets (center graph) and $CD28^{negative}$ subsets (right graph) are shown. Error bars correspond to standard deviations, and bars represent means from nine independent biological replicates (healthy donors) and six independent replicates (patients).

B–D Same as in (A) but representing CD4 T cells expressing only one cytokine (B), two (C) or three cytokines simultaneously (D). Error bars correspond to standard deviations, and bars represent means from five independent biological replicates (patients).

Data information: Relevant statistical comparisons are shown within the graphs by the test of Kruskal–Wallis.

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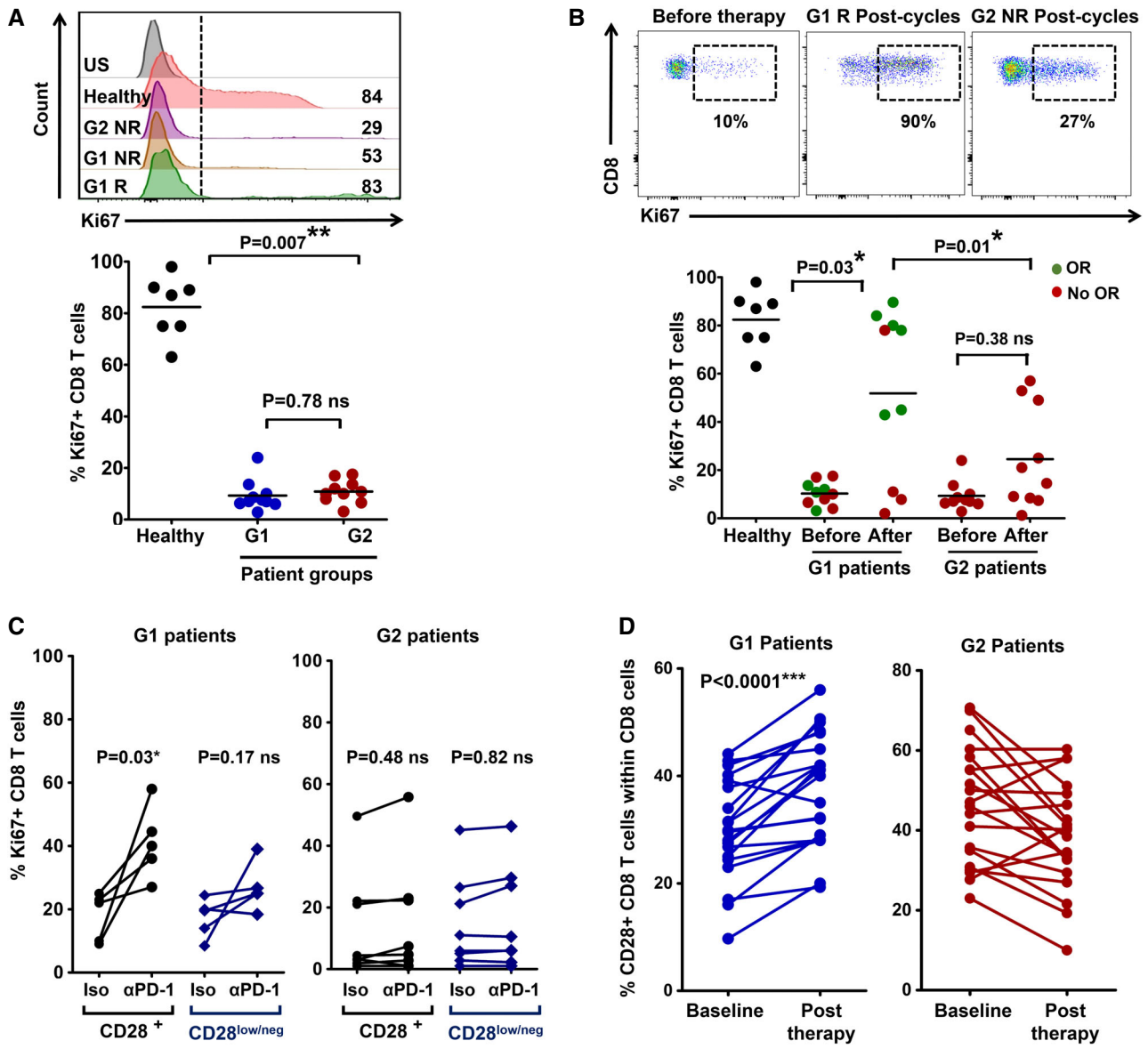

Figure 5. CD8 dysfunctionality recovers in G1 patients undergoing immunotherapy.

A Upper flow cytometry histograms, expression of the proliferation marker Ki67 in CD8 T cells from the indicated patients or healthy donor before the start of immunotherapy, stimulated *ex vivo* by A549-SC3 cells. Numbers indicate mean fluorescence intensities. G1 R and G1 NR, responder and non-responder G1 patient, respectively; G2 NR, non-responder G2 patient. US, unstained control. Below, same as above but as a dot plot graph with percentage of proliferating Ki67$^+$ CD8 T cells from the indicated groups (n = 7–10). Relevant statistical comparisons are shown with the test of Mann–Whitney.

B Upper flow cytometry density plots, expression of Ki67 in *ex vivo*-stimulated CD8 T cells from the indicated patients before and after the start of immunotherapies. NR, non-responder patient; R, responder patient. Below, dot-plots of the percentage of Ki67$^+$ proliferating CD8 T cells after *ex vivo* activation by A549-SC3 cells. CD8 T cells were obtained from samples of G1 or G2 patients before immunotherapy and after three cycles of anti-PD-1 therapy (n = 7–10). Relevant statistical comparisons are shown with the test of Mann–Whitney. Green, objective responders (OR) and red, no ORs.

C Same as in (A) but in the presence of an isotype control antibody or an anti-PD-1 antibody molecularly equivalent to pembrolizumab. Relevant statistical comparisons are shown with comparisons carried out with paired Student's t-test.

D Change in CD8 CD28$^+$ T cells from baseline to post-therapy in G1 patients (left) or in G2 patients (right). Statistical comparisons were carried out with paired Student's t-test.

confirmed that PD-1/LAG-3 co-upregulation contributed to keeping systemic CD4 and CD8 T cells from G2 patients in a proliferative dysfunctional state following stimulation, and that this T-cell dysfunctionality can be reverted by co-blockade of both immune checkpoints.

Objective responders are found within G1 patients with PD-L1-positive tumors

Objective response rates in G1 patients were about 50%. Hence, our results indicated that functional systemic CD4 responses

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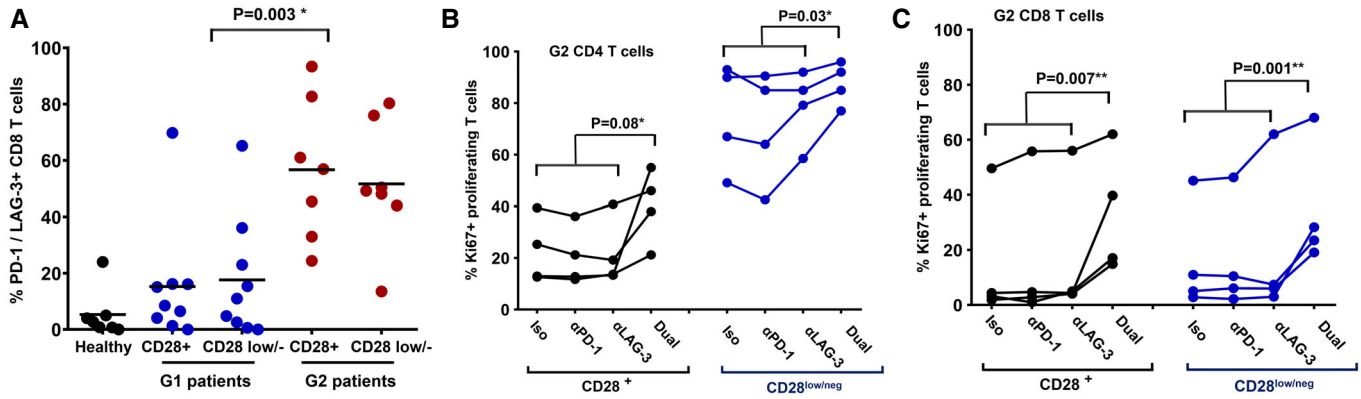

Figure 6. PD-1/LAG-3 co-blockade recovers proliferative capacities of CD4 and CD8 T cells from G2 patients.

A Scatter plots of PD-1/LAG-3-expressing CD8 T cells after activation by A459-SC3 cells in a sample of G1 (n = 9) and G2 (n = 7) patients within CD28^{+} and CD28^{negative} populations as indicated in the figure. Relevant statistical comparisons are shown with the test of Kruskal-Wallis.

B, C Dot-plot representing the percentage of proliferating CD4 T cells (B) and CD8 T cells (C) from a sample of G2 patients before starting immunotherapy, activated *ex vivo* by A549-SC3 cells in the presence of the indicated antibodies. "Dual" represents the addition of both anti-PD-1 and anti-LAG-3 antibodies. Appropriate statistical comparisons are shown within the graph with two-way paired ANOVA. Data from CD28^{+} and CD28^{negative} subsets are represented as indicated.

were necessary but not sufficient for clinical efficacy. As NSCLC patients with high PD-L1 tumor expression benefit from anti-PD-L1/PD-1 blockade therapies (Borghaei *et al*, 2015), we assessed PD-L1 tumor expression and its association to responses in G1 and G2 patient cohorts for whom PD-L1 tumor expression could be determined. G1 patients with PD-L1-positive tumors had a PFS of 70% (> 5%; P = 0.007; Fig 7A). The same benefit was observed when the stratification was extended to include patients with unknown PD-L1 tumor status in our cohort (Fig 7B).

Discussion

Tumor intrinsic and extrinsic factors contribute to the efficacy of PD-L1/PD-1 blockade therapies. So far, not a single factor has been associated with objective responses or progression, suggesting that multiple mechanisms influence clinical responses.

Because PD-L1/PD-1 blocking antibodies are systemically administered, these therapies cause systemic changes in immune cell populations (Kamphorst *et al*, 2017a; Krieg *et al*, 2018). Some of these changes may reflect the efficacy of immunotherapy in patients and could be used for patient stratification. Several studies have been performed to monitor systemic dynamics of immune cell populations, some of them retrospectively and by high-throughput techniques (Hui *et al*, 2017; Kamphorst *et al*, 2017b; Krieg *et al*, 2018). We evaluated responses from fresh blood samples because freezing PBMCs led to a significant alteration in the distribution of immune cell types, and distorted expression patterns of cell surface markers. Hence, sample manipulation had a significant impact on our results, which limited our study to prospective data.

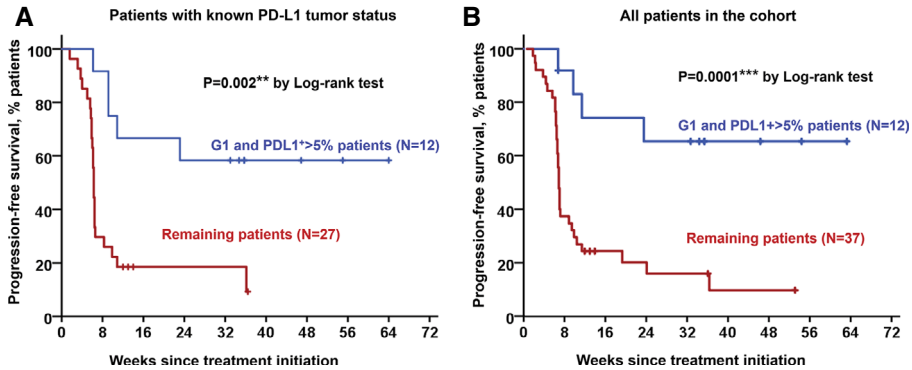

Figure 7. Objective responders are found within G1 patients with PD-L1^{+} tumors.

A Kaplan–Meier plot for PFS in patients undergoing immune checkpoint inhibitor therapies stratified by G1/PD-L1^{+} tumors (blue) and remaining patients for whom their PD-L1 tumor status is known (red).

B Same as in (A) but including all patients in the study cohort. Remaining patients (red) also included G1 patients with PD-L1 low/negative tumors, G1 patients with unknown PD-L1 tumor status, and G2 patients with either PD-L1^{+} or PD-L1-negative tumors.

Source data are available online for this figure.

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Here, we found that the proliferative functionality of systemic CD4 immunity is required for clinical responses to PD-L1/PD-1 blockade therapy. Indeed, it was a differential baseline factor in our cohort of NSCLC patients progressing from conventional therapies. Hence, patients with non-dysfunctional CD4 responses contained all objective responders with a response rate of about 50% (G1 patients), while no objective responses were observed in patients with dysfunctional CD4 T cells (G2 patients). CD4 T-cell dysfunctionality in G2 patients was reflected as strongly impaired proliferation after stimulation, high co-expression of LAG-3/PD-1, and resistance to *ex vivo* and *in vivo* PD-1 monoblockades. As both responders and non-responders contained comparable proportions of lung cancer-specific CD4 and CD8 T cells in our cohort of patients before the start of therapy, the experimental evidence pointed to the baseline intrinsic functionality of CD4 immunity as the key factor in our study. Systemic $CD28^+$ CD4 T cells in G2 patients were not truly exhausted or *bona fide* anergic T cells. No constitutive high-level expression of PD-1 and LAG-3 was observed unless stimulated. They were proficient in multicytokine expression following stimulation. Indeed, CD4 T cells from both G1 and G2 patient cohorts were skewed toward Th17-expressing phenotypes compared to healthy donors. All these characteristics were indicators of systemic CD4 T-cell proliferative dysfunctionality in G2 patients.

Importantly, patients with functional CD4 immunity could be easily identified by having a high proportion of circulating CD4 T_{HD} memory cells. ROC analysis provided a cut-off value of $> 40\%$ CD4 T_{HD} to identify objective responders from freshly analyzed blood samples. It is worth noting that the cut-off value was reduced to 20% in a validation cohort that was independently processed and analyzed by a very different procedure. Importantly, patient classification in G1 or G2 cohorts and their association with clinical responses agreed independently of the protocol utilized. We are well aware that quantification of CD4 T_{HD} cells could be used as a baseline factor for clinical stratification. Proper validation of CD4 T-cell profiling will require protocol standardization for sample manipulation and analyses. In fact, G1 patients with PD-L1-positive tumors exhibited response rates of 70%, which strongly highlights the role of CD4 immunity in clinical responses. However, the main goal of the current study was to understand the contribution of systemic T-cell immunity to PD-L1/PD-1 blockade therapies, rather than providing a predictive biomarker.

The requirement of functional systemic immunity has been previously demonstrated in murine models for the efficacy of other immunotherapy approaches (Spitzer *et al*, 2017), as well as the importance of CD4 T cells for anti-PD-1 immunotherapy (Markowitz *et al*, 2018). These studies are in agreement with our present data in human patients undergoing PD-L1/PD-1 blockade therapies. Indeed, the appearance of a specific murine subtype of CD4 T cell was the main correlator with efficacious responses by administration of anticancer cell immunoglobulins (Spitzer *et al*, 2017). These results together with our data strongly support the need for proficient CD4 responses to achieve efficacious responses.

Immune checkpoint inhibitor therapy aims to recover CD8 cytotoxic responses (Ahmadzadeh *et al*, 2009). To our surprise, all systemic CD8 T cells in patients before the start of immunotherapies were also dysfunctional. Nevertheless, the proliferative capacities of CD8 T cells were recovered during immunotherapy but only in patients with functional CD4 immunity. This was reflected by an expansion of $CD28^+$ cells in agreement with data in murine models (Kamphorst *et al*, 2017b). CD8 dysfunctionality in G2 patients was again correlated with PD-1/LAG-3 co-upregulation. Both CD4 proliferative dysfunctionality and CD8 proliferative dysfunctionality in G2 patients were reversible *ex vivo* by PD-1/LAG-3 co-blockade.

An increasing number of studies are linking PD-1/LAG-3 co-expression in T cells to resistance to anti-PD-L1/PD-1 therapies (Mishra *et al*, 2016; Huang *et al*, 2017; Williams *et al*, 2017; Johnson *et al*, 2018). Our study prompts the clinical evaluation of patients with systemic CD4 T-cell dysfunctionality by PD-1/LAG-3 dual-blockade strategies.

Materials and Methods

Study design

The study was approved by the Ethics Committee at the Hospital Complex of Navarre. Informed consent was obtained from all subjects and all experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Samples were collected by the Blood and Tissue Bank of Navarre, Health Department of Navarre, Spain. Thirty-nine patients diagnosed with non-squamous and 12 with squamous NSCLC were recruited at the Hospital Complex of Navarre (Table EV1). Patients had all progressed to first-line chemotherapy or concurrent chemo-radiotherapy. Eligible patients were 18 years of age or older who agreed to receive immunotherapy targeting PD-1/PD-L1 following the current indications (Table EV1). Tumor PD-L1 expression could be quantified in 39 of these patients before the start of therapies. Measurable disease was not required. The exclusion criteria consisted of concomitant administration of chemotherapy or previous immunotherapy treatment. NSCLC patients had an age of 65 ± 8.9 ($N = 51$). Age-matched healthy donors were recruited from whom written informed consent was also obtained, with an age of 68.60 ± 8 (mean $\pm$ SD, $N = 40$).

Therapy with nivolumab, pembrolizumab, and atezolizumab was provided following current indications (Herbst *et al*, 2016; Horn *et al*, 2017; Rittmeyer *et al*, 2017). 4 ml peripheral blood samples were obtained prior and during immunotherapy before administration of each cycle. PBMCs were isolated as described (Escors *et al*, 2008) and T cells analyzed by flow cytometry. The participation of each patient concluded when a radiological test confirmed response or progression, with the withdrawal of consent or after death of the patient. Tumor responses were evaluated according to RECIST 1.1 (Eisenhauer *et al*, 2009) and Immune-Related Response Criteria (Wolchok *et al*, 2009). Objective responses were confirmed by at least one sequential tumor assessment.

Flow cytometry

Surface and intracellular flow cytometry analyses were performed as described (Karwacz *et al*, 2011; Gato-Canas *et al*, 2017). T cells were immediately isolated and stained. 4 ml blood samples were

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collected from each patient, and PBMCs were isolated by FICOL gradients right after the blood extraction. PBMCs were washed and cells immediately stained with the indicated antibodies in a final volume of 50 μl for 10 min in ice. Cells were washed twice, resuspended in 100 μl of PBS, and analyzed immediately. The following fluorochrome-conjugated antibodies were used at 1:50 dilutions unless otherwise stated: CD4-FITC (clone M-T466, reference 130-080-501, Miltenyi Biotec), CD4-APC-Vio770 (clone M-T466, reference 130-100-455, Miltenyi Biotec), CD4-PECy7 (clone SK3, reference 4129769, BD Biosciences), CD14-VF450 (1:500 dilution, clone 61D3, TONBO), CD3-APC (clone REA613, reference 130-113-135, Miltenyi Biotec), CD27-APC (clone M-T271, reference 130-097-922, Miltenyi Biotec), CD27-PE (clone M-T271, reference 130-093-185, Miltenyi Biotec), CD45RA-FITC (reference 130-098-183, Miltenyi Biotec), CD62L-APC (reference 130-099-252, Miltenyi Biotech), CD28-PECy7 (clone CD28.2, reference 302926, BioLegend), PD-1-PE (clone EH12.2H7, reference 339905, BioLegend), CD8-FITC (clone SDK1, reference 344703, BioLegend), CD8-APC-Cy7(clone RFT-8, reference A15448, Molecular probes by Life technologies), CD57-PE (clone HCD57, reference 322311, BioLegend), H2AX-FITC (1:100 dilution, clone 2F3, reference 613403, BioLegend), LAG-3-PE (clone 11C3C65, reference 369306, BioLegend), IL-2 Alexa Fluor 647 (1:100 dilution, clone MQ1-17H12, reference 500315, BioLegend), IFN γ-APC (1:100 dilution, clone 4S.B3, reference 50256, BioLegend), IFN γ-FITC (1:100 dilution, clone 4S.B3, reference 502506, BioLegend), IL-17A-BV421 (1:100 dilution, clone BL168, reference 512322, BioLegend), IL-17A-Violet 667, clone CZ8-23G1, reference 130-120-554, Miltenyi Biotec), IL-4-PE (1:100 dilution, reference 130-091-647, Miltenyi Biotec), and IL-10-APC (1:100 dilution, reference 130-096-042, Miltenyi Biotec).

Cell culture

Human lung adenocarcinoma A549 cells were a kind gift of Prof Ruben Pio and authenticated by his group, and were grown in standard conditions. They were confirmed to be mycoplasma-free by PCR. These cells were modified with a lentivector encoding a single-chain version of a membrane-bound anti-OKT3 antibody (Arakawa *et al*, 1996). The lentivector expressed the single-chain antibody construct under the control of the SFFV promoter and puromycin resistance from the human ubiquitin promoter in a pDUAL lentivector construct (Karwacz *et al*, 2011). The single-chain antibody construct contained the variable light and heavy OKT3 immunoglobulin sequences separated by a G-S linker fused to a human IgG1 constant region sequence followed by the PD-L1 transmembrane domain.

Monocyte-derived DCs were generated from adherent mononuclear cells in the presence of recombinant GM-CSF and IL-4 as described (Escors *et al*, 2008). DCs were loaded with A549 protein extract obtained after three cycles of freezing/thawing. Loading was carried out overnight, and DCs were matured with 10 ng/ml of IFN-γ before adding T cells in a 1:3 ratio as described (Escors *et al*, 2008).

When indicated, PD-1 (clone EH12.2H7, BioLegend) and LAG-3 (clone 17B4, BioLegend) blocking antibodies were added to cell cultures at a final concentration of 5 μg/ml. When appropriate, T cells were stimulated with plate-bound anti-CD3/anti-CD28 antibodies as described (Liechtenstein *et al*, 2014).

Anti-PD-1 antibody production and purification

To generate an antibody molecularly equivalent to the published sequence of pembrolizumab, cDNAs encoding the published amino acid sequences of the heavy and light immunoglobulin chains (Scapin *et al*, 2015) were cloned and expressed in Chinese hamster ovary (CHO) cells. Supernatants were collected and antibodies purified by affinity chromatography following standard procedures.

Data collection and statistics

T-cell percentages were quantified using FlowJo (Lanna *et al*, 2014, 2017). The percentage of CD4/CD8 T_{HD} (CD28 and CD27 double-negative) and poorly differentiated T cells ($CD28^+$ $CD27^+$) were quantified prior to therapy (baseline) and before administration of each cycle of therapy within CD4 and CD8 cells. Gates in flow cytometry density plots were established taking $CD27^+$ $CD28^+$ T cells as a reference. Data were recorded by M.Z. and separately analyzed thrice by M.Z. and H.A. independently. Cohen's kappa coefficient was utilized to test the inter-rater agreement in classification of immunological profiles ($\kappa = 0.939$).

The mode of action, pharmacokinetics, adverse events, and efficacies of the three PD-L1/PD-1 blocking agents are comparable in NSCLC, which act through the interference with the inhibitory interaction between PD-L1 and PD-1 (Herbst *et al*, 2016; Horn *et al*, 2017; Rittmeyer *et al*, 2017). Treatments administered to the patients were allocated strictly on the basis of their current indications and independently of any variable under study. All data were pre-specified to be pooled to enhance statistical power, and thereby reducing type I errors from testing the hypotheses after *ad hoc* subgrouping into specific PD-L1/PD-1 blockers. The number of patients assured statistical power for Fisher's exact test of 0.95 and superior for Student's *t* and Mann–Whitney tests (G*Power calculator; Faul *et al*, 2009), taking into account that the expected proportion of responders is around 25–35% without stratification (Herbst *et al*, 2016; Horn *et al*, 2017; Rittmeyer *et al*, 2017). Two pre-specified subgroup analyses in the study were contemplated: the first, baseline T-cell values and the second, post-first cycle T-cell changes from baseline. The study protocol contemplated the correlation of these values with responses using Fisher's exact test, paired Student's *t*-tests/repeated-measures ANOVA (if normally distributed) or *U* of Mann–Whitney/Kruskal–Wallis (if not normally distributed, or data with intrinsic high variability). Two-tailed tests were applied with the indicated exceptions (see below).

The percentage of T-cell subsets in untreated cancer patients was normally distributed (Kolmogorov–Smirnov normality test), but not in age-matched healthy donors. Hence, to compare T-cell values between two independent cancer patient groups, two-tailed unpaired Student's *t*-tests were used, while comparisons between healthy subjects and cancer patients were carried out with the Mann–Whitney *U*. Percentages of T-cell populations in treated patients were not normally distributed, so response groups were compared with either Mann–Whitney (comparisons between two independent groups) or Kruskal–Wallis for multi-comparison tests if required. Two-tailed paired *t*-tests were carried out to compare changes in the proportion of $CD28^+$ CD8 T cells between baseline and post-therapy paired groups, and to compare Ki67 expression in T-cell subsets activated with A549-SC3 cells subjected to PD-1 or

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The paper explained

Problem
Over 70% of lung cancer patients progressing from conventional therapies do not respond to PD-L1/PD-1 blockade therapies. The reasons behind this failure are currently unclear.

Results
We studied systemic CD4 immunity as a differential factor for clinical responses to PD-L1/PD-1 blockade therapies. Patients with high percentages of systemic highly differentiated memory CD4 T cells contained all responders. In contrast, patients with low percentages of this subset did not respond to therapy. These patients were refractory to immunotherapy, and their systemic CD4 T cells failed to proliferate following activation. Furthermore, they responded by strongly co-upregulating PD-1 and LAG-3. We demonstrated that these T cells could overcome their proliferative dysfunctionality by PD-1/LAG-3 co-blockade with antibodies.

Impact
Profiling of CD4 T-cell subsets can help identifying patients with a high probability of responding to immunotherapy, especially in combination with PD-L1 tumor expression. Patients with dysfunctional CD4 immunity before starting PD-L1/PD-1 blockade could undergo alternative therapies such as combinations with LAG-3 blocking agents.

LAG-3 blockade. For comparison of paired samples with anti-PD-1/anti-LAG-3 combinations, two-way ANOVA tests with a random criterium (subjects) were used. Fisher's exact test was used to assess the association of the baseline values of T_{HD} cells with clinical responses. The same tests were performed to assess associations between G1/G2 groups with the indicated prognostic variables.

Progression-free survival (PFS) was defined as the time from the starting date of therapy to the date of disease progression or the date of death by any cause, whichever occurred first. PFS was censored on the date of the last tumor assessment demonstrating absence of progressive disease in progression-free and alive patients. PFS rates at 12 and 28 weeks were estimated as the proportion of patients who were free-of-disease progression and alive at 12 and 28 weeks after the initiation of immunotherapies. Patients who dropped out for worsening of disease and did not have a 28-week tumor assessment were considered as having progressive disease. Overall response rate (ORR) was the proportion of patients who achieved best overall response of complete or partial responses.

PFS was represented by Kaplan–Meier plots and log-rank tests utilized to compare cohorts. Hazard ratios were estimated by Cox regression models. Receiver operating characteristic (ROC) analysis was performed with baseline T_{HD} numbers and response/no response as a binary output. Statistical tests were performed with GraphPad Prism 5 and SPSS statistical packages.

Validation dataset

Data from a set of 32 patients were validated in parallel by independent handling, processing, staining, flow cytometry data collection, and analysis. The validation dataset was generated by a technician working in unrelated research themes (A.B.). A very different protocol was used to quantify CD4 T_{HD} cells in the validation set compared to the discovery cohort. For the validation dataset, isolated PBMCs were resuspended in TeXmacs serum-free medium (Miltenyi) and plated on 6-well cell culture plates. Myeloid cells were allowed to adhere overnight, and non-adherent cells were collected, centrifuged, and resuspended and T cells stained with the appropriate antibodies for flow cytometry analyses. ROC analysis was used to establish the cut-off value for the relative percentage of CD4 T_{HD} cells to discriminate G1 versus G2 patients in the validation cohort. *Post hoc* Cohen's kappa coefficient test was used to test the agreement between the discovery cohort versus the validation cohort on classification of G1/G2 patients.

Expanded View for this article is available online.

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Author contributions
MZ designed and carried out experiments, collected data, and analyzed data. HA designed and carried out experiments, collected data, and analyzed data. GF-H recruited patients, collected data, and analyzed clinical data. MJG-G, MG, and AB carried out experiments, collected data, and analyzed data. MM, BH, LT, IM, MJL, AFL, and RV recruited patients, collected data, and analyzed clinical data. RV supervised the clinical staff, recruited patients, and analyzed clinical data. GK conceived the project, supervised non-clinical researchers, analyzed data, and wrote the paper. DE conceived the project, supervised non-clinical researchers, analyzed data, and wrote the paper. All authors participated in the writing of the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

For more information
(i) https://www.cancerresearchuk.org/about-cancer/cancer-in-general/treatment/immunotherapy
(ii) https://www.aecc.es/es/red-social/testimonios/tratamientos-inmunoterapia
(iii) https://www.cancer.org/treatment/treatments-and-side-effects/treatment-types/immunotherapy.html

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