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Tesi sperimentale:

Analysis of potential immunological markers associated with anti-PD-1 therapy in non- small cell lung cancer patients

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1. INTRODUCTION

1.1 Immune checkpoints' role in immune response mechanism

The progress of immuno therapy strategies for oncology is based on the insight that the development of cancer, which involves progressive mutations, is monitored by the immune system. A process termed immunoediting or immune surveillance eliminates most incipient tumors. A subset of cancer mutations generate protein coding sequence changes called neoantigens, which can be processed into peptide antigens; these molecules are presented by the major histocompatibility complex (MHC) and recognized as foreign by T cells. Neoantigens are the primary targets for immunoediting (1). The ultimate amplitude and quality of the T cell-mediated responses, initiated through antigen recognition by the T cell receptor (TCR), are regulated by a balance between co-stimulatory and inhibitory molecules, called immune checkpoints. In particular, inhibitory immune checkpoints are physiologically involved in maintaining self-tolerance, T-cell homeostasis and protect tissue from damage during infections (Fig. 1).

The immune response to cancer evolves over many years and ultimately fails. One reason is that the expression of immune-checkpoint proteins can be dysregulated by tumors as an important immune resistance mechanism (2). The antitumor immune response has many characteristics of a chronic immune response to viral infections, with T cell inhibition mediated by the overexpression of multiple inhibitory immune checkpoint receptors on lymphocytes, such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), T cell immunoglobulin and mucin domain 3 (Tim-3; also known as HAVCR2), lymphocyte activation gene 3 (Lag-3), T cell immunoglobulin and ITIM domain (TIGIT), among others (1). In addition, tumors that evade immunoediting often express molecules that are able to inhibit the antitumor immune response, including programmed cell death 1 ligand 1 (PD-L1), whose upregulation creates a feedback loop which generates continuous PD-1 signaling thus maintains immune suppression.

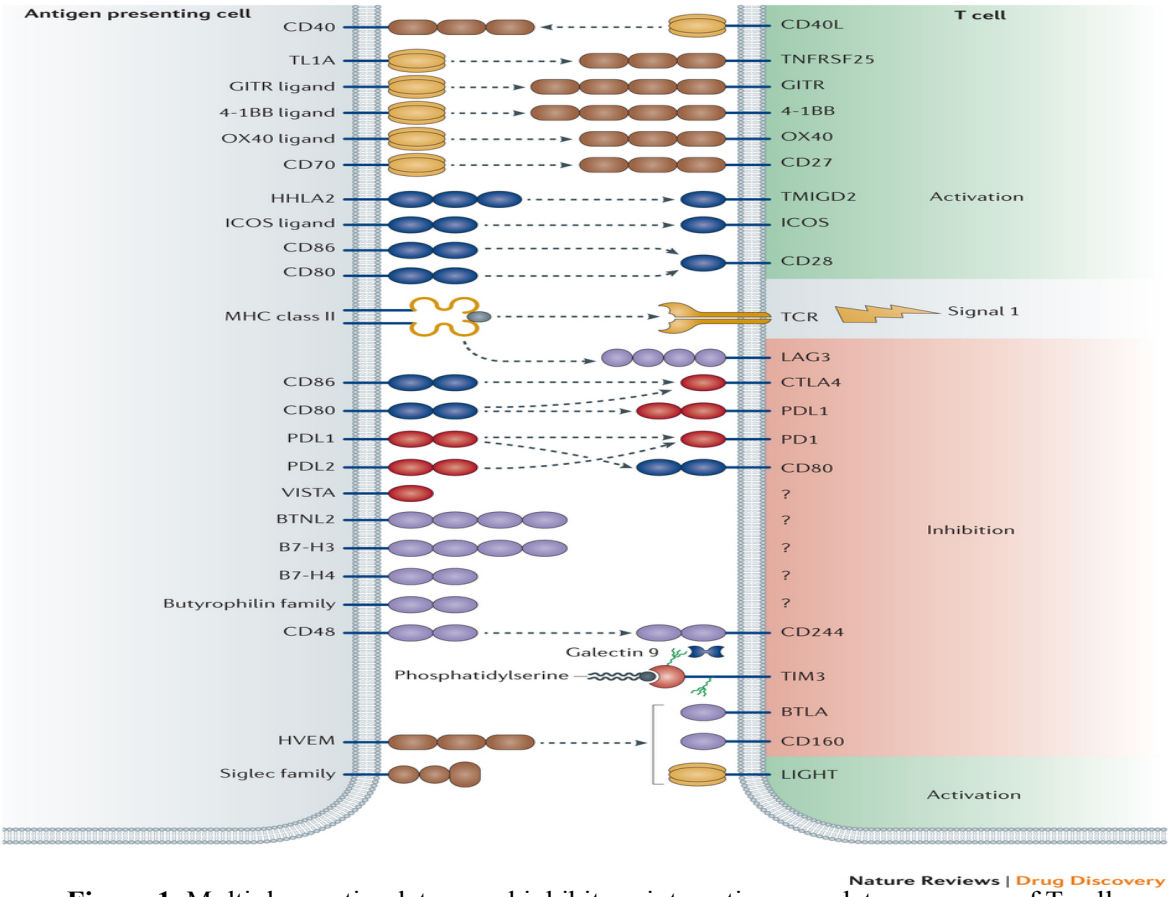


Figure 1. Multiple co-stimulatory and inhibitory interactions regulate responses of T cells. [Mahoney KM Nat Rev Drug Discov 2015]

1.2 Biological role of PD-1

PD-1 is a surface receptor upregulated on immune cells immediately after activation (3). It is expressed by subsets of tolerant T cells, regulatory T (Treg) cells, T follicular helper (TFH) cells, T follicular regulatory (TFR) cells and memory T cells but it could also be expressed on B cells, Natural Killer cells (NKs), some myeloid cells, and even on tumor cells (4). PD-1 is a crucial regulator of adequate T cell responses. It is upregulated on naive T cells following initial antigen-mediated TCR engagement and has a key role in effector T cell activation, differentiation and in the development of immunological memory (5,6). Furthermore, PD-1 is essential for the generation and selection of high-quality, high-affinity antibodies by regulating the properties and abundance of antigen-stimulated $CD4^+$ T cells (7).

The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM); these domains act by recruiting phosphatases, including SHP2 (also known as PTPN11) and SHP1, when PD-1 engaged with its ligands (8).

SHP2 and SHP1 dephosphorylate kinases and antagonize positive signals that occur through the TCR and CD28 stimulation, affecting downstream signaling pathways. In particular, they can prevent lymphocyte-specific protein tyrosine kinase (LCK)-mediated phosphorylation of the protein kinase ZAP70, affecting the TCR signaling pathway. In addition, PD-1 can inhibit CD28-induced activation of Phosphatidylinositide 3 Kinase (PI3K), leading to reduced AKT (also known as PKB, Protein Kinase B) and Mammalian Target of Rapamycin (mTOR) activation, implicating PD-1 in survival, proliferation and altered metabolism. PD-1 can also modulate the Ras pathway influencing the cell cycle. Together, these alterations result in decreased activation of different transcription factors as NFAT, NF-kB and AP-1 (5).
Of note Hui and his colleagues demonstrated that PD-1 recruits preferentially SHP2 and that the co-receptor CD28 is the main substrate for PD-1-SHP2 dephosphorylation. Interestingly, although costimulation via CD28 is usually correlated with naïve T cell priming, there is increasing evidence that it may have a role at later stages of immune response in chronic viral infection and in cancer (9).
Alternative ways of PD-1-mediated inhibition are to increase transcription factor BATF, involved in downregulation of effector programs, and to affect T cell motility, stability and/or duration of T cell – antigen-presenting cell (APC) interactions (6,10) (Fig. 2).
PD-1 could be expressed on activated $CD8^+$ T cells and $CD4^+$ T cells, including Tregs: interestingly, PD-1 upregulated on Treg cells and its interaction with PD-1 ligand on effector T cells may represent one cause for the potent T cell suppression and proposes the role of PD-1 on Treg cells (11).

PD-1 could interact with two different ligands: PD-L1 (also known as B7-H1 and CD274) and PD-L2 (also known as B7-DC and CD273), both B7 family members. PD-L1 is widely expressed by many different cell types and is found on both haematopoietic cells (including T cells, B cells, dendritic cells (DCs) and macrophages) and on non-haematopoietic cells (including vascular and stromal endothelial cells, pancreatic islet cells, placental syncytiotrophoblasts and keratinocytes) but notably also on tumor cells. Pro-inflammatory signals, such as interferon (IFN)-γ, produced by activated T cells, are involved in the upregulation of PD-L1 on tumor cells and on cells of the tumor microenvironment (12). By contrast, PD-L2 expression is much more restricted and is expressed predominantly by DCs, macrophages and B cell populations (6).

B7 family members are negative regulators of immune response, physiologically important in peripheral tolerance. For example, it has been demonstrated that PD-L1 on pancreatic islets could prevent diabetes inducing an inhibitory signal to self-reactive $CD4^+$ T cells (13).

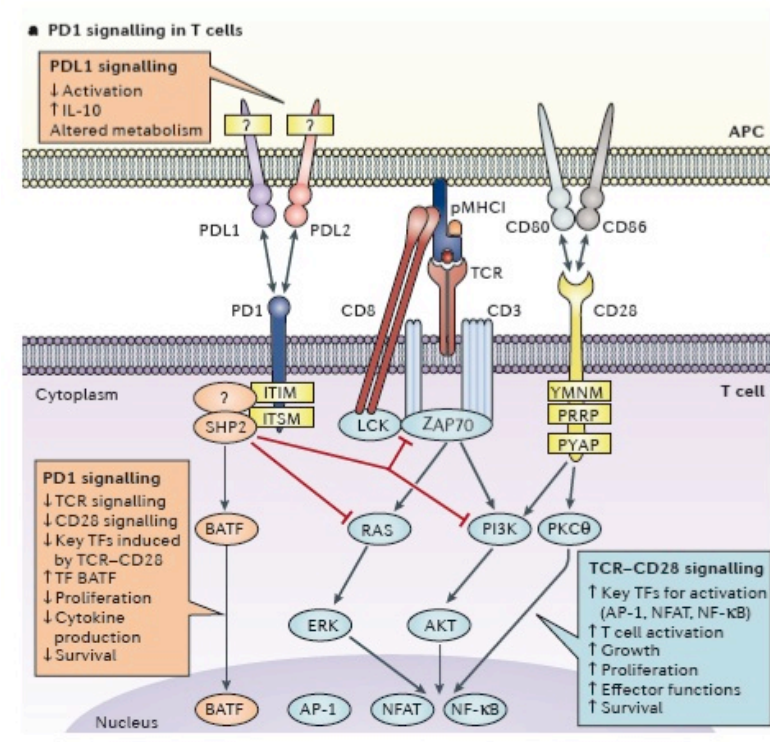


Figure 2. Mechanism of PD-1 signaling in T cells.
Signalling motifs are indicated in yellow boxes; circles indicate key proteins involved in signalling pathways and main transcription factors. Abbreviations: ITIM, immunoreceptor tyrosine-based inhibitory motif; ITSM, immunoreceptor tyrosine-based switch motif; TCR, T cell receptor; pMHCI, peptide-Major Histocompatibility Complex class I; SHP2, Src homology region 2 domain-containing phosphatase; PI3K, Phosphatidylinositide 3 Kinase ; ZAP70, Zeta-chain-associated protein kinase 70; LCK, lymphocyte-specific protein tyrosine kinase; BATF, basic leucine zipper transcription factor, ATF-like; AP-1, activator protein 1; NFAT, nuclear factor of activated T cells and NF-κB, nuclear factor-κB. [Sharpe AH, Pauken KE Nat Rev Immunol 2018]

Inhibitory signals are used in many ways to maintain balance in the immune system. Perturbing the PD-1 pathway can profoundly impact host physiology. Mice genetically deficient in *Pdcd1* (which encodes PD-1) develop accelerated autoimmunity, as lupus-like disease, arthritis and cardiomyopathies (14,15). Conversely, the persistent antigen presentation and inflammatory signals occurring during chronic infections and cancer

induce continue activation of immune cells and upregulation of PD-1 on their surface. This persistent stimulation causes an altered functional state of T cells, called exhaustion.

Several studies demonstrate that exhausted T cells are not inert and that they can be reinvigorated (10,16). Thus, blocking the PD-1 pathway can improve T cell functions and reduce viral load and tumor burden (6).
Another important mechanism involved in PD-1 expression is the epigenetic regulation (occurring during differentiation): Ghoneim et al. demonstrated that de novo DNA methylation of *Pdcd 1* locus is involved in exhaustion and inhibits anti-PD-1-mediated reinvigoration (17).
Increasing evidence of a connection between PD-1 expression and metabolism are currently emerging. TCR and CD28 signaling usually induce enhance glucose and glutamine metabolism. PD-1 engagement can modulate metabolic reprogramming during early phases of T cell activation by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation (18). It has also been demonstrated that in tumor microenvironment occurs a metabolic competition through glucose sequestration, thus inducing hyporesponsive T cells and tumor progression (19). Recent studies demonstrated that mitochondrial activation by bezafibrate, an agonists of peroxisome proliferator–activated receptor gamma (PPARγ) coactivator 1-a (PGC-1a), had synergistic effects with a PD-1–blockade in a mouse tumor model, since T cell activation and differentiation are associated with mitochondrial energy metabolism (20).

1.3 Biological role of others immune checkpoints

CTLA-4. Together with PD-1 is the inhibitory immune checkpoint molecule most actively studied in the field of clinical cancer therapy. It is expressed by T cells and $CD4^+$ Tregs. In particular, CTLA-4 is a target gene of Foxp3, the transcription factor characteristic of Treg cell lineage (21). CTLA-4 counteracts CD28 pathway and produces an inhibitory signal, through competition with the costimulatory molecule CD28 for the B7 ligands B7-1 (CD80) and B7-2 (CD86), for which CTLA-4 has higher avidity and affinity. It has a role in early stages of activation in secondary lymphoid organs and its expression level depends on the amplitude of the initial TCR-mediated

signaling. In addition, CTLA-4 also attenuates T-cell activation in peripheral tissues given that B7 ligands are constitutively expressed by antigen-presenting cells (APC) to varying degrees, but can also be expressed by activated T cells (22). In naive T cells CTLA-4 molecules are stored in intracellular vesicles, but after TCR engagement they are immediately transported to cell surface (2). Although the exact pathway of CTLA-4 is still under debate, different studies suggest that activation of the protein phosphatases, SHP2 and PP2A, is important in counteracting kinase signals that are induced by TCR and CD28 (23). However, CTLA-4 also acts through the sequestration of CD80 and CD86 from CD28 engagement. The central role of CTLA-4 for keeping T cell activation under control is dramatically demonstrated by the fatal autoimmunity observed in CTLA-4-deficient mice, due to hyperactivation of lymphocytes (24,25).

CTLA-4 had been the first immune checkpoint receptor to be clinically targeted. Ipilimumab (an anti-CTLA-4 monoclonal antibody) is the first-generation immune checkpoint inhibitor, approved by US Food and Drug Administration (FDA) in 2011. Different studies demonstrated its efficacy in melanoma patients, with 10% of objective clinical response (26) and improved OS (27). Despite the remarkable progress achieved with monotherapies, there is a urgent need to improve efficacy across tumor types, also because the high immune-related toxicities that occur with this drug (26). To date several clinical trials propose combination therapies with ipilimumab, in lower doses than monotherapy, and other immune checkpoint blockers, in order to optimize antitumor treatment (28).

Tim-3. Tim-3 is an inhibitory receptor first identified as a transmembrane protein expressed on $CD4^+$ T helper type 1, but then described also on $CD8^+$ T cells, on $CD4^+$ Treg cells and on innate immune cells (DC, NK cells, monocytes)(29,30). The known ligands of Tim-3 are galectin-9 (Gal-9), high mobility group protein B1 (HMGB1), carcinoembryonic antigen cell adhesion molecule 1 (Ceacam-1), and phosphatidylserine (PtdSer) (29). Current data show that the Tim-3 cytoplasmic tail contains five conserved tyrosine residues that can be phosphorylated by either Src kinases. Tim-3 interacts with multiple components of the TCR complex and influences the balance of Bat-3 (HLA-B associated transcript 3) versus Fyn, both of which bind to the same region on the cytoplasmic tail of Tim-3. Ligand binding triggers the dissociation of Bat-3 from the cytoplasmic domain of Tim-3, thus allowing Fyn to bind at the same site and promote

the inhibitory function of Tim-3 (29). This receptor plays a positive role in protection from autoimmunity but it also contributes dampening immune response against tumor and chronic infections. Tim-3 is highly co-expressed with PD-1 on a subset of $CD8^+$ T cells exhibiting dysfunctional or exhausted phenotype, but its alone presence is not sufficient to induce T cell exhaustion process (30). However, expression of Tim-3 could be sufficient to drive resistance to anti PD-1/PD-L1 therapy and for this reason combinational therapies are necessary in the future.
Furthermore, although Tim-3 is primarily considered as a marker of T-cell activation and exhaustion, Tim-3 also has a role to attenuate NK cell cytotoxicity (31).

Lag-3. It's another checkpoint inhibitory receptor, upregulated on $CD4^+$ T cells, in particular on Tregs, on $CD8^+$ T cells and on a subset of NK cells (32). Lag-3 binds to MHC class II molecules with higher affinity than CD4 co-receptor. Currently, additional ligands for Lag-3 are under research. Along this line, it has been suggested that LSECtin, a member of the DC-SIGN family of molecules expressed in the liver and also on many tumors, is another ligand for Lag-3 (33). The cytoplasmic tail of Lag-3 contains a unique KIEELE motif that is essential for the inhibitory function of Lag-3 (29).
In mouse model, Lag-3 expression seemed to be strongly correlated with the severity of infection (34). As described for Tim-3, also co-expression of Lag-3 with PD-1 is characteristic of dysfunctional or exhausted $CD8^+$ T cells (35). Dual blockade of Lag-3 and PD-1 could potentially reverse exhaustion and anergy among tumour-specific $CD8^+$ T cells and virus-specific $CD8^+$ T cells in the setting of chronic infection (2).

TIGIT. T cell immunoglobulin and ITIM domain is a co-inhibitory receptor, member of CD28 family, expressed on activated T cells but also found on NK cells, memory T cells, a subset of Treg cells as well as follicular T helper (Tfh) cells. The main ligand for TIGIT is CD155 (PVR) but it could also bind CD112 (PVRL2, nectin-2), both expressed on APCs, T cells and various cell types including tumor cells (29).
Interestingly, CD226 (DNAM-1) and CD96 (Tactile) interact with the same ligands and define a pathway, together with TIGIT, in which CD226 delivers a positive co-stimulatory signal, in association with the integrin LFA-1 (36). On the contrary, CD96 and TIGIT deliver inhibitory signals (37) by ITIM motifs in their cytoplasmic tails. CD155 and TIGIT are homodimers on the cell surface, and dimerization is essential for

their appropriate function (29). As CD155 is highly expressed on cancer cells, the binding of TIGIT negatively regulates antitumor responses. As mentioned above combined immune checkpoint blockade therapies may restore antitumor immunity in a more efficient way. Thus, TIGIT not only synergizes with PD-1 but also with Tim-3 in impairing protective antitumor responses (38,39). Interestingly, a recent study demonstrated that the blockade of TIGIT prevents NK exhaustion and elicits tumor-specific T cell immunity in an NK cell-dependent manner (40).

1.4 Phenotypic, functional and molecular features of T-cell exhaustion

As we described before, PD-1 is a crucial regulator of correct immune responses. Normally, following the peak of effector expansion, the foreign antigen is cleared and PD-1 expression is down regulated. After the clearance of antigen, most activated specific T cells die, but a subset persists and transitions into the memory T cell pool. By contrast, during chronic infection and cancer, antigen and inflammation persist after the effector phase and PD-1 expression remains high, inducing an altered differentiation and functional state, called exhaustion.

Exhausted T cells were first described in chronic murine lymphocytic choriomeningitis virus (LCMV) and then observed also in human infections and cancers. Features of exhausted T cells are overexpression of multiple inhibitory receptors, including PD-1, Tim-3, Lag-3, 2B4, CD160 and TIGIT, altered metabolism and progressive loss of effector function, which typically arise in a hierarchical manner (10). The first steps are the reduced IL-2 production, proliferation and cytolytic activity, followed by defects in producing various cytokines, such as IFN-γ and tumor necrosis factor (TNF)-α, and chemokines as well as in degranulation (10).

Of note, although PD-1 has been predominantly associated with T exhaustion, it is important to remember that it is mainly an effector marker, not a specific exhaustion molecule. Thus, high amounts of PD-1 do not necessarily correlate with more exhausted phenotype (41).

Different studies demonstrated that the severity of exhaustion strongly depends on the level of antigen stimulation and that exhausted T cells did not persist in antigen-free

recipients (16,42). Early studies in mouse model revealed that $CD8^+$ T cells infected with LCMV only for a week, keep the ability to form fully functional memory T cells when the antigen is removed. By contrast, if the antigen presentation persists for 2-4 week, T cell exhaustion develops and becomes established. At this time, removing antigens will not be sufficient to restore T cell functionality and memory differentiation (43,44).

While memory $CD8^+$ T cells can be maintained efficiently long term without antigen stimulation via IL-7- and IL-15-driven mediated self-renewal, exhausted T cells show a downregulation of CD127 (α-chain of IL-7 receptor) and CD122 (β-chain of IL-2 and IL-15 receptors), and are unable to persist in antigen-independent manner.

Exhausted T cells are not inert, since they are characterized by a hyporesponsive state but not complete functional unresponsiveness, thus exhaustion is not irreversible. It has been demonstrated that the blockade of PD-1/PD-L1 axis could partly reverse exhaustion, restoring effector functions, proliferation, and enhancing viral and tumor control (45). Interestingly, recent studies demonstrated that the potential of anti–PD-1/PD-L1 therapy to restore antiviral and antitumor T cell responses depends on CD28 expression by T cells (46). Blockade of CD28-binding ligands (CD80, CD86) has also been shown to completely reduce the ability of PD-1/PD-L1 blockers to prevent T cell exhaustion (46).

In mouse model, two different subsets of $CD8^+$ exhausted T cells could be defined based on the potential rescue by PD-1/PD-L1 checkpoint blockers. In chronic LCMV infection, a pool of PD-1^{low} T-bet^{high} T cells, named progenitor subset, demonstrated higher potential of reinvigoration, with higher residual proliferation capacity and effector cytokines production, while PD-1^{high} $Eomes^{high}$ T cells were defined as terminally differentiated, since they did not respond to anti-PD-L1 (47). However, these distinct subsets based on Eomes, T-bet and PD-1 expression could not be transferred to humans, since heterogeneity within $CD8^+$ exhausted T cells is still unclear.

Another important transcription factor involved in T cell exhaustion is B lymphocyte-induced maturation protein-1 (Blimp-1), a negative regulator of B and T terminal differentiation. Upregulation of Blimp-1 is associated with high levels of various inhibitory receptors as PD-1, Lag-3 and CD160 in exhausted T cells and its deletion could restore their expression and induce memory differentiation (48).

Nevertheless, various studies are focusing on the way blocking PD-1/PD-L1 pathway could affect transcriptional programs, cellular and epigenetic changes of exhausted T cells. Pauken et al demonstrated that anti-PD-L1 treatment of exhausted T cells in chronic infected mice induced a transcriptional program more similar to effector than memory T cells, largely driven by cell-cycle pathway, metabolic alterations and effector genes transcription (42). Moreover, exhausted T cells treated with anti-PD-L1 showed increased IL-7 signaling. Nevertheless, if antigen persisted even after cessation of blockade, exhausted T cells with or without PD-L1 treatment showed similar transcriptomic patterns, due to inability of the checkpoint inhibitor to restore memory-like phenotype or reprogram the epigenetic landscape of exhausted T cells into effector or memory T cells.

In conclusion, there are increasing evidence suggesting that exhausted T cells are a distinct lineage of $CD8^+$ T cells, since they acquire a distinct epigenetic profile that couldn't be modify by PD-1/PD-L1 blockade. For this reason immune checkpoint inhibitors' efficacy may be restricted, as epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade (42).

1.5 PD-1 expression on NK cells

Natural killer lymphocytes are cells of the innate immunity that play a fundamental role in antiviral and antitumor responses (49).

Since their discovery in the 1970s, different studies in mice and humans demonstrated an anti-tumor activity of NK cells. For example, the infiltration of NK cells into renal cell cancer (RCC) is associated with a favorable prognosis (50) and in melanoma has been shown to positively correlate with melanocytic lesion regression (51).

The process of NK cell activation is the result of a fine balance between signals mediated by an array of triggering and inhibitory surface receptors (52-54). NK cell receptors involved in tumor cell killing include the HLA class I-specific inhibitory receptors (i.e. KIRs and CD94/NKG2A) and major activating NK receptors (including NKp30, NKp46, NKp44, NKG2D and DNAM-1). In the absence of inhibitory signals the interaction between activating receptors and their specific ligands on tumor cells

results in NK cell triggering and target cell lysis. The main cellular ligands of activating NK receptors include MICA/B, ULBPs (recognized by NKG2D) (55,56), Nectin-2 and PVR (recognized by DNAM-1) (36), B7H6 (recognized by NKp30) (57,58) and the isoform of the mixed-lineage leukemia-5 protein (MLL5) (recognized by NKp44) (59). In most instances, these ligands are not (or only marginally) expressed by normal resting cells while they become highly expressed on tumor cells.

It has recently been shown that also NK cells can express PD-1 and can acquire an exhausted phenotype. In multiple myeloma (MM) patients, in which disease progression is associated with a gradual decline of immune cell function, has been demonstrated a role of PD-1/PD-L1 pathway in the NK cell immune response against MM and that an anti-PD-1 antibody could enhance NK function against tumor cells (60).

Another paper reports that PD-1 was upregulated on circulating NK cells of patients with Kaposi sarcoma (KS) and associated with strongly altered functional capacities. PD-1 was expressed in a sub-population of activated, mature $CD56^{dim}CD16^{pos}$ NK cells with otherwise normal expression of NK surface receptors. $PD\text{-}1^{pos}$ NK cells from KS patients were hyporesponsive *ex vivo* following direct triggering of NCR or CD16 activating receptors, or short stimulation with NK cell targets. $PD\text{-}1^{pos}$ NK cells failed to degranulate and release IFN-γ, but exogenous IL-2 or IL-15 restored this defect (61). The expression of PD-1 on mature $CD56^{dim}CD16^{pos}$ NK cells subset had been confirmed also in peripheral blood NK cells from ovarian cancer patients. However, the proportions of such cells were much higher in the ascitic fluid, suggesting that the tumor microenvironment may be responsible for the *de novo* expression of PD-1 on NK cells. Interestingly, all individuals with $PD\text{-}1^{+}$ NK cells in their peripheral blood were seropositive for cytomegalovirus (CMV) (62).

Finally, $CD56^{bright}CD16^{-}$ NK cells have been reported to display high levels of PD-1 in patients with lymphoma, suggesting the possibility of inhibition by malignant B cells and PDL-1/PD-L2-expressing tumor associated macrophages (63).

Therapeutic PD-1 blockade may be a strategy for circumventing tumor escape not only from the T cell-mediated, but also the NK cell-mediated immune surveillance.

1.6 PD-1/PD-L1 inhibition in antitumor therapy and potential biomarker associated with response

Since the interaction of PD-1/PD-L1 molecules has a major role in the inhibition of effector T cell functions (2), targeting the immuno-regulatory axis PD-1/PD-L1 represents the most significant approach to restore T cell–mediated antitumor immunity. Recently blockade of this pathway has become a corner stone in the current management of several different cancers, in particular advanced non-small cell lung cancer (NSCLC) and melanoma. Several compounds designed to disrupt this axis are currently available in clinical practice as Immune Checkpoint Inhibitors (ICI). Such agents include the anti-PD-1 monoclonal antibodies nivolumab and pembrolizumab, as well as the anti-PD-L1 monoclonal antibodies atezolizumab, avelumab and durvalumab (64-68).

In spite of the impressive results obtained from these therapies, a large proportion of patients still not experience clinical benefit.

For this reason a great effort has been put to identify the most appropriate candidates for PD-1/PD-L1 blockade among patients. However, the identification of robust biomarkers able to predict either clinical response or resistance remains elusive. The most deeply investigated biomarker is PD-L1, evaluated by immunohistochemistry in tumor tissue. Nevertheless, several trials showed some inconsistencies with regards to the value of PD-L1 expression as predictor of outcomes (69). Beyond PD-L1, there is an ongoing effort to identify biomarkers related to the immune system, which may provide additional mechanistic insights into the ways anti-immune checkpoint antibodies exert their effects (70-74).

Tumor-infiltrating lymphocytes (TILs) have been shown to represent a key element influencing the behavior of human tumors (75) thus the relative abundance and phenotype of specific subsets of TILs have been extensively investigated as potential biomarkers for ICIs (76-78). However, this approach is often unfeasible for advanced lung cancer patients because of the limited availability of patient material.

For this reason, a growing interest has developed in circulating immune cells as possible biomarkers for outcomes with ICIs in cancer patients. Current research on this field is active to understand whether measurements in blood may inform, or at least approximate, about the state of the immune response at the tumor site prior to treatment.

On this regard, it has been found that circulating $CD8^+PD\text{-}1^+$, but not $CD8^+PD\text{-}1^-$ T cells, were enriched in T cells specific for patient tumor-antigens and showed direct tumor recognition, implying that the circulating $CD8^+PD\text{-}1^+$ lymphocytes could partially mirror tumor-resident antitumor lymphocytes (79).

Recent studies have further demonstrated that sampling peripheral blood lymphocytes may provide insights into the ongoing immune responses induced by ICI. In murine model of melanoma it has been demonstrated that PD-L1 blockade could partially restore exhausted T cells, inducing a T cell reinvigoration identified as a *de novo* expression of the proliferation marker KI-67, as well as an increased $CD8^+$ T cell to T regulatory cells ratio (71). Accordingly, PD-1 blockade in NSCLC patients results in an increase of $KI\text{-}67^+PD\text{-}1^+CD8^+$ T cells, characterized by an effector-like phenotype ($CD45RA^-$ $CCR7^-$, $CD38^+$, HLA DR^+) (80).

Taking into account the complex interactions between the immune system and cancer, little is known about the dynamics of systemic chemokines and cytokines serum levels in anti-PD-1/ PD-L1 blockade. Several studies revealed that circulating cytokine values correlate with responses to immune checkpoint inhibitors. A recent study in NSCLC patients described that decreasing plasma CXCL2 levels and increasing plasma matrix metalloproteinase-2 (MMP2) levels, after treatment with PD-1 inhibitor, were significantly associated with improved PFS, and that most of the long responders to anti-PD-1 therapy maintained these changes in CXCL2 and MMP2 during the course of treatment (81).

Moreover, Sanmamed et al. showed serum IL-8 levels to be highly correlated with tumor burden changes in metastatic melanoma and NSCLC patients during treatment with anti-PD-1/anti-CTLA-4 therapy. They reported that overall survival (OS) was significant longer in PD-1-treated patients presenting early decreases in serum IL-8 levels than in patients showing early increases (82). Another study on advanced melanoma patients treated with nivolumab reported that pretreatment serum IFN-γ, IL-6, and IL-10 levels were significantly higher in those with tumor progression (83). The effects of anti-PD-1, anti-CTLA-4 or combination of these immune checkpoint blockers (Combo blockade) demonstrated that each form of treatment was associated with distinct pattern of systemic changes in cytokines. In addition, Das R. and colleagues described that the levels of IL1α were increased following anti-PD1 and Combo

blockade while IFN-γ-induced protein 10 (IP-10, known also as CXCL10) levels were increased following anti-PD1, anti-CTLA4, and Combo blockade (84).

1.7 Aims of the study

The goals of our study were :

1) to evaluate the signature of the immune system during nivolumab therapy in a cohort of patients affected by advanced NSCLC. To this aim, we performed a high-dimensional flow cytometry analysis to probe the distribution of lymphocyte subsets in the peripheral blood of NSCLC patients before and during anti-PD-1 immunotherapy, and to verify whether peculiar immune cell signatures could effectively be related to the clinical outcomes.

2) to profile changes in plasma levels of a range of chemokines, cytokines, and growth factors in order to correlate them with clinical outcome to nivolumab therapy. To this aim, we performed the analysis of a panel of various soluble immune mediators using Luminex Technology, and examined the association with nivolumab therapy.

2. PATIENTS AND METHODS

2.1 Study population and assessment of clinical outcomes

The study was designed as part of a mono-institutional translational research within our Institution. The study included patients receiving nivolumab for advanced NSCLC within the global Italian nivolumab Expanded Access Program (EAP), which was designed to allow patients affected by advanced NSCLC to receive nivolumab in the timespan between its registration and its availability in Italy as therapeutic standard (NCT02475382). The main eligibility criteria for the treatment with nivolumab included cytological or histological diagnosis of advanced NSCLC, progression after at least one line of platinum-based chemotherapy for advanced disease, Eastern Cooperative Oncology Group Performance Status (ECOG-PS) ≤ 2; the main exclusion criteria included the necessity to assume systemic corticosteroid treatment at a dose > 10 mg/die of prednisone (or equivalent) and the presence of uncontrolled brain metastases. Notably, the determination of PD-L1 expression was not required for the inclusion within EAP. Eligible patients received nivolumab at 3 mg/Kg every 14 days until death or unacceptable toxicity, or up to 96 weeks from the first administration; in case of progressive disease (PD), treatment continuation was allowed if clinical benefit was perceived. Those patients who were candidate to receive nivolumab within the Italian EAP at our Institution were asked to participate to an additional translational research designed to explore potential predictive biomarkers of outcomes with nivolumab; the study was approved by our local Ethical Committee.

2.2. Response assessment

The enrolled patients underwent disease assessment after every 4 administrations of nivolumab. Response assessment was performed by using the Response Evaluation Criteria in Solid Tumors (RECIST) v. 1.1; additionally, since RECIST could underestimate the activity of ICI, an additional response assessment was performed by using the immune-related response criteria (irRC) (85). In case of PD according to either RECIST or irRC, the subsequent CT scan was performed after 2 cycles instead of 4 for confirmation of progression. The best overall response (BOR)

achieved during the whole treatment with nivolumab was recorded for both RECIST and irRC according to the following categories: complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD); those patients who died before undergoing at least one radiologic response assessment were categorized as early death (ED). Based on these categories, for our analyses, patients were then clustered in: i) controlled disease (CD) group, which comprised every patient displaying CR, PR, SD, or ii) progressive disease group (PD). Notably, ED patients were not considered within the PD group, apart from one longitudinal analysis reported in Figure 8.

Overall survival (OS) time was recorded from the first administration of nivolumab to the date of death or date at last clinical examination. PFS time was recorded from the first administration to the date of progression according to RECIST (RECIST-PFS) or irRC (irRC-PFS) or date at last clinical examination. If a patient died before experiencing disease progression, PFS was set equal to OS time.

2.3. Blood collection and isolation of peripheral blood mononuclear cells

Blood samples were collected before each administration of nivolumab. Thus, we analyzed blood samples at baseline (T0) and at the subsequent collection timings (every 15 days) defined as T1, T2, T3, up to 4 therapy cycles (Fig.3). The rationale for limiting the number of samples to 4 for each patient was to evaluate the early landscape of circulating immune cells from baseline to the time of the first radiologic assessment (planned after the fourth administration of nivolumab).

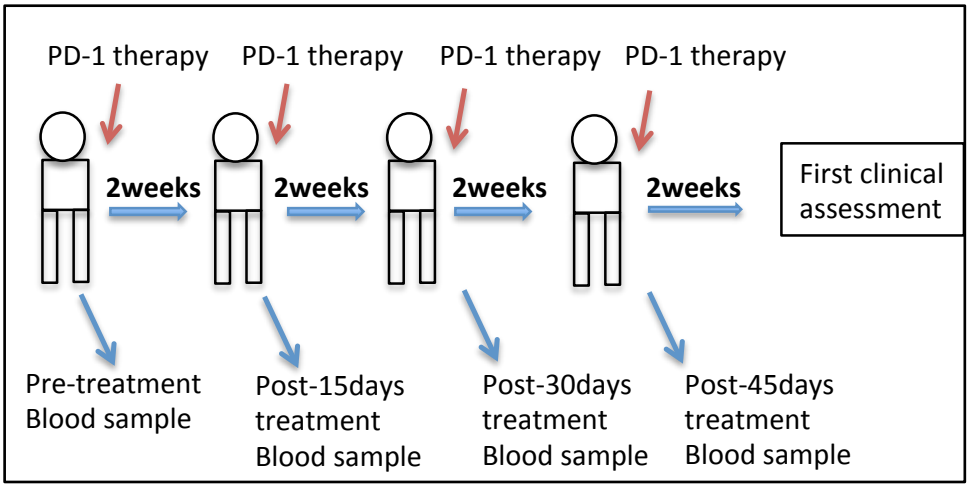


Figure 3. Study design

Blood samples were collected using EDTA as anti-coagulant. Upon collection, plasma was separated by a first centrifugation step at 1800 rpm at 4 °C for 10 min. The supernatant was carefully collected and centrifuged a second time at 1800 rpm at 4 °C for 10 min; finally, the supernatants were made to aliquots and stored in -20°C until assayed.

After plasma separation, blood samples were quickly processed by Ficoll-Hypaque (Cedarlane) density gradient centrifugation to isolate peripheral blood mononuclear cells (PBMCs). PBMCs were then counted, aliquoted and stored in liquid nitrogen for subsequent flow cytometry analyses.

2.4 Flow cytometry

To comprehensively investigate the phenotype of blood lymphocyte populations, PBMCs were thawed and incubated in complete medium (RPMI 1640 supplemented with 10% FCS, Penicillin /Streptomycin) overnight at 37°C (79). Then samples were washed, resuspended in 50 µl PBS and then stained with four different multicolor staining panels. Monoclonal antibodies used for the staining are reported in detail in Table 1. For the detection of surface markers, cells were incubated with a combination of antibodies for 20-30 min, at 4°C. For intranuclear antigens detection, cells were washed twice with PBS after surface staining and then additionally fixed and permeabilized using the Fix/Perm solution (eBioscience) for 30 min at room temperature. After washing, cells were resuspended in 1× Permeabilization Buffer (eBioscience) containing a mixture of antibodies for 40 min at room temperature.

Samples were then acquired using Gallios (Beckman Coulter) flow cytometer, and data analyzed with FlowJo 10.3 software (TreeStar Inc.). Appropriate forward/side scatter and live cell selection with LIVE/DEAD FixableAqua Dead dye (Invitrogen, L34957, dil: 1:500) or 7Amino-Actinomycin D (BD, 559925,dil 1:20) was performed to identify different peripheral lymphocyte sub-populations. Staining panels and representative gating strategies used to identify various PBMC subsets are shown in Figure 4.

Antigen	Fluorocrome	Clone	Source	Dilution	Code
7AAD			BD	1:20	559925
FixableAqua Dead dye			Invitrogen	1:500	L34957
CD127	PE/ DAZZLE	A019D5	Biolegend	1:100	351336
CD25	BV421	M-A251	BD	1:25	562442
CD3	APC-R700	UCHT1	BD	1:100	565119
CD3	VioBlue	BW264/56	MILTENYI	1:100	130-094-363
CD39	BB515	TU66	BD	1:25	565469
CD4	PC7	SFCI12T4D11	Coulter	1:200	737660
CD45	APC-H7	2D1	BD	1:100	560178
CD56	APC	AF12-7H3	MILTENYI	1:200	130-090-843
CD56	PC7	N901 (HLDA6)	Coulter	1:100	A21692
CD69	PE-CF594	FN50	BD	1:50	562617
CD8	APC	RPA-T8	BD	1:100	561952/ 561953
EOMES	FITC	WD1928	eBioscience	1:50	11-4877-42
FOX P3	AlexaFluor 647	259D/C7	BD	1:50	560889/ 560045
Granzyme B	PE- CF594	GB11	BD	1:200	562462
KI-67	Per-CP-Cy 5.5	B56	BD	1:100	561284
KLRG 1	PE-Cy 7	2F1/KLRG1	Biolegend	1:75	138415/6
PD-1	PE	J105	eBioscience	1:75	12-2799-42
TIM-3	BV421	7D3	BD	1:200	565563

Table 1. Antibodies used for flow cytometry

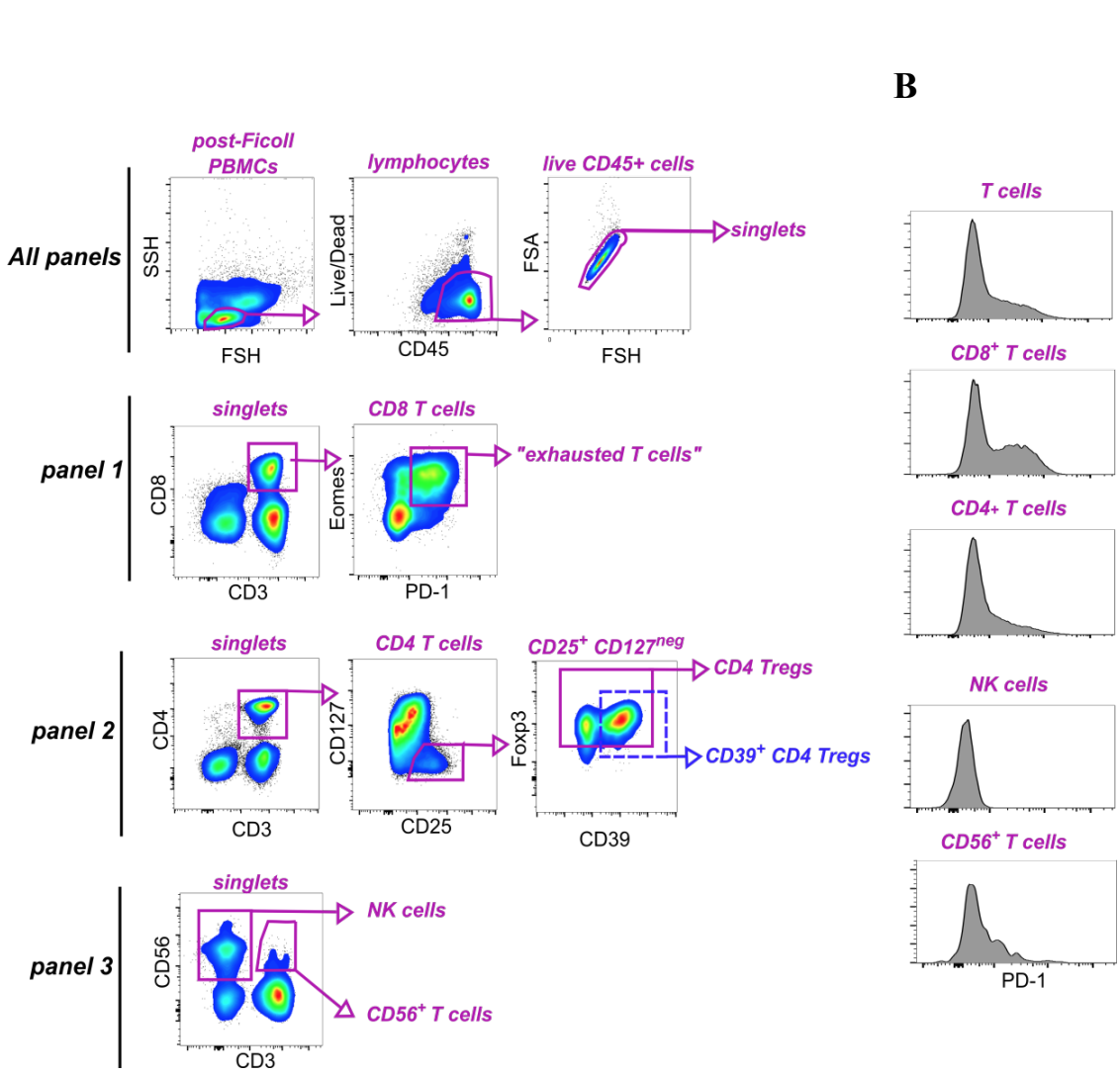


Figure 4. (**A**) Representative flow cytometry plots showing gating strategy for correlate analysis of peripheral lymphocyte subpopulations. (**B**) Histograms show frequency of PD-1^{+} cells among CD3^{+} T cells, CD8^{+} T cells, CD4^{+} T cells, NK cells, CD3^{+}CD56^{+} T cells, for one representative patient. Gating strategy was performed using Fluorescence Minus One (FMO) for each marker as control.

2.5 Plasma samples

We evaluate levels of various soluble factors including lymphokines, interferons, colony stimulating factors and chemokines through different MILLIPLEX® MAP Kits, (MAGPIX® System (Luminex® xMAP® Technology, Merck Millipore, Germany) as reported in Table 2. These panels give the opportunity to perform a simultaneous quantification of a large number of cytokines, so that we could achieve a global overview of soluble factors' distribution in our samples.

Plasma samples were thawed at 4°C overnight and then centrifuged at 2000g for 10 minutes to removed lipids. Each patient sample was run using 25 µl of plasma.

Milliplex Map Kit	Cytokines/chemokines
HCYTOMAG-60K	EGF, FGF-2, G-CSF, Fractalkine, IFNα2, IL-12 p40, IL-12 p70, IL-15, IL-1ra, IL-1α, IL-1β, IL-7, IL-8, IP-10, MCP-1, TNFβ, VEGF
HCYP3MAG-63K	CXCL11, CCL19
HCD8MAG-15K	CD137, Fas, FasL, GM-CSF, Granzyme A e B, IFNγ, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, MIP-1a, MIP-1b, Perforina, TNFa,
HTH17MAG-14K	IL-17a, IL-25, IL-21, IL-22, IL-23, IL-27, IL-33, MIP-3a,
HCYP2MAG-62K	CXCL13, CXCL12

Table 2. Human cytokine/chemokine Magnetic Bead Panels and detailed soluble factors detected.

Protein levels were expressed as OD value as measured by Microplate Reader AD200 Beckman, according to manufacturer's instruction for each plate.

2.6 Statistical analysis

Distributions of all immune biomarker measurements and patients' characteristics (gender, age at enrollment, time since diagnosis, ECOG-PS, number of previous treatments, histotype, disease stage and smoking habit) were explored and summarized using descriptive statistics. In particular, continuous variables (e.g., biomarkers and age at enrollment) were described through mean, median and range of variation (min-max). Data relative to biomarkers were also dichotomized using median values as cut-off points in order to obtain equally sized subgroups. All categorical and discrete variables (e.g., gender and ECOG-PS) were expressed in terms of absolute and relative frequencies (percentages). Differences in immune biomarker distribution in sub-groups of patients were assessed using the nonparametric Kruskal-Wallis test.
Kaplan-Meier method was applied to describe the effect of each dichotomized immune biomarker on PFS/OS probabilities while the association between all immune

biomarker and relapse/death rates was estimated by means of the Cox regression analysis and expressed as rate (hazard) ratio (HR) which represents the rate of relapse/death in the higher immune biomarker category relative to the analogous rate in the lower category.

To evaluate the association between each baseline dichotomized immune biomarker measurement and RECIST/irRC binary BOR outcome (PD vs CD), a logistic regression analysis was performed. In this setting, odds ratio (OR), namely the ratio of progressive patients' proportion in the higher immune biomarker category to the analogous proportion in the lower category, was calculated as an index of association.

Finally, in order to assess the impact of the RECIST/irRC BOR variable (PD vs CD) on the time trajectory of all individual immune biomarker measurements from T0 (cycle 1) to T3 (cycle 4), a random effects regression analysis was applied to log-transformed immune biomarker data. In this context, median ratio (MR), that is the ratio of median immune biomarker value among PD patients and the median immune biomarker value among CD patients, was used as an index of association.

In all regression settings, baseline patients' characteristics were taken into consideration as confounding factors and statistical inference on HR/OR/MR was carried out using the likelihood ratio test. Ninety-five percent confidence limits (95% CL) were also computed for all indexes.

A two-tailed p-value < 0.050 was assumed as statistically significant.

All analyses were performed using Stata (StataCorp. Stata Statistical Software. Release 13.1. College Station, TX (USA), 2013).

Data from plasma samples analysis had been performed using GraphPad Prism v7.0e (GraphPad Software Inc.). Correlation of values obtained from MILLIPLEX® MAP Kits with patients' response was performed by Two-way ANOVA (analysis of variance) statistical test.

3. RESULTS

3.1 Study population and patients outcomes

Globally, 74 patients were enrolled within our Institution. The baseline clinical and pathological characteristics are reported in Table 3 and were used as covariates in the subsequent correlation analyses. Notably, only two patients had EGFR mutation, one harboring exon 19 deletion and one harboring exon 19 deletion and exon 20 insertion, while no ALK or ROS1 rearrangement was reported. The median number of nivolumab administrations was 6 (range: 1-46).

Three patients were not evaluable for BOR assessment as the best response CT scan was not available, while one patient was not evaluable for PFS assessment, as he/she did not undergo further CT scans. Notably, one patient was considered evaluable for irRC BOR and PFS but not for RECIST BOR and PFS, due to differences between the criteria. All the patients were evaluable for OS analysis.

Patients' characteristics	*N*	%	*Mean*	*SD*	*Min-Max*
Gender					
Male	51	68.9			
Female	23	31.1			
Age			67.6	9.0	44.0-85.0
≤ 70 years	37	50.0			
> 70 years	37	50.0			
Time since diagnosis			2.4	2.1	0.0-11.3
≤ 2 years	37	50.0			
>2 years	37	50.0			
ECOG-PS					
0	27	36.5			
≥ 1	47	63.5			
Previous treatments					
1	30	40.5			
>1	43	58.1			
Histotype					
Squamous cell lung cancer	15	20.3			
Adenocarcinoma	59	79.7			
Smoking habit					
Non smoker	9	12.2			
Ex-smoker	27	36.5			
Current smoker	38	51.4			
PD-L1					
0%	40	54.1			
≥ 1%	7	9.5			
Missing	27	36.5			
Whole sample	74	100.0			

Table 3. Summary of patients' characteristics. Abbreviations: ECOG-PS, Eastern Cooperative Oncology Group Performance Status

The median OS, RECIST-PFS and irRC-PFS time were 8.60, 1.87 and 1.93 months, respectively.

The RECIST-BOR was reported as follows: PR = 10; SD = 14; PD = 30; the irRC-BOR was reported as follows: PR = 9; SD = 19; PD = 27. No complete responses were observed. Overall, 16 patients died before undergoing the first response assessment, and were defined as ED. Notably, smoking habit, PD-L1 status and disease stage were excluded from analyses since they resulted to be irrelevant, affected by missing data (36%) and with strongly imbalanced categories (lower stage = 3 pts. vs higher stage = 74 pts), respectively.

Blood specimens for PBMC isolation and immunophenotyping were available for 73 patients at baseline, while fewer samples were available at the subsequent administrations.

3.2 Peripheral blood lymphocyte sub-populations

To gain insights in the mechanisms underlying the clinical responses to anti-PD-1 therapy in cancer patients, we comprehensively evaluated the frequency and phenotype of immune cell populations potentially involved in the response to human tumors (namely T cells and NK cells) in PBMCs of patients receiving nivolumab. By the use of multicolor flow cytometry (Fig. 4A-B), we assessed the frequency of total circulating T cells and NK cells, as well as the relative abundance of $CD8^+$ and $CD4^+$ T cell subsets. Among $CD8^+$ T cells, we also quantified the frequency of *bona fide* exhausted T cells by gating on $PD\text{-}1^+$ $EOMES^+$ $CD8^+$ T cells, as previously described (71). In addition, because of a key role in the modulation of immune responses, we investigated the impact of regulatory $CD4^+$ $CD25^+$ $CD127^-$ $Foxp3^+$ T cells (defined as Tregs), including those co-expressing CD39 ($CD39^+$Tregs) (86) in our samples. Given the cytotoxic potential of $CD3^+CD56^+$ T cells, we also identified and monitored this subset in our cohort of patients.

3.3 Baseline PB immune features and survival

First, the impact of the baseline immunological status (pre-therapy) on OS, RECIST-PFS and irRC-PFS upon nivolumab treatment was evaluated. Figure 5 (caterpillar plot) summarizes the effect of each biomarker on patient's life expectancy through HR (Hazard Ratio) point estimates and corresponding 95%CL, obtained from the Cox regression analysis. HR is the ratio of the death/relapse rate in higher immune biomarker levels to the analogous rate in lower levels. When HR > 1, higher immune biomarker levels (i.e., greater than median value) are correlated with higher death/relapse rates.

Our results suggest that higher levels of almost all the effector T cell subsets, namely $CD3^+$, $CD8^+$ and $CD4^+$ T cells were correlated with longer OS and PFS, (HR < 1 for both indicators). Along this line, also higher percentage of circulating exhausted $CD8^+$ T cells was associated, albeit not reaching statistical significance, with longer OS (HR = 0.66; 95%CL = 0.37-1.18) and PFS (HR = 0.85; 95%CL = 0.50-1.44) (by IRRC only). As far as activated T cells are concerned, the role of $CD3^+CD56^+$ T cells was not clear. Within our cohort, patients displaying percentage of $CD3^+CD56^+$ T cells higher than the median were associated with longer OS (HR = 0.72; 95%CL = 0.41-1.27), but with shorter PFS (HR = 1.09; 95%CL = 0.65-1.82; HR = 1.11, 95%CL = 0.66-1.86, by RECIST and IRRC respectively). Similarly, with regards to total Treg cells and $CD39^+$ Treg cells, we did not find any significant correlation between their amounts and OS or PFS, but only a trend towards higher baseline frequency of $CD39^+$ Tregs in patients characterized by longer OS (HR = 0.84, 95%CL = 0.47-1.51) and PFS (0.84 RECIST; 0.88 IRRC).

Given that high value of $CD8^+$ T cells to Treg ratio have been linked with higher rate of response to immune checkpoint inhibitors (71), we also assessed whether patients with high ($CD8^+$/$CD39^+$ Treg) ratio showed better OS and/or clinical benefit. Surprisingly, we did not find any improvement in terms of survival for patients with higher baseline CD8/$CD39^+$ Treg ratio in our cohort upon treatment; rather, higher values were associated with lower OS (HR = 1.96, 95%CL = 1.04-3.72) and RECIST-PFS (HR = 1.53, 95%CL = 0.85-2.75). This may be due to a trend towards a higher baseline frequency of $CD39^+$ Tregs in patients characterized by longer overall survival.

Next, in order to define whether the survival advantage that results from possessing higher frequency of effector T cells could be actually related to a higher proportion of PD-1^+ cells within each subset, we also analyzed the expression of this immune biomarker on the various sub-populations previously defined (Fig. 4B). Despite some studies have reported that therapeutic anti–PD-1 administration hampers detection of PD-1 expression on peripheral blood cells by commercially available antibodies, we did not notice any technical issue on this regard. In contrast with what was expected (10,87), higher expression levels of PD-1 on $CD3^+$ cells and on $CD8^+$ T cells were significantly associated with poor OS and PFS, and a similar trend was observed on $CD3^+CD56^+$ T cells (HR = 1.39, 95%CL = 0.79-2.45, HR = 1.42, 95%CL = 0.84-2.37, HR = 1.57, 95%CL = 0.94-2.6 for OS, PFS RECIST and PFS IRRC respectevely). On the contrary, no correlation between PD-1 levels on $CD4^+$ T cells and survival was detected (OS: HR = 0.75, 95%CL = 0.41-1.34; PFS: HR = 0.92, 95%CL = 0.52-1.60; HR = 0.96, 95%CL = 0.55-1.67, by RECIST and IRRC respectively) (Fig. 5). Overall, these data suggest that higher baseline levels of PD-1 expression on effector T cells may limit clinical efficacy of nivolumab treatment.

Concerning the innate immune response, high frequencies of NK cells were correlated with lower OS (HR = 2.72, 95% CL = 1.46-5.08) and PFS (HR = 1.88, 95% CL = 1.08-3.26; HR = 2.22, 95% CL = 1.25-3.96, by RECIST and IRRC respectively). Moreover, we found that a discrete subset of NK cells actually expressed PD-1 on their surface **(0,6-10%)**. Nonetheless, the role of PD-1 expressed by NK cells was unclear, since we did not find a significant correlation with OS (HR = 0.85, 95% CL = 0.48-1.52), whereas only a slight association with PFS (HR = 0.8, 95% CL = 0.46-1.36; HR = 0.74, 95% CL = 0.43-1.29, by RECIST and IRRC respectively) (Fig. 5).

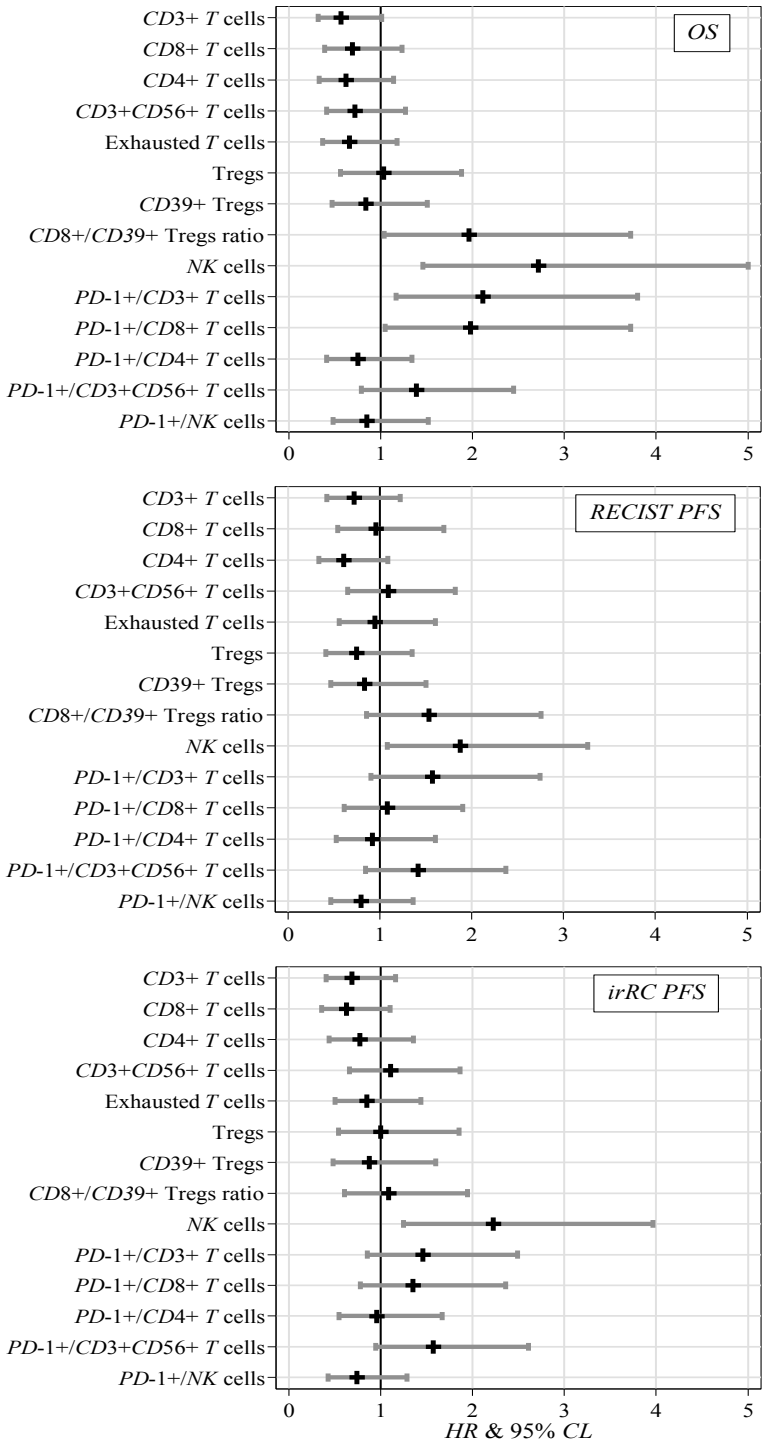
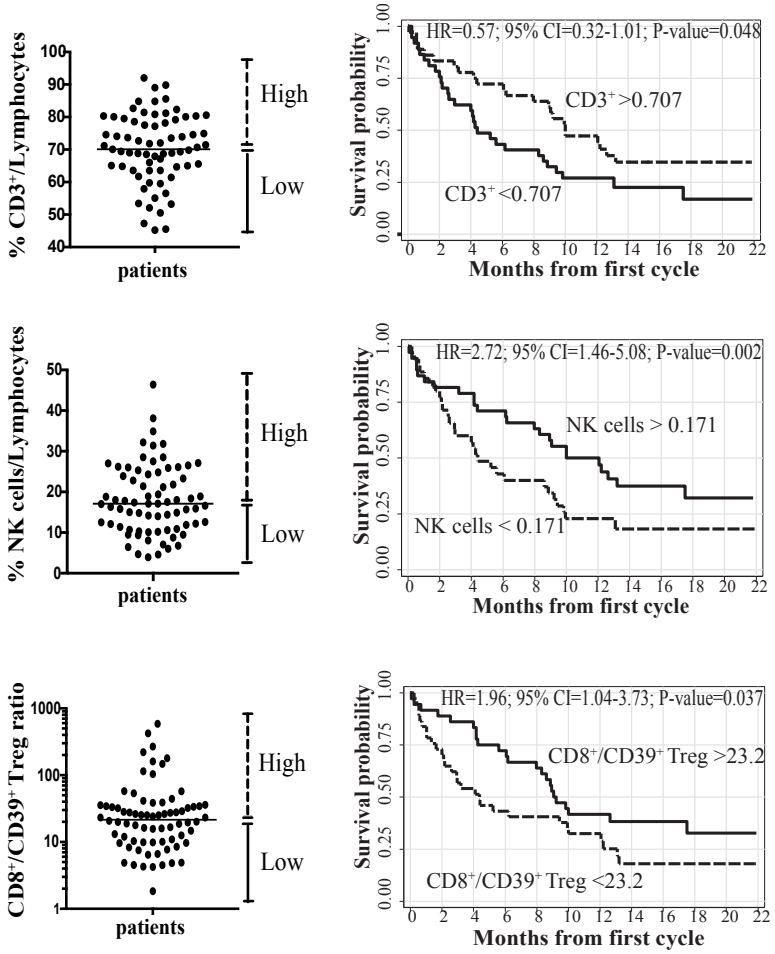


Figure 5. Caterpillar plots showing the impact of each immune biomarker evaluated at baseline on overall survival (OS), RECIST and irRC progression-free survival (PFS). Hazard ratio (HR, represented by a plus sign) and corresponding 95% confidence limits (95%CL, represented by horizontal "whiskers") were derived from a Cox regression analysis adjusted for gender, age at enrollment, time since diagnosis, ECOG-PS, number of previous treatments and histotype. Vertical line at HR = 1 divides HR associated with a better prognosis (left side) from those associated with a worsen prognosis (right side).

Our data were further confirmed using multivariate analyses (Cox curves) (Fig.6). Patients were categorized in high and low groups based on the median cut-off value of the each marker. Although statistical significance was not observed for several of the analyzed markers, relative amounts of circulating $CD3^+$ cells and NK cells could predict patients' overall survival time ($p = 0.048$ and $p = 0.002$, respectively). These data reinforce the notion that the relative abundance of $CD3^+$ T cells and NK cells could represent, respectively, positive and negative prognostic markers of survival in our cohort. Moreover, the CD8/$CD39^+$Treg ratio emerged as a biomarker able to predict the clinical outcome, since higher values (> 23.2) at baseline were associated with shorter OS ($p = 0.037$)(Fig. 6A).

We also noticed that baseline PD-1 expression was generally correlated with poor clinical outcomes. In particular, high expression of PD-1 on $CD3^+$ cells (> 29.3%) and on $CD8^+$ T cells (>32.8%) were both significantly associated with shorter OS ($p = 0.013$ and $p = 0.033$, respectively)(Fig. 6B).

A



B

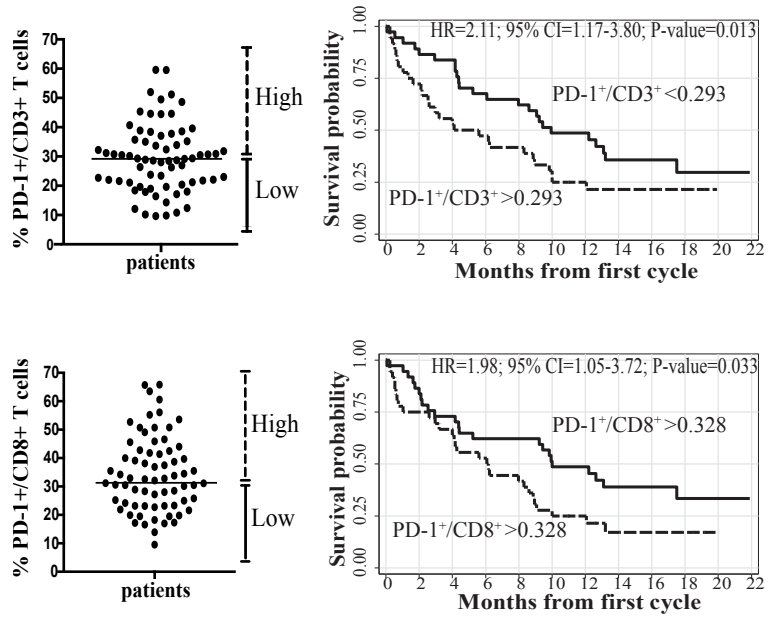


Figure 6. (A) Kaplan-Meier survival curves illustrating the prognostic effect on OS of $CD3^+$T cells, $CD56^+CD3^-$ NK cells and $CD8^+/CD39^+$Treg ratio for patients with higher (dotted lines) and lower (solid lines) cell counts. **(B)** Kaplan–Meier survival curves for patients with high or low expression of PD-1 on $CD3^+$ T cells and on $CD8^+$ T cells. The median values used as thresholds for categorizing immune biomarkers are indicated in the left plots of each curve. HR= Hazard Ratio

3.4 Baseline PB immune features and radiological response

Next, we wondered whether, beside OS, immunological signatures measured at baseline could also be prognostic of clinical response (i.e. RECIST and irRC) to nivolumab treatment. Notably, analyses using irRC criteria didn't show any relevant difference from RECIST. As show in Figure 7, in agreement with our data correlating immune biomarkers and survival, higher frequency of total $CD3^+$ lymphocytes (HR= 0.92, 95% CI= 0.26-3.21) and total $CD4^+$ T cells (HR=0.28, 95% CI= 0.07-1.06) were also associated with controlled disease. Along the same line, higher amounts of both total Tregs and $CD39^+$ Tregs cells were significantly correlated with clinical benefit (T regs: HR=0.38, 95% CI= 0.09-1.55; $CD39^+$Tregs: HR=0.33, 95% CI=0.08-1.32). Conversely, higher frequency of NK cells was associated with a progressive disease, in line with results correlated to survival (HR=1.77, 95% CI=0.52-5.99).

Moreover, consistent with previous observation regarding the negative impact of PD-1 expression on patients' OS, we found that PD patients significantly differed from CD patients by displaying higher level of PD-1 on almost all lymphocyte sub-populations examined ($CD3^+$, $CD4^+$, $CD8^+$, $CD3^+CD56^+$) (Fig.7). Thus, again suggesting that PD-1 expression on circulating T lymphocytes, at baseline, might negatively impact on patient's clinical response and survival. In Figure 7 we reported results from analysis of each biomarker; OR is to be interpreted as the ratio of the proportion of progressive patients in higher immune biomarker levels to the analogous proportion in lower levels.

Surprisingly, in contrast to what was found by analyzing OS, we found that higher frequency of $CD8^+$ T cells and of $CD3^+CD56^+$ T cells significantly correlated with progressive disease (HR=2.78, 95% CI=0.74-10.43; HR=2.14, 95% CI= 0.65-7.07, respectively).

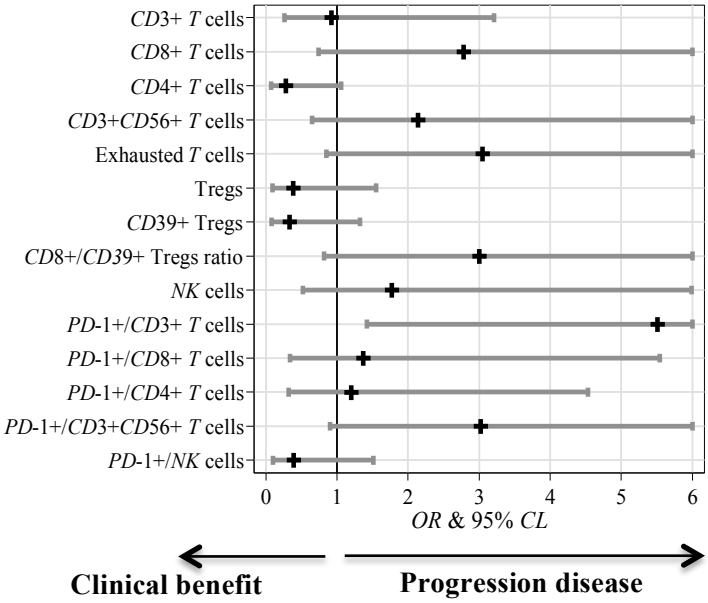


Figure 7. Caterpillar plot showing the effect of each immune biomarker evaluated at baseline on PD vs CD in relation with best overall response (BOR), defined according to RECIST criteria. Odds ratio (OR, represented by a plus sign) and corresponding 95% confidence limits (95%CL, represented by horizontal "whiskers") were derived from a logistic regression analysis adjusted for gender, age at enrollment, time since diagnosis, ECOG-PS, number of previous treatments and histotype. OR is to be interpreted as the ratio of the proportion of progressive patients in higher immune biomarker levels to the analogous proportion in lower levels. Vertical line at OR = 1 divides OR associated with a responsive disease (left side) from those associated with a progressive disease (right side).

Of note, by repeating the same multivariate Cox regression analysis including also early-death (ED) patients within the PD group (Fig. 8), we confirmed that there was a trend toward clinical benefit in patients who had higher frequencies of $CD3^+$ T cells, $CD4^+$ T cells, total and $CD39^+$ Tregs, while higher amount of $CD8^+$ T cells, NK cells and overall PD-1 expression were associated with a poor outcome. In particular, high frequency of $CD4^+$ T cells was correlated with controlled disease, while PD-1 expression on $CD3^+$ T cells and on $CD3^+$ $CD56^+$ T cells, as well as a high $CD8^+/CD39^+$ Treg ratio, were correlated with progression disease.

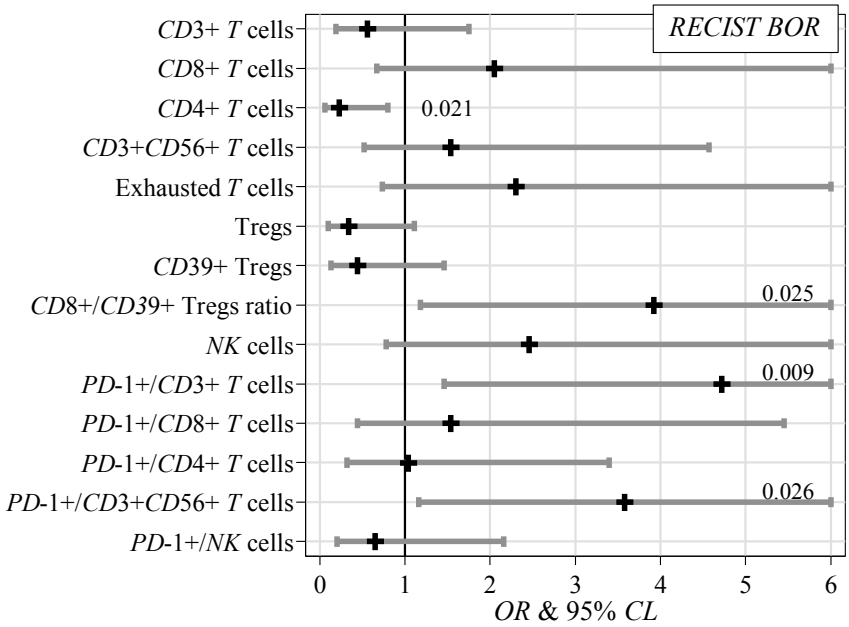


Figure 8. Caterpillar plot showing the effect of each immune biomarker, evaluated at baseline on PD+ED patients vs CD patients. Odds ratio (OR, represented by a plus sign) and corresponding 95% confidence limits (95%CL, represented by horizontal "whiskers") were derived from a logistic regression analysis adjusted for gender, age at enrollment, time since diagnosis, ECOG-PS, number of previous treatments and histotype. Vertical line at OR = 1 divides OR associated with a responsive disease (left side) from those associated with a progressive disease (right side). Statistically significant P-values derived from the likelihood ratio test are reported in the graph.

We further investigated each of the above-mentioned parameters with univariate analysis and similar results were obtained. Remarkably, while not reaching statistical significance on multivariate model, univariate analysis showed that the impact of exhausted T cells on the response rate was remarkable. Of note, although exhausted T cells were supposed to be the target sub-population of ICI, the baseline frequency was significantly higher in PD patients than in CD patients (Fig. 9 p= 0.046).

This data suggests that the amount of exhausted T cells may be considered a potential predictive factor of disease control to nivolumab treatment in NSCLC.

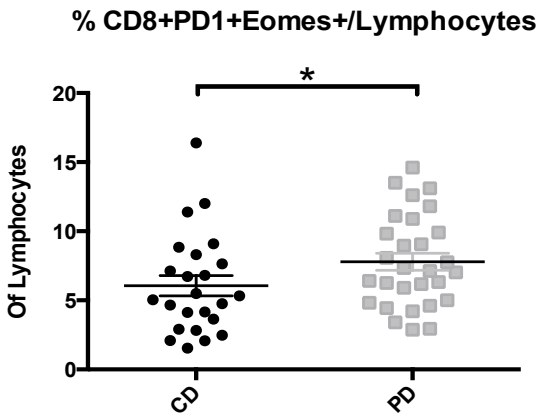


Figure 9. Distribution of $CD8^{+}PD\text{-}1^{+}Eomes^{+}$ T cells according to binary (CD vs PD) best overall response (BOR). Statistical comparison between the two distributions was performed using Kruskall-Wallis test (KW P-value). *p<0.05

3.5 Changes in PB lymphocytes during anti-PD-1 therapy

Finally, we aimed at identifying whether variations in immunological biomarkers, assessed during the treatment, could be associated with disease control. To this end, we performed a longitudinal analysis of blood samples obtained at baseline and before each treatment cycle, up to six weeks. Remarkably, those patients who died before the first response assessment, categorized as early death (ED), were excluded from longitudinal analyses as they could not undergo all the four pre-planned blood sample collections. We found that in PD patients the frequencies of $CD8^+$ T cells, exhausted T cells, and $CD3^+CD56^+$ T cells were significantly higher than median values of the CD group. Figure 10 shows the relationship between CD vs PD best overall response (BOR) on the longitudinal profile of all immune biomarkers. MR represents the ratio of each immune marker median value in PD to CD groups. These data, again, highlighted the putative role of exhausted T cells as a prognostic biomarker.

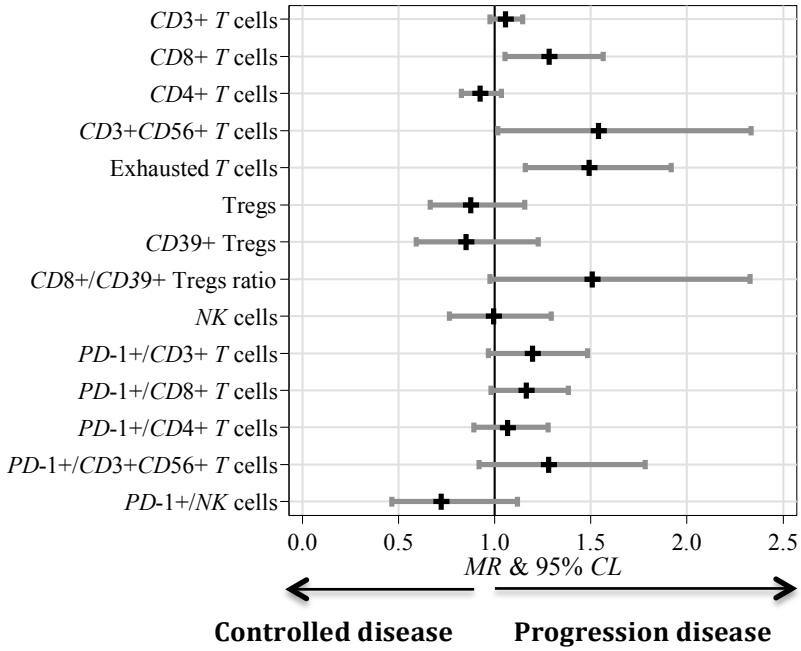


Figure 10. Caterpillar plot showing the relationship between the binary (CD vs PD) best overall response (BOR) on the longitudinal profile of all immune biomarkers. Median ratio (MR, represented by a plus sign) and corresponding 95% confidence limits were derived from a random effects regression analysis adjusted for gender, age at enrollment, time since diagnosis, ECOG-PS, number of previous treatments and histotype. Vertical line at MR = 1 divides MR associated with a responsive disease (left side) from those associated with a progressive disease (right side).

Thus, when longitudinally analyzed in each group of patients, we found that the frequency of exhausted T cells among total lymphocytes was lower in CD than PD patients. Interestingly, in CD patients level of exhausted T cells immediately decreased from baseline after the first cycle of therapy (p-value = < 0,0001), then reaching plateau as long as at day 45 of treatment (Fig. 11). Conversely, it is worth noting that levels of exhausted T cells in PD patients did not show significant variations across the different time points. Moreover exhausted T cell levels were consistently higher in PD than in CD patients during treatment.

Similarly, the difference of $CD8^+$ T cells and of $CD3^+CD56^+$ T cells frequencies between CD and PD patients persisted during therapy (p-value = 0.016 and 0.045 respectively, data not shown)

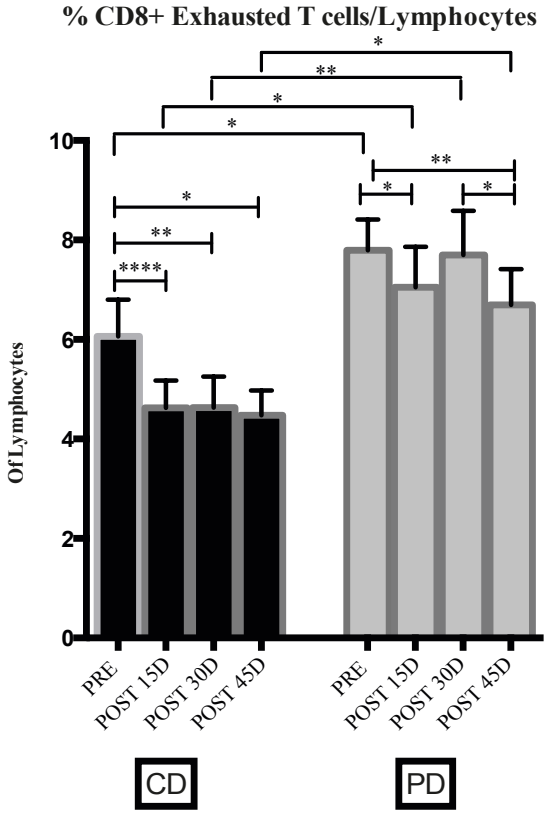


Figure 11. Frequency of $CD8^+$ exhausted T cells at four different time points (Pre-treatment, post 15 days, post 30 days and post 45 days) in PD and in CD patients. Significative correlation of exhausted population between CD and PD groups is reported in the upper parte of the figure, for each time point. Statistical significance was calculated using Mann-Whitney test. At the bottom are reported differences inside each group during therapy. Statistical significance was calculated using paired nonparametric Wilcoxon Test. *p<0.05, **p <0.01, ****p<0.0001.

3.6 CD69 expressing NK cells are higher in Controlled Disease patients

Our previous results showed that higher frequency of NK cells at baseline was correlated with poor response. We further analyzed the expression of CD69 activation marker on peripheral blood NK cells derived from NSCLC patients treated with nivolumab.

We found that, at baseline, CD patients showed a significant higher percentage of $CD69^+$ NK cells. Moreover, after the first therapy cycle, $CD69^+$ NK cell levels were consistently higher in CD than in PD patients (Fig. 12).

These results suggest that $CD69^+$ NK cells could represent potential biomarker candidate specific to early response to anti-PD-1 immunotherapy.

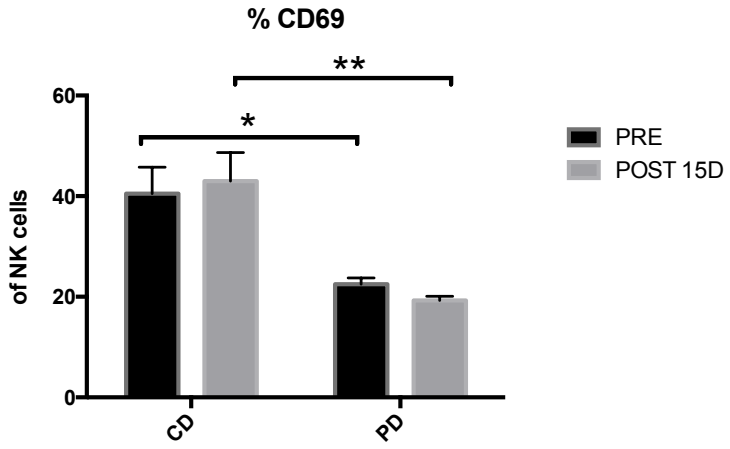


Figure 12. Frequency of $CD69^+$ NK cells at two different time points (Pre-treatment, post 15 days) in CD and in PD patients. Statistical significance was calculated using Two-way ANOVA test. *p <0.05, ** p<0.01

3.7 Soluble factors in plasma samples during anti-PD-1 therapy

As checkpoint blockade led to modifications in circulating immune cells, we hypothesized that these therapies could also lead to detectable changes in systemic levels of cytokines. Accordingly, we evaluated changes in the plasma levels of a panel of 46 cytokines/chemokines/growth factors in 10 CD and 10 PD patients at baseline (Pre-treatment), post 15 days and post 45 days of treatment, using Luminex analysis.

Of the analytes tested, our results showed that IFN-γ-induced protein 10 (IP-10) and monocyte chemoattractant protein 1 (MCP-1 also known as CCL2) significantly increased during treatment in CD patients (Fig. 13). Similarly, albeit not reaching statistical significance, we found higher plasma levels of both vascular endothelial growth factor (VEGF) and epithelial growth factors (EGF) in CD patients during therapy (data not shown).

These data suggest that the modulation of IP-10 and MCP-1 soluble factors in NSCLC patients during nivolumab therapy may represent novel circulating biomarker of controlled or uncontrolled disease.

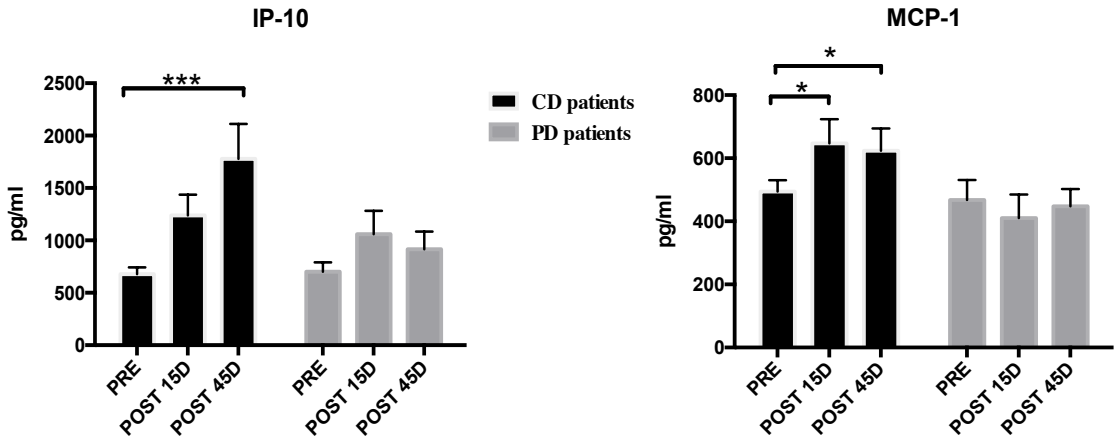


Figure 13. Changes in plasma cytokines and chemokines evaluated in CD and PD NSCLC patients, treated with nivolumab, at different time points: Pre-treatment (T0), after 15 days (T1) and 45 days (T3) from the beginning of therapy. Statistical analysis was performed using Two-way ANOVA test. *p<0.05, ***p<0.001

4. DISCUSSION

In recent years, therapies that block immune checkpoints, thereby enhancing antitumor immune responses, have revolutionized the treatment of many solid malignancies, including melanoma and NSCLC. Although these tumors are reported to be high immuno-genic, the immune system often fails to eliminate cancer cells. It is now clear that tumors co-opt certain immune-checkpoint pathways as a major mechanism of immune resistance.

Among the most important and promising immunological approaches to re-activate antitumor immunity has emerged the blockade of PD-1/PD-L1 axis with monoclonal antibodies.

Patients and tumors may respond unpredictably to immunotherapy partly owing to heterogeneity of the immune composition and phenotypic profiles of TILs within individual tumors and between patients. Thus, a significant number of patients treated fails to have a clinical benefit from immunotherapies and the molecular mechanism underling this negative response is still unknown. Identification of predictive biomarkers is a priority in the field of cancer immunotherapy, in order to monitor or predict patients' response to appropriate treatment.

While immunotherapy with PD-1/PD-L1 blocking agents has achieved impressive results in the management of advanced NSCLC, reliable biomarkers of efficacy are still required. This study was designed to determine whether the proportions of circulating T cells sub-populations could represent potential predictors of outcomes for NSCLC patients receiving nivolumab.

With regards to baseline values, our data revealed that patients owning higher frequency of $CD3^+$ T cells and of distinct T cell subsets (i.e $CD4^+$, $CD8^+$, and $CD3^+CD56^+$ T cells) showed a survival advantage upon nivolumab treatment. Conversely, relative high proportions of NK cells, as well as a higher $CD8^+/CD39^+$ Treg ratio, were associated with shorter OS and PFS; along this line, also possessing high levels of PD-1 appeared to play a role in limiting patients' survival.

These data are in line with what observed by Mazzaschi and coworkers reporting that in NSCLC a low expression of PD-1 among $CD8^+$ TILs was associated with prolonged progression-free survival (PFS) during treatment with nivolumab (78).

Consistently to what found by analyzing OS, our data showed that baseline higher frequencies of total $CD3^+$ T cells, $CD4^+$ T cells, total Tregs and $CD39^+$ Tregs were correlated with controlled disease, whereas higher proportions of NK cells, as well as a high $CD8^+/CD39^+$Treg ratio, were found in PD patients. A remarkable exception regards the amount of $CD8^+$ T cells that, in contrast to what found by analyzing patients survival, was significantly correlated with progressive disease.

These data suggest that while some sub-populations associated with survival were also associated with radiological response in a consistent fashion (ie: $CD3^+$ and $CD4^+$ effector T cells were associated with improved response and survival, while NK cells were associated with worse response and survival), other sub-populations appeared to behave differently, in terms of response and survival. This apparent inconsistency might be explained by the peculiar mechanism of action of immune checkpoint inhibitors, which might achieve prolonged survival while their effect in terms of response might be underestimated by radiological response evaluation criteria (this is especially true with regards to RECIST).

Interestingly, in line with data regarding the inverse correlation between overall PD-1 expression and OS, we found that at baseline higher PD-1 levels on T cells were strictly associated with poor outcome. This data suggests that baseline PD-1 amount on lymphocytes may represent a predictive biomarker correlated with both survival and response.

Remarkably, univariate analysis showed that the frequency of exhausted T cells at baseline was higher in PD patients, thus highlighting the possible role of this $CD8^+$ T cell sub-population as a predictive factor associated with response.

It should be stressed that our present analysis was done on PB lymphocytes and thus our results might not completely mirrored what found analyzing $CD8^+$ T cells present at the tumor site. Indeed, data collected from metastatic melanoma samples suggest that a higher proportion of exhausted $CD8^+$ among TILs predicts response to PD-1/PD-L1 blockade, implying that the relative composition of TIL populations might impact on the outcomes achieved by immune checkpoint inhibitors (77).

Our present data suggest that levels of PB exhausted T cells during nivolumab therapy may be associated with clinical outcome, though this association needs to be confirmed by larger studies.

Along this line, longitudinal peripheral blood samples from patients revealed that, both at baseline and before each treatment cycle, the frequency of exhausted T cells was higher in PD as compared to CD patients. Moreover, in CD group the amount of exhausted T cells declined soon after the first therapy cycle and then remained stable until the fourth administration, whereas in PD patients exhausted T cells levels alternatively increased and decreased at different time points.
These results support the notion that levels of circulating exhausted T cells might be used to identify patients who will experience clinical disease control with nivolumab. However, additional studies with detailed analysis of exhausted T population levels in peripheral blood during late weeks after PD-1 blockade are needed.
Thus, if these results will be confirmed and extended also at later time points, peripheral blood analysis of exhausted T cell subsets may provide a valuable strategy to predict responses to PD-1–targeted therapy that may assist in the management of lung cancer patients.

Although T cells are believed to play the primary role in the anti-tumor immunity of checkpoint blockade therapies, the potential role of non-T cell population cannot be excluded. In this study we found that CD patients showed higher frequency of $CD69^+$ NK cells than PD patients both at baseline and after the first cycle of nivolumab therapy. As CD69 is an activation marker, it's reasonable to think that $CD69^+$ NK cells might represent a more functional subset, playing a role in antitumor immune response. Thus, we could assume that higher frequency of $CD69^+$ NK cells potentially correlates with clinical benefit to anti-PD-1 treatment.
Notably, it had been reported that also NK cells could express PD-1 and that in vitro blockade of PD-1 pathway reverted the NK cell functional defects induced by PD-1/PDL-1 interactions (62). Therefore, immune checkpoint inhibitors might act also on NK cells, helping to circumvent tumor escape and boost T cell-mediated antitumor immune activity by enhancing NK-cell trafficking and effector functions against the tumor.

In this study, we also assessed variations in plasma levels of several chemokines and cytokines in NSCLC patients treated with nivolumab. Although the majority of analyzed soluble factors didn't show any relevant variation, possible due to the fact that they probably act locally in the tumor microenvironment, we found an increase of

plasma IP-10 and MCP-1 levels from baseline to T1 (post 15 days) and T3 (post 45 days) time points in CD patients. Under proinflammatory conditions, IP-10 is secreted in response to IFN-γ from a variety of cells, such as leukocytes, activated neutrophils, eosinophils, monocytes, epithelial cells, endothelial cells, stromal cells (fibroblasts) and keratinocytes. This crucial regulator of the interferon response preferentially attracts activated Th1 lymphocytes to the area of inflammation. IP-10 is also a chemo-attractant for monocytes, T cells and NK cells (88).
In our study, higher plasma levels of IP-10, found in CD patients could be in agreement with a model in which nivolumab induces IP-10 production by myeloid cells at inflammatory sites in tissue in order to recruit immuno-competent T cells to the tumor microenvironment (89).

MCP-1 is produced by many cell types, including endothelial, fibroblasts, epithelial cells. However, monocyte/macrophages are found to be the major source of MCP-1, which regulates the migration and infiltration of monocytes, memory T lymphocytes, and NK cells (90). MCP-1 has been shown to exert both pro- and anti-tumor effects. Elevated MCP-1 expression levels in the tumor microenvironment as well as high circulating concentrations of this chemokine have been associated with poor prognosis in breast carcinoma patients. Moreover, MCP-1 has also been shown to mediate the recruitment of specific monocyte populations that support the establishment of metastatic disease. Conversely, MCP-1 may also recruit antitumor immune cells to tumor bed and is required for efficient immunosurveillance, implying that the inhibition of MCP-1 may support metastatic spread (91).
MCP-1 is known to be released during immune responses causing stimulation of both macrophages and T cells (92,93). Thus, in the current study, the upregulation of MCP-1 found in patients with clinical benefit could represent an “anti-PD-1-induced” immune response.
Taken together, since IP-10 and MCP-1 can be easily measured by minimally invasive blood sampling, they could be potential prognostic biomarkers for monitoring of clinical outcomes in NSCLC patients receiving PD-1 inhibitor therapy.

We are aware that the possible conclusions of our study might be limited by the relatively small number of evaluated patients, especially when the longitudinal assessment is taken into account, as some patients did not undergo all the pre-planned

sample collections due to early treatment discontinuation; in spite of this, our research might provide an insight on the dynamic immunologic mechanisms leading to different efficacy of PD-1 blocking agents and suggests a potential predictive role of both circulating immune cell sub-populations and plasma factors during treatment with nivolumab for advanced NSCLC.

5. BIBLIOGRAPHY

1. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. Nat Rev Drug Discov 2015;14(8):561-84.
2. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12(4):252-64.
3. Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. EMBO J 1992;11(11):3887-95.
4. Yao H, Wang H, Li C, Fang JY, Xu J. Cancer Cell-Intrinsic PD-1 and Implications in Combinatorial Immunotherapy. Front Immunol 2018;9:1774.
5. Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. Nat Immunol 2013;14(12):1212-8.
6. Sharpe AH, Pauken KE. The diverse functions of the PD1 inhibitory pathway. Nat Rev Immunol 2018;18(3):153-67.
7. Kawamoto S, Tran TH, Maruya M, Suzuki K, Doi Y, Tsutsui Y, et al. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. Science 2012;336(6080):485-9.
8. Okazaki T, Maeda A, Nishimura H, Kurosaki T, Honjo T. PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proc Natl Acad Sci U S A 2001;98(24):13866-71.
9. Hui E, Cheung J, Zhu J, Su X, Taylor MJ, Wallweber HA, et al. T cell costimulatory receptor CD28 is a primary target for PD-1-mediated inhibition. Science 2017;355(6332):1428-33.
10. Pauken KE, Wherry EJ. Overcoming T cell exhaustion in infection and cancer. Trends Immunol 2015;36(4):265-76.
11. Park HJ, Park JS, Jeong YH, Son J, Ban YH, Lee BH, et al. PD-1 upregulated on regulatory T cells during chronic virus infection enhances the suppression of CD8+ T cell immune response via the interaction with PD-L1 expressed on CD8+ T cells. J Immunol 2015;194(12):5801-11.
12. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. J Exp Med 2000;192(7):1027-34.
13. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. J Exp Med 2006;203(4):883-95.
14. Nishimura H, Nose M, Hiai H, Minato N, Honjo T. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. Immunity 1999;11(2):141-51.
15. Nishimura H, Okazaki T, Tanaka Y, Nakatani K, Hara M, Matsumori A, et al. Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice. Science 2001;291(5502):319-22.
16. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 2015;15(8):486-99.

17. Ghoneim HE, Fan Y, Moustaki A, Abdelsamed HA, Dash P, Dogra P, et al. De Novo Epigenetic Programs Inhibit PD-1 Blockade-Mediated T Cell Rejuvenation. Cell 2017;170(1):142-57 e19.
18. Patsoukis N, Bardhan K, Chatterjee P, Sari D, Liu B, Bell LN, et al. PD-1 alters T-cell metabolic reprogramming by inhibiting glycolysis and promoting lipolysis and fatty acid oxidation. Nat Commun 2015;6:6692.
19. Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, et al. Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. Cell 2015;162(6):1229-41.
20. Chowdhury PS, Chamoto K, Kumar A, Honjo T. PPAR-Induced Fatty Acid Oxidation in T Cells Increases the Number of Tumor-Reactive CD8(+) T Cells and Facilitates Anti-PD-1 Therapy. Cancer Immunol Res 2018;6(11):1375-87.
21. Walker LS. Treg and CTLA-4: two intertwining pathways to immune tolerance. J Autoimmun 2013;45:49-57.
22. Wei SC, Duffy CR, Allison JP. Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. Cancer Discov 2018;8(9):1069-86.
23. Rudd CE, Taylor A, Schneider H. CD28 and CTLA-4 coreceptor expression and signal transduction. Immunol Rev 2009;229(1):12-26.
24. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity 1995;3(5):541-7.
25. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, et al. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. Science 1995;270(5238):985-8.
26. Phan GQ, Yang JC, Sherry RM, Hwu P, Topalian SL, Schwartzentruber DJ, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci U S A 2003;100(14):8372-7.
27. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363(8):711-23.
28. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 2013;369(2):122-33.
29. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. Immunity 2016;44(5):989-1004.
30. Avery L, Filderman J, Szymczak-Workman AL, Kane LP. Tim-3 co-stimulation promotes short-lived effector T cells, restricts memory precursors, and is dispensable for T cell exhaustion. Proc Natl Acad Sci U S A 2018;115(10):2455-60.
31. Ndhlovu LC, Lopez-Verges S, Barbour JD, Jones RB, Jha AR, Long BR, et al. Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. Blood 2012;119(16):3734-43.
32. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, et al. Role of LAG-3 in regulatory T cells. Immunity 2004;21(4):503-13.

33. Xu F, Liu J, Liu D, Liu B, Wang M, Hu Z, et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. Cancer Res 2014;74(13):3418-28.
34. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol 2009;10(1):29-37.
35. Richter K, Agnellini P, Oxenius A. On the role of the inhibitory receptor LAG-3 in acute and chronic LCMV infection. Int Immunol 2010;22(1):13-23.
36. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. J Exp Med 2003;198(4):557-67.
37. Chan CJ, Martinet L, Gilfillan S, Souza-Fonseca-Guimaraes F, Chow MT, Town L, et al. The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. Nat Immunol 2014;15(5):431-8.
38. Kurtulus S, Sakuishi K, Ngiow SF, Joller N, Tan DJ, Teng MW, et al. TIGIT predominantly regulates the immune response via regulatory T cells. J Clin Invest 2015;125(11):4053-62.
39. Chauvin JM, Pagliano O, Fourcade J, Sun Z, Wang H, Sander C, et al. TIGIT and PD-1 impair tumor antigen-specific CD8(+) T cells in melanoma patients. J Clin Invest 2015;125(5):2046-58.
40. Zhang Q, Bi J, Zheng X, Chen Y, Wang H, Wu W, et al. Blockade of the checkpoint receptor TIGIT prevents NK cell exhaustion and elicits potent anti-tumor immunity. Nat Immunol 2018;19(7):723-32.
41. Thommen DS, Koelzer VH, Herzig P, Roller A, Trefny M, Dimeloe S, et al. A transcriptionally and functionally distinct PD-1(+) CD8(+) T cell pool with predictive potential in non-small-cell lung cancer treated with PD-1 blockade. Nat Med 2018;24(7):994-1004.
42. Pauken KE, Sammons MA, Odorizzi PM, Manne S, Godec J, Khan O, et al. Epigenetic stability of exhausted T cells limits durability of reinvigoration by PD-1 blockade. Science 2016;354(6316):1160-65.
43. Brooks DG, McGavern DB, Oldstone MB. Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. J Clin Invest 2006;116(6):1675-85.
44. Angelosanto JM, Blackburn SD, Crawford A, Wherry EJ. Progressive loss of memory T cell potential and commitment to exhaustion during chronic viral infection. J Virol 2012;86(15):8161-70.
45. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, et al. Restoring function in exhausted CD8 T cells during chronic viral infection. Nature 2006;439(7077):682-7.
46. Kamphorst AO, Wieland A, Nasti T, Yang S, Zhang R, Barber DL, et al. Rescue of exhausted CD8 T cells by PD-1-targeted therapies is CD28-dependent. Science 2017;355(6332):1423-27.
47. Paley MA, Kroy DC, Odorizzi PM, Johnnidis JB, Dolfi DV, Barnett BE, et al. Progenitor and terminal subsets of CD8+ T cells cooperate to contain chronic viral infection. Science 2012;338(6111):1220-5.
48. Crotty S, Johnston RJ, Schoenberger SP. Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation. Nat Immunol 2010;11(2):114-20.

49. Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. Nat Immunol 2008;9(5):503-10.
50. Eckl J, Buchner A, Prinz PU, Riesenberg R, Siegert SI, Kammerer R, et al. Transcript signature predicts tissue NK cell content and defines renal cell carcinoma subgroups independent of TNM staging. J Mol Med (Berl) 2012;90(1):55-66.
51. McKay K, Moore PC, Smoller BR, Hiatt KM. Association between natural killer cells and regression in melanocytic lesions. Hum Pathol 2011;42(12):1960-4.
52. Moretta A, Bottino C, Vitale M, Pende D, Cantoni C, Mingari MC, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. Annu Rev Immunol 2001;19:197-223.
53. Moretta A, Vitale M, Sivori S, Morelli L, Pende D, Bottino C. Inhibitory and activatory receptors for HLA class I molecules in human natural killer cells. Chem Immunol 1996;64:77-87.
54. Lanier LL. NK cell recognition. Annu Rev Immunol 2005;23:225-74.
55. Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 1999;285(5428):727-9.
56. Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, et al. ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. Immunity 2001;14(2):123-33.
57. Brandt CS, Baratin M, Yi EC, Kennedy J, Gao Z, Fox B, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J Exp Med 2009;206(7):1495-503.
58. Pogge von Strandmann E, Simhadri VR, von Tresckow B, Sasse S, Reiners KS, Hansen HP, et al. Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. Immunity 2007;27(6):965-74.
59. Baychelier F, Sennepin A, Ermonval M, Dorgham K, Debre P, Vieillard V. Identification of a cellular ligand for the natural cytotoxicity receptor NKp44. Blood 2013;122(17):2935-42.
60. Benson DM, Jr., Bakan CE, Mishra A, Hofmeister CC, Efebera Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. Blood 2010;116(13):2286-94.
61. Beldi-Ferchiou A, Lambert M, Dogniaux S, Vely F, Vivier E, Olive D, et al. PD-1 mediates functional exhaustion of activated NK cells in patients with Kaposi sarcoma. Oncotarget 2016;7(45):72961-77.
62. Pesce S, Greppi M, Tabellini G, Rampinelli F, Parolini S, Olive D, et al. Identification of a subset of human natural killer cells expressing high levels of programmed death 1: A phenotypic and functional characterization. J Allergy Clin Immunol 2017;139(1):335-46 e3.
63. Vari F, Arpon D, Keane C, Hertzberg MS, Talaulikar D, Jain S, et al. Immune evasion via PD-1/PD-L1 on NK cells and monocyte/macrophages is more prominent in Hodgkin lymphoma than DLBCL. Blood 2018;131(16):1809-19.

64. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. N Engl J Med 2015;373(17):1627-39.
65. Brahmer J, Reckamp KL, Baas P, Crino L, Eberhardt WE, Poddubskaya E, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. N Engl J Med 2015;373(2):123-35.
66. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. N Engl J Med 2016;375(19):1823-33.
67. Herbst RS, Baas P, Kim DW, Felip E, Perez-Gracia JL, Han JY, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet 2016;387(10027):1540-50.
68. Rittmeyer A, Barlesi F, Waterkamp D, Park K, Ciardiello F, von Pawel J, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet 2017;389(10066):255-65.
69. Lisberg A, Garon EB. The Value of PD-L1 Testing in Non-Small-Cell Lung Cancer. JAMA Oncol 2016.
70. Tumeh PC, Harview CL, Yearley JH, Shintaku IP, Taylor EJ, Robert L, et al. PD-1 blockade induces responses by inhibiting adaptive immune resistance. Nature 2014;515(7528):568-71.
71. Twyman-Saint Victor C, Rech AJ, Maity A, Rengan R, Pauken KE, Stelekati E, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. Nature 2015;520(7547):373-7.
72. Dronca RS, Liu X, Harrington SM, Chen L, Cao S, Kottschade LA, et al. T cell Bim levels reflect responses to anti-PD-1 cancer therapy. JCI Insight 2016;1(6).
73. Hannani D, Vetizou M, Enot D, Rusakiewicz S, Chaput N, Klatzmann D, et al. Anticancer immunotherapy by CTLA-4 blockade: obligatory contribution of IL-2 receptors and negative prognostic impact of soluble CD25. Cell Res 2015;25(2):208-24.
74. Powles T, Eder JP, Fine GD, Braiteh FS, Loriot Y, Cruz C, et al. MPDL3280A (anti-PD-L1) treatment leads to clinical activity in metastatic bladder cancer. Nature 2014;515(7528):558-62.
75. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313(5795):1960-4.
76. Uryvaev A, Passhak M, Hershkovits D, Sabo E, Bar-Sela G. The role of tumor-infiltrating lymphocytes (TILs) as a predictive biomarker of response to anti-PD1 therapy in patients with metastatic non-small cell lung cancer or metastatic melanoma. Med Oncol 2018;35(3):25.
77. Daud AI, Loo K, Pauli ML, Sanchez-Rodriguez R, Sandoval PM, Taravati K, et al. Tumor immune profiling predicts response to anti-PD-1 therapy in human melanoma. J Clin Invest 2016;126(9):3447-52.
78. Mazzaschi G, Madeddu D, Falco A, Bocchialini G, Goldoni M, Sogni F, et al. Low PD-1 Expression in Cytotoxic CD8(+) Tumor-Infiltrating Lymphocytes Confers an Immune-Privileged Tissue Microenvironment in NSCLC with a Prognostic and Predictive Value. Clin Cancer Res 2018;24(2):407-19.

79. Gros A, Parkhurst MR, Tran E, Pasetto A, Robbins PF, Ilyas S, et al. Prospective identification of neoantigen-specific lymphocytes in the peripheral blood of melanoma patients. Nat Med 2016;22(4):433-8.
80. Kamphorst AO, Pillai RN, Yang S, Nasti TH, Akondy RS, Wieland A, et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. Proc Natl Acad Sci U S A 2017;114(19):4993-98.
81. Matsuo N, Azuma K, Hattori S, Ohtake J, Kawahara A, Ishii H, et al. Association between soluble immune mediators and tumor responses in patients with nonsmall cell lung cancer treated with anti-PD-1 inhibitor. Int J Cancer 2019;144(5):1170-79.
82. Sanmamed MF, Perez-Gracia JL, Schalper KA, Fusco JP, Gonzalez A, Rodriguez-Ruiz ME, et al. Changes in serum interleukin-8 (IL-8) levels reflect and predict response to anti-PD-1 treatment in melanoma and non-small-cell lung cancer patients. Ann Oncol 2017;28(8):1988-95.
83. Yamazaki N, Kiyohara Y, Uhara H, Iizuka H, Uehara J, Otsuka F, et al. Cytokine biomarkers to predict antitumor responses to nivolumab suggested in a phase 2 study for advanced melanoma. Cancer Sci 2017;108(5):1022-31.
84. Das R, Verma R, Sznol M, Boddupalli CS, Gettinger SN, Kluger H, et al. Combination therapy with anti-CTLA-4 and anti-PD-1 leads to distinct immunologic changes in vivo. J Immunol 2015;194(3):950-9.
85. Kim HK, Heo MH, Lee HS, Sun JM, Lee SH, Ahn JS, et al. Comparison of RECIST to immune-related response criteria in patients with non-small cell lung cancer treated with immune-checkpoint inhibitors. Cancer Chemother Pharmacol 2017;80(3):591-98.
86. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, et al. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110(4):1225-32.
87. Chen L, Han X. Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. J Clin Invest 2015;125(9):3384-91.
88. Liu M, Guo S, Stiles JK. The emerging role of CXCL10 in cancer (Review). Oncol Lett 2011;2(4):583-89.
89. Choueiri TK, Fishman MN, Escudier B, McDermott DF, Drake CG, Kluger H, et al. Immunomodulatory Activity of Nivolumab in Metastatic Renal Cell Carcinoma. Clin Cancer Res 2016;22(22):5461-71.
90. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res 2009;29(6):313-26.
91. Li M, Knight DA, L AS, Smyth MJ, Stewart TJ. A role for CCL2 in both tumor progression and immunosurveillance. Oncoimmunology 2013;2(7):e25474.
92. Lanca T, Costa MF, Goncalves-Sousa N, Rei M, Grosso AR, Penido C, et al. Protective role of the inflammatory CCR2/CCL2 chemokine pathway through recruitment of type 1 cytotoxic gammadelta T lymphocytes to tumor beds. J Immunol 2013;190(12):6673-80.
93. Monti P, Leone BE, Marchesi F, Balzano G, Zerbi A, Scaltrini F, et al. The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity. Cancer Res 2003;63(21):7451-61.

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