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A harmonized approach towards immuno-monitoring of myeloid-derived suppressor cells for prediction of clinical outcomes in melanoma patients treated with ipilimumab

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ABBREVIATIONS

A2aR: adenosine A_{2A} receptor

ACT: adoptive cell transfer

APC: antigen presenting cell

Arg-1: arginase 1

BM: bone marrow

BM-MDSC: BM-derived myeloid-derived suppressor cells

BRAF: v-Raf murine sarcoma viral oncogene homolog B

BTLA: B- and T-lymphocyte attenuator

CAT: computered axial tomography

CCL2: chemokine (C-C motif) ligand 2

CCL22: chemokine (C-C motif) ligand 22

CCR4: chemokine (C-C motif) receptor 4

CD: cluster of differentiation

CDKN2A: cyclin-dependent kinase inhibitor 2A

CIK: cytokine-induced killer

CIP: cancer immunoguiding program

CRP: C-reactive protein

CTLA-4: cytotoxic T-lymphocyte antigen 4

CV: coefficient of variation

CXCL1: chemokine (C-X-C motif) ligand 1

CXCL9: chemokine (C-X-C motif) ligand 9

CXCL10: chemokine (C-X-C motif) ligand 10

DC: dendritic cell

DCM: dead-cell marker

EDTA: ethylenediaminetetraacetic acid

FBS: fetal bovine serum

FcR: Fc receptor

FDA: Food and Drug Administration

FMO: fluorescence minus one

FoxP3: forkhead box P3

FSC: forward scatter

G-CSF: granulocyte-colony stimulating factor

GM-CSF: granulocyte macrophage-colony stimulating factor

G-MDSC: granulocytic MDSC

HD-PMN: high-density polymorphonuclear cells

HLA: human leukocyte antigen

HIV: human immunodeficiency virus

HS: human serum

I-MDSC: immature myeloid-derived suppressor cells

ICI: immune checkpoint inhibitor

ICOS: inducible T-cell costimulator

IDO: indoleamine 2 3-dioxygenase

IFN-α: interferon-α

IFN-γ: interferon-γ

Ig: immunoglobulin

IL: interleukin

IL4Rα: α chain of IL-4 receptor

IMDM: Iscove's modified Dulbecco's medium

iNOS: inducible nitric oxide synthase

ir-ADR: immune-related adverse drug reaction

LAG-3: lymphocyte-activation gene

LAK: lymphokine-activated killer

L-arg: L-arginine

LDH: lactate dehydrogenase

LD-PMN: low-density polymorphonuclear cells

L/D: Live/Dead®

Lin: lineage

mAb: monoclonal antibody

MAGE: Melanoma-associated antigen

MAPK: mitogen-activated protein kinase

MART: melanoma antigen recognized by T cells

MDSC: myeloid-derived suppressor cell

MEK: mitogen-activated protein kinase kinase

MFI: mean fluorescence intensity

MHC-I: major histocompatibility complex-I

MHC-II: major histocompatibility complex-II

MITF: microphtalmia-associated transcription factor

MMP9: matrix metalloproteinase 9

M-MDSC: monocytic-MDSC

NADPH: nicotinamide adenine dinucleotide phosphate

NK: natural killer

NKT: natural killer T cells

NO: nitric oxide

NOS: nitric oxide synthase

NY-ESO-1:

OS: overall survival

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

PD-1: programmed death-1

PD-L1: programmed death ligand 1

PD-L2: programmed death ligand 2

PFS: progression free survival

PGE2: prostaglandin E2

PI3K: phosphoinositide 3-kinase

PTEN: Phosphatase and tensin homolog

PMN: polymorphonuclear cells

RAG: recombination activation gene

RAGE: receptor for advanced glycation endproducts

RCC: renal cell carcinoma

ROS: reactive oxygen species

RNS: reactive nitrogen species

RPMI: Roswell Park Memorial Institute medium

SSC: side-scatter

TCR: T cell receptor

TDF: tumor-derived factor

TGF-β: tumor growth factor-β

Th: T helper

TIL: tumor-infiltrating lymphocytes

TIM-3: T cell immunoglobulin and mucin-domain-containing molecule-3

TNM: TNM classification of malignant tumours

Treg: regulatory T cell

UV: ultra violet

VEGF: vascular endothelial growth factor

W: week

ABSTRACT

Ipilimumab is a monoclonal antibody against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) that belongs to a new class of immunotherapeutic drugs called immune checkpoint inhibitors (ICI) which prevent the feedback inhibition of activated T cells and hold great promise to treat cancer. Indeed, it has been demonstrated that ipilimumab increases overall survival (OS) of metastatic melanoma patients, but a durable 5-year survival benefit is observed in a proportion of patients ranging from 12% to 49% and, the treatment has mild to severe immune-related adverse events (irADR). In this context, the development of reliable biomarkers is of great importance to select patients with higher possibility to benefit from this treatment. In this respect, immune profiling could play a significant role, and, in particular, the monitoring of myeloid-derived suppressor cells (MDSC), which are predictive of OS and response to chemotherapy in many types of cancer, and thus represent a promising biomarker also for response to ICI. However, the validation of the predictive significance of MDSCs is challenged by the phenotypic complexity of these cells, that lacks an international consensus on the minimal requirements for MDSC monitoring.

To meet this request, we organised the first proficiency panel to harmonize human MDSC phenotyping in collaboration with the Cancer Immunoguiding Program. Hence, we proceeded to the first phase, that consisted in phenotyping three batches of PBMC distributed to the 23 participating laboratories. We also analysed data from the 23 laboratories and observed that the quantification of MDSCs across different laboratories was affected by high variance, and we identified some parameters responsible for the high heterogeneity of results. Results of this first step will set the basis for the second step which is expected to reduce inter-laboratory variance.

From this experience, we developed a standardized approach to monitor the circulating levels of four MDSC subsets in melanoma patients undergoing ipilimumab treatment. These results were included in a wide dataset together with other tumor-associated and immunological parameters (TIPs) and used to identify early predictors of OS and toxicity through a multivariate non-parametric statistical approach.

We followed the variations of TIPs during ipilimumab treatment and identified two immune profiles predictive of OS. The immune profile of patients with better prognosis included: lower baseline levels of IL-6, CRP and VEGF and higher post-treatment frequencies of $CD3^+$ T cells and $CD4^+/CD279^+$ T cells. On the other hand, patients with worse prognosis presented higher post-treatment levels of MDSC1 and 2 and of $CD8^+/PD\text{-}1^+$ T cells. In addition, the development of grade 3 irADR was negatively associated to the levels of $CD3^+$ and $CD4^+/PD\text{-}1^+$ T cells and of eosinophils, while positively associated to an increased variance of MDSC4. In conclusion, we demonstrated that the monitoring of the immunological correlates has the potential to identify patients with better prognosis following ipilimumab treatment, thus guiding a more rational use of this therapy.

RIASSUNTO

Ipilimumab è un anticorpo monoclonale diretto contro cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) e appartiene a una nuova classe di farmaci immunoterapeutici chiamati immune checkpoint inhibitors (ICI). Questi farmaci prevengono la fisiologica inibizione dell'attivazione dei linfociti T e costituiscono una promettente terapia per la cura del cancro. Infatti è stato dimostrato che ipilimumab è in grado di aumentare la sopravvivenza dei pazienti affetti da melanoma metastatico. Tuttavia il farmaco produce una sopravvivenza stabile a 5 anni in una parte variabile dei pazienti trattati (12-49%) e inoltre può comportare effetti collaterali con eziologia di tipo immunologico (irADR) di grado medio o alto. Risulta quindi importante identificare biomarcatori che siano in grado di discriminare i pazienti con più alta probabilità di trarre beneficio da questo tipo di trattamento. A tal fine può essere importante tracciare un profilo immunologico del paziente, e in particolare monitorare i livelli circolanti di cellule soppressorie di derivazione mieloide (MDSC). Infatti i livelli circolanti di queste cellule sono stati associati a una minor sopravvivenza e risposta a trattamenti chemioterapici in diversi tipi di cancro, e quindi possono avere un ruolo potenzialmente predittivo anche nei confronti della risposta agli ICI. Tuttavia la validazione del potere predittivo delle MDSC si deve scontrare con la complessità fenotipica di queste cellule e con la mancanza di un consenso internazionale circa la definizione da applicare per il loro monitoraggio.

Per cercare di dare una risposta a questi problemi, abbiamo organizzato in collaborazione con il Cancer Immunoguiding Program, un pannello internazionale di armonizzazione del fenotipo delle MDSC. Attualmente è stata completata la prima fase che consisteva nel fenotipizzare tre campioni di cellule mononucleate da sangue periferico da parte dei 23 laboratori partecipanti. Analizzando i dati provenienti da questi 23 laboratori abbiamo osservato che la quantificazione delle MDSC presentava un'alta varianza e abbiamo identificato alcuni dei parametri responsabili di tale eterogeneità. I risultati di questa prima fase sono le fondamenta per la progettazione della seconda fase da cui ci aspettiamo una riduzione della varianza tra i diversi laboratori.

L'esperienza maturata con il pannello di armonizzazione ci è servita per sviluppare un metodo standardizzato per il monitoraggio di quattro sottopopolazioni di MDSC circolanti

in pazienti affetti da melanoma e in trattamento con ipilimumab. Questi risultati assieme ai valori di altri parametri, associati al tumore o al profilo immunologico del paziente (TIPs), sono stati elaborati con metodi statistici multivariati e non-parametrici atti ad individuare dei marcatori precoci di sopravvivenza e tossicità in risposta al trattamento con ipilimumab.

Grazie al monitoraggio dei livelli di questi parametri durante la terapia con ipilimumab, è stato possibile individuare due profili predittivi di sopravvivenza. I pazienti con migliore prognosi hanno minori livelli basali di IL-6, CRP e VEGF e maggiori livelli di linfociti T, in particolare della sottopopolazione $CD4^+/CD279^+$ al termine della terapia. Al contrario, una ridotta sopravvivenza si accompagna a maggiori livelli di MDSC1-2 e di linfociti T $CD8^+/PD\text{-}1^+$ post-trattamento. Inoltre lo sviluppo di irADR di grado 3 è inversamente correlato con i livelli di linfociti T ($CD3^+$ e $CD4^+/PD\text{-}1^+$) e di eosinofili, mentre è direttamente correlato con un aumento nella varianza dei valori di MDSC4. In conclusione questo studio ha dimostrato che le modificazioni del profilo immunologico associate al trattamento con ipilimumab possono dare informazioni sulla prognosi del paziente e quindi indirizzare le scelte cliniche verso un uso più razionale di questo tipo di terapia.

INTRODUCTION

A historical overview of the tumor immunology: from the concept of immunosurveillance to the cancer immunoediting theory

The idea that the immune system can control growing tumors was formally enunciated by Burnet and Thomas in the hypothesis of cancer immunosurveillance[1]. According to this theory, malignant transformation naturally occurs in the body as a result of genetic changes, but tumors do not become clinically detectable because they are eliminated by the immune system which recognises the new antigenic determinants expressed on their surface. The higher incidence of cancer in immunodeficient or transplanted patients constituted an early evidence supporting the immunosurveillance theory[2,3]. However, the long-term follow up of these patients revealed that this high risk was partially explained by an impaired natural protection against oncogenic virus[4], and similar findings were observed also in mice with induced immunodeficiency[5]. In the same years, the hypothesis of immunosurveillance was further challenged by the work of Stuntman and colleagues demonstrating that athymic nude mice did not developed spontaneous or chemical-induced tumors with a higher rate than immunocompetent controls[6]. At that time, these results constitutes an important proof against the hypothesis of cancer immunosurveillance, but it is now clear that those results were affected by a major pitfall. Indeed, nude mice are not completely immunodeficient, as they were considered at that time, but, on the contrary, they still possess myeloid cells, natural killer cells (NK) and B lymphocytes, which can thus provide a reduced, but sufficient, anti-tumor immunity. Some decades afterwards, the improved knowledge in the field of tumor immunology and important technological discoveries brought about a renewed interest in the theory of cancer immunosurveillance. In particular, the development of transgenic and knoc-out mice lacking NK, T and B lymphocytes finally provided strong and convincing data in favour of cancer immunosurveillance[7-9]. Indeed, RAG1-2$^{-/-}$ mice failed to control both chemical-induced and natural occurring carcinogenesis[10] and, since the impairment of their immune system is confined only to lymphocytes, these results constitutes an important evidence that these cells play a major role in the surveillance of nascent tumors. In fact, previous studies have already demonstrated that IFN-γ, a cytokine produced by T cells, block the growth of transplanted tumors[11] but Shankaran *et al*., definitively proved that lymphocytes and IFN-γ block the formation of chemical-induced tumors in a synergic fashion[10] . The clinical relevance of tumor-infiltrating T lymphocytes (TIL) was recently investigated by Galon and colleagues in the context of human colorectal carcinoma in which they demonstrated that a massive infiltration of T cells is associated

with better prognosis of patients and it constitutes a discriminating parameter for the choice of the therapeutic approach applied after the resection of primary colorectal cancer[12].

Albeit from the late 1990s the hypothesis of immunosurveillance lived a period of renaissance, this concept finds its intrinsic caveat in the fact that microscopic neoplasia can often overwhelm the immune control and thus become clinically detectable. In fact, a number of experiments revealed that the immune system exerts both a host-protective and tumor-sculpting effect on developing malignancies (reviewed in Dunn[7]). A striking evidence of this phenomenon is that tumors formed in immune-competent mice fails to be immunogenic when transplanted into a second immune-competent host, while tumors developed in an immunodeficient animal are then effectively rejected upon transfer in an immune-competent recipient[10]. As a consequence, Robert Schreiber and colleagues believed that the original immunosurveillance theory was no longer appropriate to fully describe the interaction between immunity and tumors, and they proposed to use the broader term "cancer immunoediting" to explain the ambivalent behaviour of the immune system towards cancer[7].

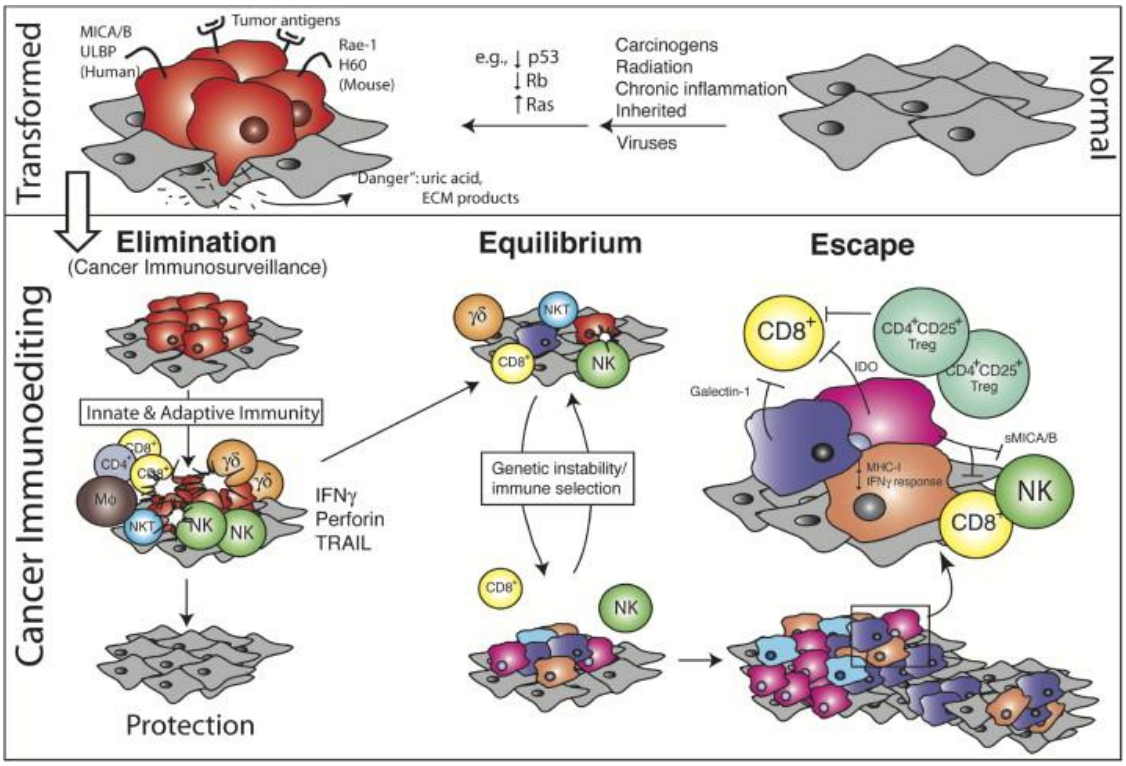


Figure 1: The three Es of cancer immunoediting
The three Es of cancer immunoediting refers to 'Elimination', 'Equilibrium', and 'Escape'. Elimination: the development of tumor is kept in check by the immunosurveillance. Equilibrium: tumor cells that have survived the immune surveillance are in balance with immunity of the host. Escape: tumor cells grow and overwhelm the control of the host immune system. (Dunn *et al.* Immunity, 2004)

According to the "cancer immunoediting" theory, the crosstalk between cancer and immune system results from three processes: *E*limination, *E*quilibrium and *E*scape (Figure 1). The original idea of immunosurveillance is included in the elimination phase in which the growth of a growing tumor is controlled by the immune system. This phase corresponds to the theory of immunosurveillance, if the tumor is completely eliminated by the immune system. However, if the elimination of the tumor is incomplete, then a second phase takes place, called the equilibrium phase, in which the genetic instability of malignant cells gives rise to low-immunogenic clones that have a higher probability to survive in an immune-competent host and are therefore favoured in the Darwinian selection of tumor variants. In the equilibrium phase, the tumor is not yet clinically apparent because the immune system is still able to control the growth of the majority of malignant cells. If this equilibrium phase breaks, the third phase of cancer immunoediting, called escape phase, takes place. This phase can be achieved only if cancer cells become able to suppress the immune system and can therefore expand in uncontrolled manner becoming clinically detectable (reviewed in[7]). The concept of cancer immunoediting is now considered a pillar of tumor immunology and, indeed, in 2011 Hanahan and Weiberg acknowledged immune escape as an important hallmark of cancer[13].

The concept of tumor microenvironment

Different types of leukocytes infiltrate the tumors having a beneficial as well as a deleterious action on disease progression, but the tumor microenviroment is far from being composed only of immune cells[14]. Indeed, stromal cells, like fibroblasts, shape and sustain the complex architecture of cancer, and vascular endothelial cells formed blood vessels supporting the nutritional requirements of the growing mass. Even if these ancillary cells do not belong to the hematopoietic lineage, they are included in the definition of immune contexture because they can take part in the modulation of immunity within the tumor mass[15]. The purpose of this section is to give a brief overview of the nature and the function of the players composing the tumor microenvironment with a special focus on their ability to modulate immunity in favour of malignant cells.

Most of the immunological contexture is composed of infiltrating leukocytes belonging both to innate and acquired immunity (reviewed in [14]). The leukocytes mainly involved in tumor eradication are T lymphocytes and they are characterized by high specificity of action due to their antigen specificity. T cells can be classified in two main subsets according to expression of the two co-receptors CD4 and CD8: CD4 is expressed both by T helper (Th) and regulatory T cells (Treg), while CD8 is a marker of cytotoxic T lymphocytes (CTL)[16]. CTL are able to destroy malignant cells

upon recognition of antigens presented on MHC I molecules by the cancer cells[16]. However, as antigen presentation by tumor cells is often inefficient[17], the effective recognition of antigens by $CD8^+$ cells could occur also via cross-presentation on MHC I molecules by antigen presenting cells [18]. On the other hand, tumor antigens could be presented to T lymphocytes also by MHC II molecules, recognized by $CD4^+$ Th cells which activate a variety of immune cells through ligand-dependent interactions and secretion of cytokines[16]. Immune infiltrates characterized by high $CD8^+$ cells and memory T cells are prognostic of better diseases control while association between $CD4^+$ cells and clinical outcome is more controversial (reviewed in [15]). Albeit a T cell infiltrate is generally associated with better prognosis, studies on melanoma revealed that TIL have blunted functions that can be rescued after a period of *in-vitro* culture[19]. Indeed, several mechanisms are known to limit T cell functions in the tumor milieu. The main mechanism of negative regulation of T cells is the induction of exhaustion[20], a process characterized by expression of multiple inhibitory receptors on T cells which impair their activation and effector functions[21], and generation of regulatory T cells capable of directly suppressing the T cell response[22].

The innate immunity could contribute to tumor elimination through short-lived responses mediated mainly by NK and natural killer T cells (NKT) (reviewed in [23]). However, tumors are able to impair also the action of innate cytotoxicity and indeed intra-tumoral NK cells are often anergic[24].

Myeloid cells are the leukocytes characterized by the strongest ambivalence. In fact, on one side myeloid dendritic cells and M1 macrophages are responsible of efficient antigen presentation and contribute to the immune attack towards malignant cells, but on the other side several myeloid cells can prevent or suppress anti-tumor immunity (reviewed in [25]). M2 macrophages, plasmacitoid dendritic cells and myeloid-derived suppressor cells (MDSC) are key suppressive players and, albeit each subset is provided with specific suppressive machinery, they share some characteristics like the expression of ligands for immune checkpoint molecules (i.e PDL-1), the production of suppressive cytokines (i.e. IL-10) and the activation of indoleamine-2,3-dioxygenase (IDO) (reviewed in [14]).

An important contribution to the immunological contexture is given by stromal cells like fibroblast, vascular endothelial cells and extracellular matrix. These components shape the architecture of cancer and can impede immune infiltration through a direct physical barrier[26] or can contribute to immune evasion actively participating to immune suppression[27]. For example, a recent study by Zhang *et al*. demonstrated that fibrocytes represents a novel subsets of MDSCs present in the blood of cancer patients[28].

The coordination of this dynamic interplay between immune players, stromal cells and cancer is orchestrated by a variety of cytokines and chemokines. High expression of CX3CL1, CXCL9 and CXCL10 is associated with infiltration of effector and memory T cells[29] while the CCR4 - CCL22 axis drives the trafficking of suppressor cells[30]. Moreover, a recent study indicates that production of reactive nitrogen species within the tumor microenvironment results in nitration of CCL2 which results in a hindered T cell infiltration[31]. On the other hand, cytokines are more involved in shaping the functional properties of the immune contexture. There are cytokines with direct suppressive function like IL-10 and TGF-β, while cytokines classically involved in inflammation, like GM-CSF or IL-6, could mediate immune suppression through induction of MDSCs[25,32].

Immunotherapy of cancer

The increasing comprehension of the mechanisms involved in the relationship between cancer and the immune system shed light on the possibility to manipulate these interactions in favour of cancer therapy. The early attempts to exploit an immune reaction to cure cancer came from the studies using Coley's toxin or Bacillus Calmette-Guerin[33,34] while the first immunotherapy approach consisted in the administration of cytokines like IL-2 or IFN-α which either sustain T cells proliferation[35] or have a broader anti-proliferative effect on malignant cells[36]. A rush in the development of immunotherapy began in 1970s thanks to the improved knowledge of tumor immunology and important technological discoveries which guided the development of modern immunotherapeutics consisting in antibodies, cytokines, vaccines and cellular therapy (reviewed in [37]).

The revolution started with the discovery of the hybridoma technology in the 1970s[38]. This technique permits a large scale production of epitope-specific murine antibodies (monoclonal antibodies – mAb) that could be used either for cancer treatment or to implement diagnostics. The first therapeutic mAbs were targeted to tumor antigens and induced effective complement- and cell-dependent lysis of the tumors but the results were characterized also by unexpected toxicity (reviewed in [37]) due to inter-species cross-reaction. The production of fully human mAbs solved this problem and nowadays many antibodies of this kind are commonly used as drugs[39]. The distribution of mAbs depend on the degree of vascularisation of the tumor but, when this requirement is fulfilled, they can function also as carriers for radioisotopes, toxins or chemotherapy. Recently, research went beyond tumor-specific mAbs and developed a new class of mAbs targeted to regulators of immune response called immune checkpoints[40]. These molecules are exposed on T cells and can act either as negative or positive regulators of T cell response. Ipilimumab and pembrolizumab, two mAbs blocking negative regulators of T cells are

the first immune checkpoint inhibitors approved by the Food and Drug Administration (FDA)[41,42], while other mAbs with immune-stimulatory functions are under development (reviewed in [43]).

The discovery of tumor-associated antigens resulted in different strategies of therapeutic vaccination for cancer (reviewed in [44]). The road toward an efficient vaccination protocols is an example of how advancements in tumor immunology are translated to clinical protocols in order to implement cancer immunotherapy. Indeed, the first vaccines were based on administration of whole tumor cells derived either from autologous or allogeneic tumors. These cells were inactivated by radiation and their immunogenic potential was enhanced by chemical treatment or genetic manipulation in order to secrete immune activating cytokines like GM-CSF and IL-2[44]. However, the efficacy of these treatments was not confirmed by phase III clinical studies, and therefore subsequent efforts have been invested to enhance the immunogenic potential of vaccines. In particular, many studies focused on boosting antigen presentation of tumor antigens, through the use of activated and polarised dendritic cells pulsed with autologous tumor lysate in order to convey an efficient and specific response against the tumors[45,46]. Sipuleucel T is a vaccine composed of autologous dendritic cells pulsed with prostatic acid phosphatase antigen and activated with GM-CSF is the only vaccination protocol approved by FDA for cancer treatment[47].

The experience reached in the field of cancer vaccines offered a paradigm that could be applied to the other immunotherapeutic approaches: an effective immune rejection of cancer can be obtained only by properly stimulating and activating the immune effector cells, and the general level of immune suppression must be taken into account, and eventually ablated, to reach the proposed therapeutic effects.

Following this rationale, cancer vaccination can be effective when the immunity of the patient is functional, but if a deeper impairment is present, adoptive cell transfer (ACT) should be preferred in order to supply the suppressed natural immunity with new functional effector cells[48]. The first attempts to generate a *de-novo* immune response against cancer were pioneered by S. Rosenberg and collaborators who isolated TIL from melanoma biopsies and re-infused these cells into patients after a period of *in-vitro* activation, thus obtaining a complete and durable eradication of the tumor in some patients[49]. Following this experience, over the years many other groups contributed to implement ACT with natural occurring TILs or with autologous lymphocytes bearing an engineered T cell receptor with higher affinity for cancer cells[50]. The same procedures have been applied also to NK and NKT cells which can be activated *in-vitro* with specific cocktails of cytokines and re-injected as lymphokine-activated killer (LAK) or cytokine-induced killer (CIK), respectively[51,52]. The major obstacle of these approaches is that patients must undergo a

preparative lymphodepletion and must be infused with high doses of IL-2, to sustain the efficacy of ACT, and this treatment has a high toxicity and must be managed only by highly specialized centres[48].

A novel strategy to implement ACT is to eradicate the suppressive network present in the tumor by combinatorial approaches like adding a cycle of chemotherapy, radiation or targeted therapy to deplete suppressive leukocytes prior to ACT (reviewed in [53,54]). Another strategy to boost the activation of transferred T cells is to combine ACT with immune checkpoint blockade, and the efficacy of this approach is currently under clinical investigation[54].

Myeloid-derived suppressor cells as key players in the immune suppression of tumors

The first studies describing myeloid cells endowed with suppressive capability dated back to the late 1990s[55,56], and they were followed by a number of studies further describing the suppressive potential of myeloid cells characterised by different stages of maturation and distinct phenotypes (reviewed in [57]). These cells were often addressed as "immature myeloid cells" or "suppressive myeloid cells" but neither of these names seemed accurate. In 2008 a group of leading scientists in the field proposed to find a new acronym to unequivocally identify cells of myeloid origin, displaying an impaired differentiation and endowed with immune-suppressive functions which were often induced under pathological conditions, especially in cancer[58]. As a result, the acronym myeloid-derived suppressor cells (MDSC) which is now in use.

It has been demonstrated that MDSCs can be induced by some pathological conditions, from infection to autoimmune diseases and their level seems to increase also with age[59], but MDSCs were originally identified in cancer[25]. Indeed, our group has demonstrated that MDSCs can be induced *in-vitro* from bone marrow progenitors by adding a cocktail of cytokines, commonly secreted by the tumors[32]. Nevertheless, there are some factors that seem to be more involved in the development of MDSCs and they comprehend a number of pro-inflammatory cytokines (GM-CSF, IL-6, S100A8, S100A9, prostaglandins) but also angiogenic factors like vascular endothelial growth factor (VEGF). Several groups demonstrated the link between tumor-derived factors and MDSC expansion[60-62], and several associations between the levels of pro-inflammatory factors and the circulating levels of MDSCs have been reported also in patients with cancer (reviewed in [63]).

Phenotype of mouse MDSCs

MDSCs were originally identified in mice by using the combination of the myeloid markers CD11b and Gr-1[64]. Further studies elucidated that CD11b+/Gr-1+ cells are not a homogeneous cell population, but rather a heterogeneous collection of myeloid cells, endowed with different suppressive ability. At present, it is well known that there are at least two main subsets of MDSCs present in tumor-bearing mice, one monocytic and one granulocytic, and these populations can be properly identified by using a set of accessory markers. In particular the differential expression of Ly6C and Ly6G, the two isoforms of Gr-1, allow the identification of granulocyic MDSC (G-MDSC) as $CD11b^{+}/Gr\text{-}1^{high}/Ly6C^{-}/Ly6G^{high}$ and monocytic MDSCs (M-MDSC) as $CD11b^{+}/Gr\text{-}1^{int}/Ly6C^{high}/Ly6G^{-}$ [57]. The appropriate definition of these subsets is important in view of their differential localization and suppressive power. Indeed, several studies demonstrated that G-MDSC are mostly located in the secondary lymphoid organs while M-MDSC constitutes the

majority of immune suppressive cells at the tumor site. This is intriguing considering that M-MDSC are endowed with a higher suppressive capability than G-MDSCs, on a per cell basis[65-67]. Other potential markers have been proposed to identify the two main subsets, like CD124, CD115, CD40, CD49d, CD244, but as these markers are shared also by inflammatory monocytes and neutrophils, a suppressive assay is always required for a complete definition of MDSCs[57]. A good surrogate for phenotypic definition of MDSCs is the expression of the α chain of interleukin-4 receptor (IL4Rα or CD124) which has been implicated also in MDSC function and survival[68,69] and it is express also by human MDSCs[70,71].

Phenotype of human MDSCs

The phenotypic characterization of human MDSCs is hampered by the lack of a specific marker, and by the absence of a homologue of the murine Gr-1; therefore, pan-myeloid markers like CD11b or CD33, are used to define MDSCs in humans, together with lineage-specific markers. Indeed, three main classes of MDSCs with distinct lineage-commitments have been identified in the blood of cancer patients: monocytic, granulocytic and immature MDSCs. M-MDSCs are characterized by the expression of CD14 and frequently presented a downregulation of HLA-DR and enhanced IL4Rα expression[70,72]. G-MDSCs are characterised by the expression of markers specific of the polymorphonuclear lineage like CD15 and CD66b and could be detected both in low- and high-density polymorphonuclear cells (LD-PMN, HD-PMN)[73,74]. This definition is based on the behaviour of PMN during density gradient separation: LD-PMN co-stratify with peripheral blood mononuclear cells (PBMC) while HD-PMN have a higher density and therefore lay below the PBMC layer, on the top of erythrocytes. G-MDSC belonging to LD-PMN are generally defined as $CD14^-/CD15^+/CD66b^+/CD11b^+/SSC^{int}$ [75] while those included in HD-PMN are characterized by expression of IL4Rα and high side scatter (SSC)[70]. Immature MDSCs (I-MDSCs) are a cell subset that do not express the markers of mature immune cells like lymphocytes, monocytes and granulocytes[76,77]. As a consequence, they stain negative for an antibody cocktail containing markers of mature leukocytes (Lineage cocktail) and they also show a low expression of HLA class II, which is peculiar of immature cells. I-MDSCs ($Lineage^-/HLA\text{-}DR^-/CD33^+/CD11b^+$) circulating in the peripheral blood of patients with solid tumors have a phenotype similar to promyelocytic-like suppressive cells induced *in-vitro* from healthy bone marrow using a cocktail of GM-CSF and G-CSF[78].

The expansion of a number of MDSC phenotypes has been described in cancer patients, all of them falling in one of the three above mentioned categories and showed in Figure 2. This result depends on the lack of a standard maker combination to define human MDSCs, which led to

different combinations of common myeloid markers to define myeloid cells populations as MDSCs. It is tempting to speculate that some of the subsets are partially or completely overlapping inside each category, but so far this possibility has never been proved. This situation is further complicated by the plasticity of MDSCs which can express also other markers (like S100A8, S100A9, CD79) in response to the tumor-derived factors to which they are exposed (reviewed in [63]). Hence, the definition of human MDSC phenotype is an issue that must find a correct answer, also in view of the potential role of these cells as biomarker of response to cancer treatment. To address this problem, the Cancer Immunoguiding Program recently organised an international proficiency panel to harmonize the immunophenotyping of circulating human MDSC using cryopreserved samples.

	MDSC phenotype	Tumor
Monocytic MDSCs	$CD14^+$ HLA-$DR^{low/-}$	Melanoma
		Head and neck cancer
		Lung cancer
		Hepatocellular carcinoma
		Renal cell carcinoma
		Bladder carcinoma
		Prostate cancer
		Hematological malignancies
		Gastrointestinal cancer
	$CD14^+$ IL4Rα^+	Melanoma
		Gastrointestinal cancer
		Renal cell carcinoma
	$CD14^+$ HLA-DR^- IL4Rα^+	Glioblastoma
	$CD34^+$ $CD14^+$ $CD11b^+$ $CD33^+$	Ovarian cancer
	$CD11b^+$ $CD14^+$ $CD33^+$	Lung cancer
	$CD33^+$ HLA-DR^-	Renal cell carcinoma
Immature MDSCs	Lin^- HLA-DR^-	Renal cell carcinoma
	Lin^- HLA-DR^- $CD33^+$	Hepatocellular carcinoma
		Renal cell carcinoma
		Lung cancer
	Lin^- HLA-DR^- $CD33^+$ $CD11b^+$	Melanoma
		Glioblastoma
		Lung cancer
		Breast cancer
		Gastrointestinal cancer
		Pancreatic carcinoma
Granulocytic MDSCs	Lin^- $CD33^+$ $CD11b^+$ $CD15^+$	Pancreatic carcinoma
	Lin^- HLA-DR^- $CD33^+$ $CD11b^+$ $CD14^-$ $CD15^+$	Gastrointestinal cancer
		Melanoma
	$CD11b^+$ $CD14^-$ $CD33^+$	Head and neck cancer
		Lung cancer
		Hematological malignancies
	$CD11b^+$ $CD14^-$ HLA-DR^- $CD33^+$ $CD15^+$	Hematological malignancies
	$CD33^+$ HLA-DR^- $CD15^+$	Gastrointestinal cancer
		Renal cell carcinoma
	$CD15^+$ IL4Rα^+	Melanoma
		Gastrointestinal cancer
		Renal cell carcinoma
	$CD11b^+$ $CD15^+$ $CD66b^+$	Renal cell carcinoma
	$CD15^+$ FSC^{low} SSC^{high}	Renal cell carcinoma
	$CD15^{high}$ $CD33^+$	Bladder carcinoma
	$CD11b^+$ $CD14^-$ $CD15^+$	Renal cell carcinoma
	$CD66b^+$ SSC^{high}	Head and neck cancer
		Lung cancer
		Bladder and ureter cancer
	$CD11b^+$ $CD15^+$	Pancreatic carcinoma

Figure 2: Overview of described human MDSC phenotypes in cancer patients
The main MDSCs subsets reported in literature were classified as monocytic, granulocytic and immature and further divided on the basis of their phenotype. (Solito *et al.*, Annals of the New York Academy of Science, 2014)

Overview of the suppressive mechanisms of human MDSCs

It has been demonstrated that MDSCs are only capable to suppress the T cell response but also of influencing the behaviour of other immune players, like NK cells, in the context of a cross-talk between myeloid cells and cancer (reviewed in [25]). Besides, recent studies recognise that MDSCs have pleyotropic functions that are not limited to the immune suppression because, these cells

are involved also in the metastatic process[79], in angiogenesis[80] and in the interplay with cancer stem cells[81]. In particular, MDSCs support metastasis development in different ways: they prepare the pre-metastatic niche by creating an immune suppressed milieu[82] and by re-arranging the architecture of extracellular matrix through secretion of metalloproteinase 9[80,83], but they also exert a more direct influence on the metastatic potential of cancer cells inducing the epithelial-mesenchimal transition[84].

Concerning the immune-suppressive potential, one of their main task is to deprive the tumor micro-environment of essential amino acids, like L-arginine or L-tryptophan, through activation of Arginase-1 (Arg-1), inducible Nitric Oxide Synthase (iNOS) or Indolamine-2,3-Dyoxigenase (IDO), respectively (reviewed in [85]). L-arginine depletion reduced the expression of the ζ chain of T cell receptor thus limiting the proliferation of activated T cells[86]; on the other hand, an altered L-tryptophan metabolism leads to L-kynurenine production and activation of a down-stream pathway that is responsible for induction of regulatory T cells. Another important function is to induce an oxidative stress through a coordinated activation of Arg-1, iNOS and NADPH oxydase that leads to production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) which hamper the correct function of T cell receptor, block proliferation of T lymphocytes and alter the trafficking of these cells (reviewed in [60,63]). A third mechanism of immune suppression is the direct activation and expansion of regulatory T cells triggered by IL-10 production, activation of tolerogenic pathways, like the L-kynurenine pathway, and ligand-dependent interactions[25]. MDSCs are also able to convert Th17 cells into regulatory T cells through a mechanism that is dependent on TGF-β secretion and retinoic acid[87].

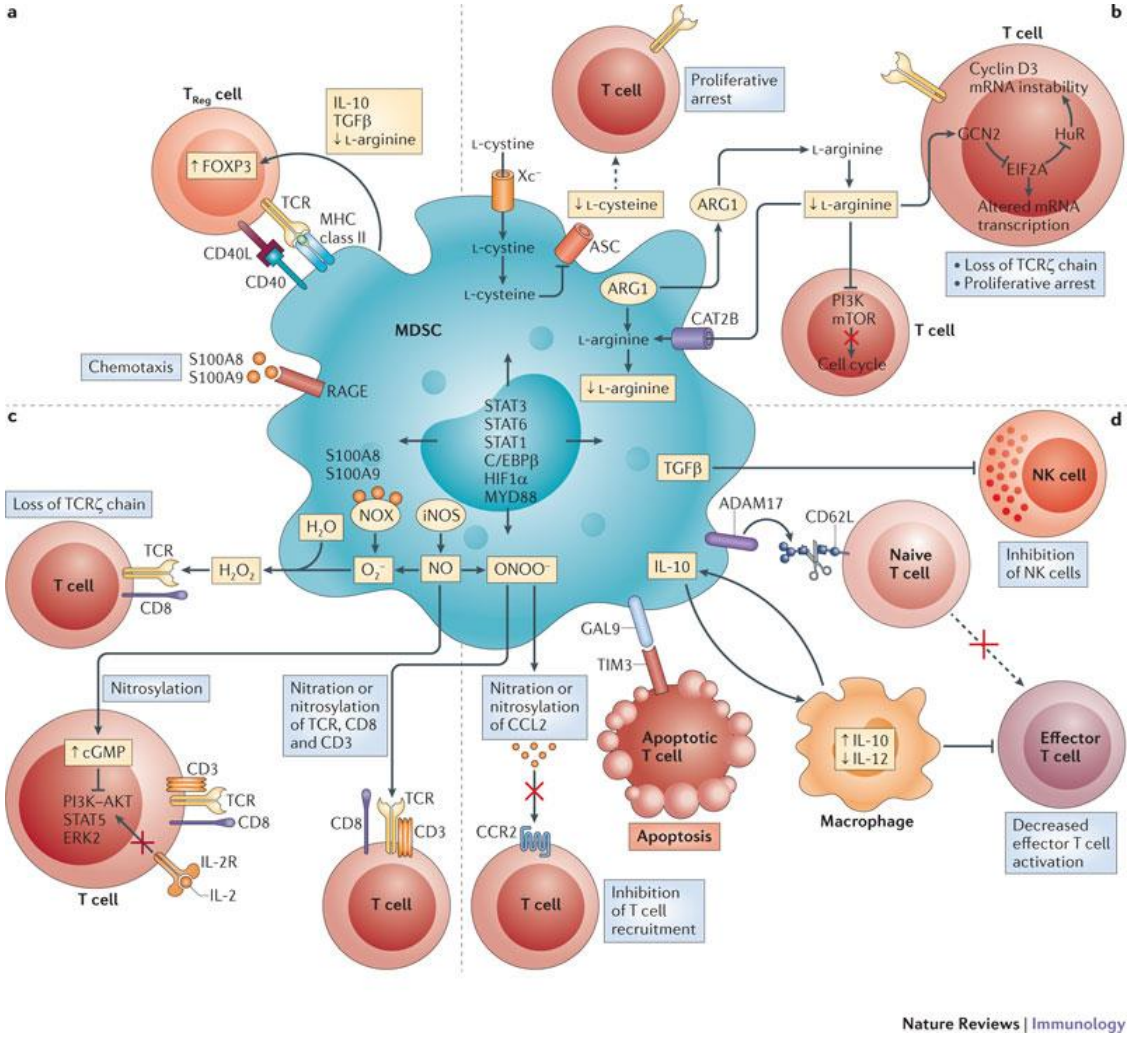


Figure 3: Principal pathways involved in immune suppression by MDSCs

Main mechanisms of suppression induced by MDSCs. a) MDSC induce regulatory T cells b) MDSCs starve T cells of amino acids that are essential for their growth and differentiation. c) MDSCs induce an oxidative stress through production of ROS and RNS. d) MDSC interfere with T cell migration and viability and they cross-talk with different immune players. (adapted from Gabrilovich *et al*., Nature Review Immunology, 2012)

MDSCs are predictors of the clinical evolution of cancer

Given the pivotal role exerted by MDSCs in the process of immune escape, it is reasonable to conceive a relationship between the expansion of these cells and the clinical evolution of cancer. Indeed, the frequency of circulating MDSCs has been frequently correlated with the stage of different solid tumors[63,87-89] and with the level of circulating tumor cells[78]. Besides, it has been demonstrated that MDSC levels are significantly decreased after tumor excision thus proving a direct association between tumor burden and the presence of these cells_ENREF_88[89-92]. As the frequencies of circulating MDSCs reflect tumor progression, they constitute a good predictor of survival or time-to-progression (reviewed in[63]). This correspondence was also used to predict response to chemotherapy[77,78,93] and the study by Walter *et al*., was the first to demonstrate a

correlation between circulating MDSCs and response to a tumor vaccine[94]. In this study, renal cell carcinoma (RCC) patients were treated with a multipeptide vaccination protocol preceded by a single-dose cyclophosphamide and six MDSCs subsets were monitored by flow cytometry: RCC patients expanded 5 out of six MDSC subsets at baseline and, of note, two MDSC populations significantly correlated with survival after vaccination[94]. Recently, several efforts have been made to find reliable biomarkers of response following immune checkpoint blockade treatment. A number of parameter were screened but, apart from lymphocytes count, the only promising biomarker is the circulating level of MDSCs (reviewed in [95]). Indeed, in this context the levels of MDSCs can have a double prognostic significance: on one side they reflect the tumor burden while, on the other hand, they can be considered as an index of the degree of immune suppression that counteracts T-cell activation triggered by ipilimumab.

Melanoma

Epidemiology, pathogenesis and staging

Melanoma is a skin cancer derived from the malignant transformation of melanocytes, the pigmented cells of the skin[96]. Albeit it accounts only for the 4% of total skin cancers, with 76.000 new diagnosis in 2014 according to the American Society of Cancer, it is characterised by high mortality (American Cancer Society) and is indeed responsible for the 80% of deaths from skin cancer and the 5-year survival is about 14%.

Melanoma is characterised by a stepwise mechanism of carcinogenesis in which each transforming event is characterized by defined genetic changes[96]. Indeed, the genetic instability of melanoma is characterised by a set of defined alterations, which could be targeted by specific inhibitors, and a multitude of random mutations that are peculiar of each patients and could give rise to a plethora of new epitopes recognised by TIL. Indeed the first protocol of adoptive cell therapy designed by Rosenberg have been carried out using TILs[49]. Melanoma carcinogenesis is divided in five steps by Clark according to histological changes which could be related to particular genetic mutations[97] (reviewed in [96]) (Figure 4). In the first step a benign nevus, composed of melanocytes, grows stimulated by a constitutive activation of the mytogen-activated protein kinases (MAPK) cascade, often resulting from mutation of the BRAF gene. The presence of nevi is considered benign as long as their growth is controlled by onco-suppressor genes like PTEN and CDKN2A, but when mutations occur at this site, the negative regulation is lost and a pre-malignant lesion develops (dysplastic nevus). Further progression of the dysplastic nevus is associated with decreased differentiation of cancer cells into melanocytes regulated by an oncogene: the microphtalmia-associated transcription factor (MITF). The last steps of carcinogenesis are characterised by enhanced motility of malignant cells, which start a vertical growth phase invading the dermis and progressively spreading to the whole body as metastatic lesions. The major alterations present in this phase affect genes involved in shaping the architecture of the tumor or coding for adhesion molecules and matrix metalloproteinases.

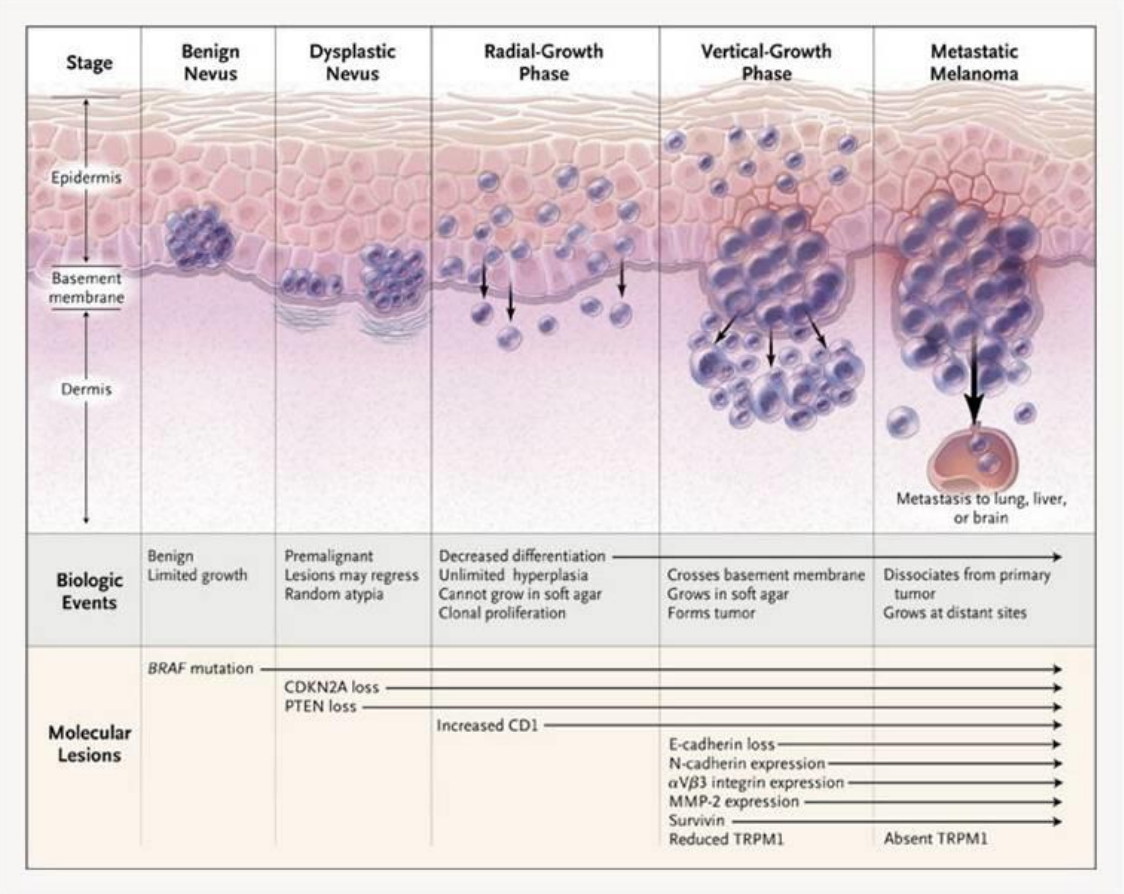


Figure 4: Molecular and biological changes occurring during oncogenesis of melanoma
Main biologic events, according to Clark's model, and molecular changes in the progression of melanoma. (adapted from Miller *et al.*, New England Journal of Medicine, 2006)

Melanomas are classified in four stages according to the TNM categories defined by the American Joint Committee on Cancer[98]. The biological parameters considered for the staging procedure are: the thickness, the ulceration and the mitotic rate of the primary lesion (T), the number and the burden of metastatic lymph nodes (N), the presence of distant metastasis and the levels of serum lactate dehydrogenase (LDH) (M). Stage I and II melanoma are characterised by lesions with different degree of thickness and ulceration but strictly confined to the skin, while stage III melanoma present metastasis to one or more lymph nodes. Stage IV melanoma, which is called also metastatic melanoma, is indeed characterised by presence of distant metastasis; the localization of the metastasis defined the sub-groups: M1a distant metastasis confined to skin or lymph nodes, M1b lung metastasis, M1c all other visceral metastasis with normal LDH levels or any distant metastasis combined with elevated LDH.

Immunological features of melanoma patients

As described above, TIL could be isolated and manipulated to destroy cancer cells, and interestingly, the efficacy of ACT using natural occurring TIL was first demonstrated in

melanoma[49]. Besides, the high genetic instability of melanoma could create a variety of new epitopes which may drive the activation of a higher number of TIL endowed with wider specificity and affinity for tumor antigens[48,99,100]. In fact, a variety of antigens have been identified on the surface of melanoma cells and they can be classified in three main groups: i) germ cell antigens like NY-ESO-1 and MAGE-1 which are normally expressed in male germ cells and silenced in somatic cells but re-expressed on malignant melanocytes; ii) differentiation antigens that characterised both normal ad melanoma cells and include tyrosinase, melan-A MART-1 and gangliosides; iii) unique antigens stemming from random mutations in melanoma cells (reviewed in [101]). The exposure of these tumor-antigens on melanoma cell surface depends on the genetic background of each cancer, and it is thus important to design personalised approaches of immunotherapy like ACT with autologous TIL or vaccination with dendritic cells pulsed with a lysate of autologous tumor[48].

Despite the evidence that immune effectors can play a significant role in controlling tumor growth both spontaneously or in response to therapeutic manipulation , it is clear that in most circumstances cancer cells survive their attack as the disease progresses. Several mechanisms underlying immune escape have been proposed in melanoma and they induce structural and functional changes both in tumor and stromal cells (reviewed in [102]). On one hand, melanoma cells become less immunogenic due to down-regulation of antigen exposure or absence of co-stimulatory molecules, and on the other side they attract suppressive leukocytes through a pattern of secreted chemokines and cytokines. In particular, increasing Treg cells infiltration was found in nevi during melanocytes'transformation, suggesting that these cells induce immunotolerance early during melanoma evolution. Moreover, tumor Ag-specific Treg were found in the blood of patients with metastatic melanoma, and they produced IL-10 and suppressed T-cell responses in a cell contact-dependent manner.

In addition, melanoma is characterised also by infiltration of innate cells, like macrophages, neutrophils and plasmacytoid DCs, all endowed with suppressive and tumor-promoting functions (reviewed in [103]). Moreover, several studies reported an expansion of circulating MDSC in melanoma patients, and melanoma cells can induce MDSCs from monocytes of healthy donors *in-vitro*[104]. $CD14^{+}/HLA\text{-}DR^{low/-}$ was the first phenotype associated to MDSC expansion in melanoma and the suppressive activity of $CD14^{+}/HLA\text{-}DR^{low/-}$ cells on T cell proliferation was dependent on transforming growth factor β[72]. Subsequent studies demonstrated that *ex-vivo* derived $CD14^{+}/HLA\text{-}DR^{low/-}$ MDSCs induced Treg[105] and activated Arg-1 and STAT-3, two important factors driving MDSC suppression[106]. Besides, in a recent publication, we demonstrated that IL4Rα, which

is not only a phenotypic but also a functional marker of murine MDSCs[69,71], was expressed on human monocytes endowed with suppressive activity, suggesting that IL4Rα could be a valuable marker also for human MDSCs[70]. Moreover, also granulocytes separated from melanoma patients showed a significant up-regulation of IL4Rα, though the presence of these cells did not correlate with a suppressive phenotype.

Melanoma often matched with induction of systemic inflammation,and indeed the levels of acute phase proteins, like C-reactive protein, or inflammatory mediators like IL-6 are strong predictors of survival in melanoma patients[102,107,108].

Serum proteins as biomarkers of melanoma

Biomarkers are tumour- or host-related factors that could be easily measured, i.e. in the serum, and are associated with tumour behaviour and patient prognosis. A good number of proteins present in the serum of melanoma patients have been correlated with tumor burden or survival and they are often used as early biomarkers for recurrence (reviewed in [109]). The strongest predictors of tumor burden and survival are LDH and S100B levels[110].

The first evidence of correlation between serum LDH levels and prognosis of melanoma dated back to 1954 and several studies confirmed this finding[109]. LDH is a sensible indicator of liver metastasis[111] and it is particularly useful to discriminate between patients with better or worse prognosis in the metastatic disease. For these reasons LDH is the only serum protein included as parameter for melanoma staging by the American Joint Committee on Cancer[98].

S100B belongs to a class of calcium-dependent proteins that are involved in signal transduction via inhibition of protein phosphorylation, regulation of enzyme activity and calcium homeostasis (reviewed in [112]). *In-vitro* studies demonstrated that S100B inhibits calcium-dependent phosphorylation of p53, resulting in impaired function of this protein and a consequent uncontrolled tumor growth[113]. S100 proteins could also exert extracellular function by binding to their receptor RAGE, but the direct consequences of this interaction are still under investigation[114]. The association between levels of S100B and melanoma are known since 1980[115], but a recent retrospective study on 670 patients with metastatic melanoma clearly identified this protein as a marker of tumor burden and as a predictor of survival[116]. Recently, a multivariate analysis on more than 1100 patients with advanced melanoma demonstrated that LDH and S100B are strong independent predictive markers for survival in these patients[110].

Proteins involved in inflammatory processes like VEGF, C-reactive protein and IL-6 have been recently identified as additional biomarkers of melanoma progression.

C-reactive protein (CRP) is an acute-phase factor produced by the liver under a variety of inflammatory and stressing conditions including cancer[117]. Elevated cytokines levels, and in particular IL-6, increase CRP production[118] and indeed, Tartour *et al.* investigated the possible correlations between IL-6, CRP levels and survival in melanoma patients treated with IL-2[119]. A trend towards association between IL-6 and CRP was observed and both parameters correlated with patients outcome. These data were further confirmed in a larger cohort where only CRP results to be an independent predictive factor for reduced survival[120]. The association between elevated levels of CRP and negative prognosis was confirmed by Allin *et al*. in a large prospective study including patients with different types of cancer and healthy donors[108]. The long follow-up phase, characterising this project, permits to highlight also a direct association between elevated baseline CRP levels in healthy individuals and increased risk of developing different types of cancer.

Interleukin-6 (IL-6) is type 2 inflammatory cytokine whose de-regulation plays a major role in inflammatory-associated diseases like autoimmune arthritis[121]. Studies on melanoma cell lines indicated that IL-6 is a negative regulator of primary melanomas whereas it stimulates the proliferation of melanoma cells isolated from metastatic disease[122]. Indeed, high levels of IL-6 have been extensively correlated with tumor burden and metastasis in melanoma patients[107] and Mouawad et al. showed that low IL-6 levels were associated with control of the disease and response to a therapeutic regimen based on cisplatin, IL-2 and IFN-α[123].

Melanoma treatment

The recommended treatment for melanoma depends on the stage of the tumor: the preferred option for early stage melanoma is surgery, possibly combined with adjuvant regimen, while a more aggressive approach, including systemic chemotherapy, radiotherapy and immunotherapy, is necessary for the management of advanced disease.

First line therapy for early stage melanoma includes surgical excision of the primary lesion, and complete lymphadenectomy recommended in the presence of metastasis to the lymph nodes. (reviewed in [124]). High risk patients often undergo also adjuvant therapy following surgical resection to minimise the risk of recurrence. If surgery and adjuvant regimens are effective for control of primary melanomas, the scenario becomes much more complex when patients presented with metastatic disease.

Interferon α2b (IFNα2b) was the first agent to significantly improve the OS of metastatic melanoma patients[125]. The mechanism of action of this cytokine is not completely understood.

The examination of tumor biopsies taken before and after therapy with IFNα2b demonstrated an enrichment in the infiltration of T lymphocytes and dendritic cells in the tumors, thus suggesting an immunological mechanism of action for this therapy[126]. For a long period several strategies aimed at improving an immune rejection of melanoma have been pursued with contrasting results. Indeed, vaccination and adoptive cell therapy with autologous TILs have demonstrated some clinical benefit but the only immunotherapeutic regimen approved until 2011 was the administration of high doses of IL-2 (reviewed in [124]). This cytokine acts sustaining T cell proliferation but its efficacy is limited to highly selected patients and it comes at the cost of high toxicity. High dose IL-2 is currently approved only for the treatment of metastatic melanoma, although it is a treatment given only in specialized and experienced centers.

Regarding chemotherapy, the alkylating agent dacarbazine, and its oral-available pro-drug temozolomide, are the only chemotherapeutic drugs approved for the treatment of metastatic melanoma (reviewed in [124]). However, only 10-15% of patients treated with dacarbazine experienced tumor regression, but this response does not increase OS [127]. Different combinatorial regimens have been tested to improve the efficacy of chemotherapy in melanoma, but with negligible benefits in terms of survival and displaying higher toxicity.

Chemotherapy is considered harmful for the immune system and it is well known that myelosuppression is one of the most frequent side effect of chemotherapeutic drugs. However, a number of studies, mostly in mouse models, suggest that conventional chemotherapy could improve immune-rejection of the tumors by inducing immunogenic cell death (ICD) of malignant cells (reviewed in [128]). ICD is characterised by alterations in the composition of the plasma membrane of dying cells and by the release of specific transductors of immunogenic signals that promote engulfment of dying cells, presentation of tumor antigens, and production of pro-inflammatory cytokines by dendritic cells, thus boosting anti-tumor response[129]. A similar induction of ICD was seen also upon administration of radiotherapy in pre-clinical models of melanoma[130] or upon infection with oncolytic-virus in humans[131]. Another possible synergy between chemotherapy and immune-rejection is the activation of genes involved in the immune response and leukocyte activation following dacarbazine administration which was demonstrated to improve the response to Melan-A vaccination[132].

Another possible synergic effect on tumor eradication can be achieved by combination of chemotherapy and immunotherapy which on one side directly kills cancer cells and on the other side activates the suppressed immune system to mount a robust anti-tumor response (reviewed in [133]). Early reports have suggested that chemotherapeutic agents administered in combination

with IL-2 or IFN can improve the response rate in melanoma patients[134,135], but an overview of 18 studies comparing standard chemotherapy to chemoimmunotherapy demonstrated that the increased response rate did not translate into a survival benefits for these patients[136]. However, the introduction of ICI opens the way for new combinatorial strategies, in particular for patients with brain metastasis who showed an increased overall response rate when treated with the combination of ipilimumab and fotemustine[137] (reviewed in[39]).

The improved knowledge of the genetic instability of melanoma and the progress of tumor immunology led to a revolution in the management of melanoma which results in the approval of two new class of drugs for the treatment of metastatic melanoma by FDA in 2011 (reviewed in[138]). The discovery of the activating mutations in the MAPK pathway, which promote uncontrolled proliferation of melanocytes, set the basis for the approval of sorafenib, a non-selective BRAF inhibitor. Unfortunately this drug failed to show any benefit[139-141], but when specific inhibitors for the kinases involved in the MAPK pathway entered into clinical trials, they showed extremely promising results (reviewed in [138]). Two BRAF inhibitors, dabrafenib and vemurafenib and one MEK inhibitor, trametinib showed significant effects in terms of tumor regression compared to dacarbazine. The toxicity profile of these drugs is milder than classical chemotherapy but the kinetics of response is characterised by high initial tumor response followed by development of resistance at a median of five to seven months. The second class of drugs that contributes to the therapeutic revolution in the field of metastatic melanoma is that of immune checkpoint inhibitors[39]. These drugs are monoclonal antibodies inhibiting key negative regulators of T cell activation like Cytotoxic T cell antigen 4 (CTLA-4) and Programmed Death 1 (PD-1). They act releasing the brake on T cells exerted by the suppressive network active at the tumor site, and therefore enhance the probability of tumor-specific T cells to attack the malignant clones. At present time, two ICI have been approved by the FDA for the treatment of metastatic melanoma: ipilimumab and pembrolizumab which are targeted to CTLA-4 and PD-1, respectively. These drugs are characterised by a low rate and a delayed onset-time of response, but also longer duration of the clinical benefits in responding patients. The encouraging data from clinical studies indicate that a combination of targeted therapy with BRAF or MEK inhibitors and ICI could feasibly maintain the high frequency of response typical of targeted therapy and prolonged the control of the disease in patients responding also to the immunotherapy approach.

Ipilimumab: a novel immune checkpoint inhibitor for the treatment of metastatic melanoma

The biology of immune checkpoints

T cell response starts with the recognition by T cell receptor (TCR) of the peptide-major hystocompatibility complex (MHC) on APC but this event is not sufficient for full activation of T cells. Indeed, the magnitude and the quality of T cell response depend on the integration between signals deriving from co-stimulatory and co-inhibitory receptors, expressed by the lymphocyte and known as immune checkpoints molecules[16]. In physiological conditions, immune checkpoints are crucial for maintaining self-tolerance and to prevent hyper-activation of adaptive response towards pathogens which could eventually damage the surrounding tissues (reviewed in[40]). Positive regulators of T cell response include ICOS, CD137 and CD28, the master positive regulator of T cell activation; in contrast, several negative regulators have been described: CTLA-4, PD-1, LAG-3, Tim-3, BTLA, A2aR (Figure 5). Amplification of signalling through inhibitory receptors participating to immune checkpoint is a useful strategy for the treatment of autoimmune arthritis and transplant rejection[142]. However, a wider field of application was found for mAb blocking these inhibitory immune checkpoint molecules for cancer treatment (reviewed in[43]). In fact, the expression of inhibitory immune checkpoints could be deregulated by tumors and their blockade unleash the potential of anti-tumor responses. Several immune checkpoint molecules represents promising targets for therapeutics, but, at the moment, only two ICI have been approved by the FDA for treatment of metastatic melanoma: ipilimumab and pembrolizumab, which are directed to CTLA-4 and PD-1 , respectively[41,42].

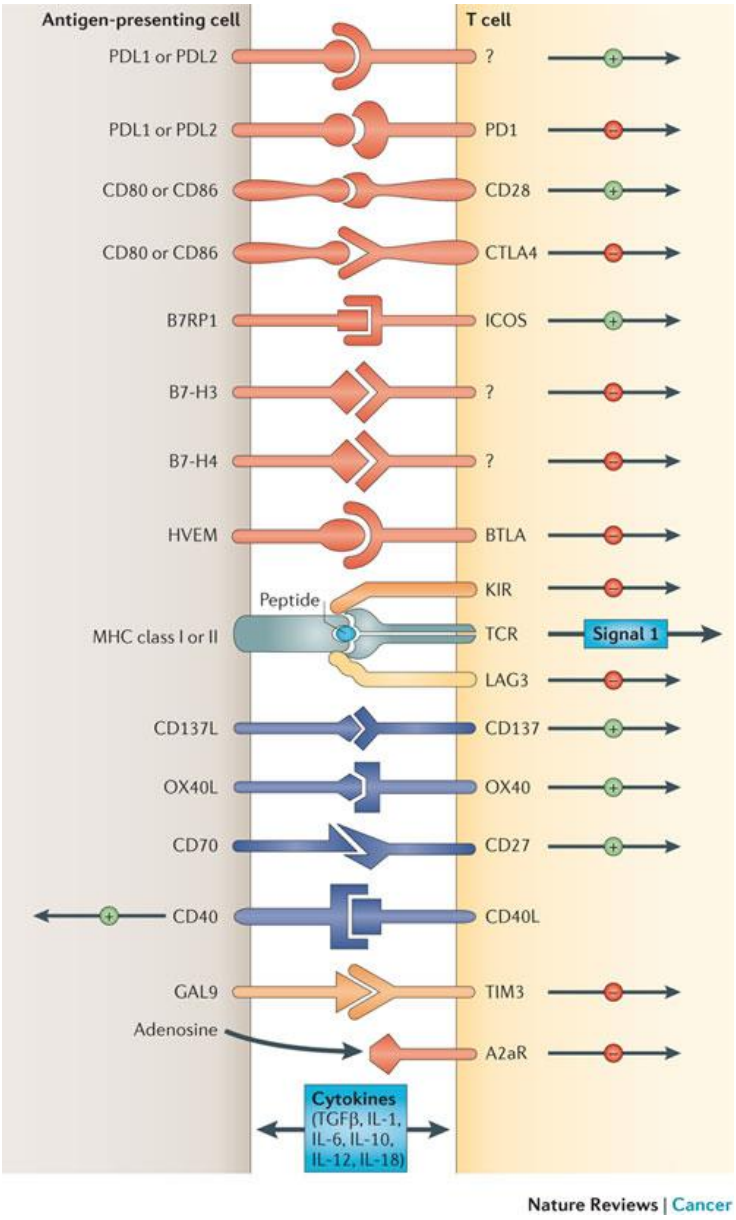


Figure 5: Immune checkpoint molecules regulate T cell responses

Multiple ligand-receptor interactions between T cells and APCs convey co-stimulatoy and co-inhibitory signals are known as immune checkpoints. A T cell response is initiated following antigen recognition by T cell receptor (Signal 1), but the onset of T cell response results from the balance of stimulatory and inhibitory signals delivered by immune checkpoint molecules. (Pardoll *et al.,* Nature Review Cancer 2012)

CTLA-4 is a key negative regulator of T cell activation, opposed to the stimulatory receptor CD28[143] (Figure 6). CTLA-4 is mainly expressed on T cells after activation[143,144], while Treg have a constitutive expression of this marker due to positive regulation of CTLA-4 expression by the Treg-associated transcription factor FoxP3[145]. Indeed, it has been demonstrated that CTLA-4 participates to the suppressive function of Tregs[146,147]. CTLA-4 presents a complex trafficking behaviour: conventional T cells store CTLA-4 in cytoplasmic vescicles and TCR stimulation promotes its exposure on the cell surface[144]; however, CTLA-4 is also repetitively endocytosed by

T cells with the result that only a small fraction of the CTLA-4 pool is exposed on the cell surface[148]. For a long time, expression of CTLA-4 was thought to be confined on T cells but recent reports described its expression also on myeloid cells[149,150] and tumors[151], albeit its functional role on these cells remained largely unknown. In contrast, a large number of mechanisms have been proposed that account for the inhibitory activity of CTLA-4 towards T cell activation and they consist of both cell-intrinsic and cell-extrinsic pathways (reviewed in [152]). Cell-intrinsic pathways include all functional mechanisms acting on the cell that express CTLA-4, namely T cells, while cell-extrinsic pathways are those in which CTLA-4 carries out its effects through other cells. The most important mechanism of action of CTLA-4 blockade lays in the inhibition of the co-stimulatory signal delivered by CD28 to T lymphocytes upon TCR engagement[143]. Indeed, CTLA-4 competes with CD28 for binding to their common ligands, CD80 and CD86, expressed by APC[153] and, since CTLA-4 possesses a higher affinity for the ligands than CD28, the inhibition is efficient and stable; recent reports demonstrated that CTLA-4 can also physically remove CD80 and CD86 form APC resulting in a further elongation of CD28 blockade[154]. In addition, CTLA-4 can oppose the co-stimulatory effect of CD28 also by delivering an intra-cellular inhibitory signal to T cell, thus decreasing the phosphorylation of several key proteins in the TCR signalling cascade[155,156].

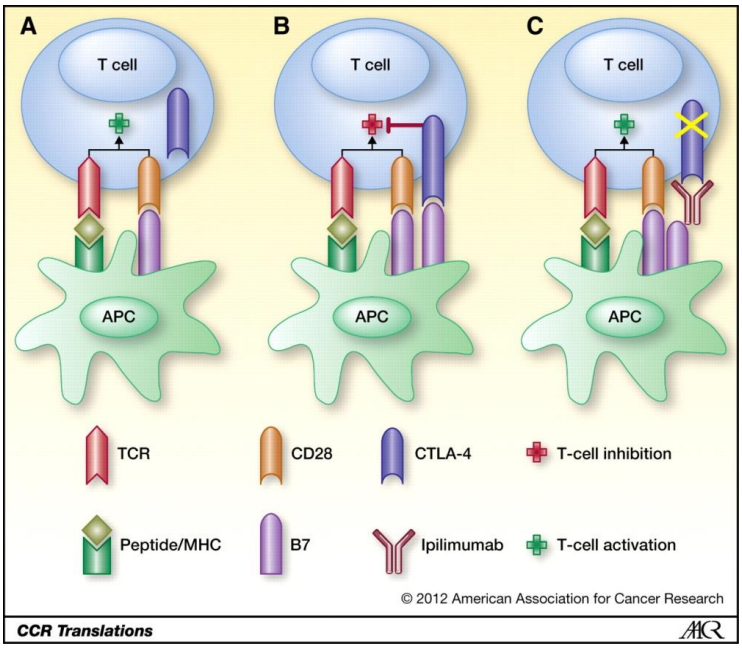


Figure 6: Ipilimumab blocks negative signaling from CTLA-4
A) T cells requires two signals for full activation: the first signal is delivered by the T cell receptor (TCR) while the second involves co-stimulation through the interaction of CD28 on T cells with B7 molecules (CD80, CD86) on APCs. B) upon T-cell activation, CTLA-4 is recruited to the plasma membrane and, binding with higher affinity than CD28 to B7 molecules, it delivers an inhibitory signal that block T cell activation. C) ipilimumab blocks CTLA-4 cell-intrinsic and cell-estrinsic functions, thus releasing the brake on T cell activation. (Postow *et al.*, Clinical Cancer Res 2012)

PD-1 is another negative regulator of T cell function whose blockade has been exploited for cancer treatment. Similar to CTLA-4, also PD-1 expression is induced upon activation on T cell surface[157] and its expression is elevated in circulating T lymphocytes from patients with chronic infection, like HIV, and cancer[158-160]. As expected, PD-1 is mostly expressed by antigen-experienced cells belonging to the memory compartment[159] and further up-regulated when memory T cells enter the effector phase[161]. Evidence of PD-1 expression have been observed also on Treg[162]. PD-1 can bind to PDL-1 and PDL-2 which could be expressed by myeloid cells and tumors (reviewed in[163]). In the tumor microenvironment, the major PD-1 ligand that is expressed is PDL-1[164] (reviewed in 163) and its expression could be further up-regulated by IFN-γ[165]. In contrast to CTLA-4 that blocks T cell activation at the time of antigen exposure in secondary lymphoid organs, the major role for PD-1 have been observed in the periphery where it limits the effector functions of previously activated T cells (reviewed in[40]). In this context, PD-1 acts by modifying the duration of T cell-APC contact[166], by inducing a functional impairment called T cell exhaustion[21] and by enhancing the proliferation of Treg[162].

In the last years, ipilimumab and pembrolizumab, two mAb targeting CTLA-4 and PD-1, respectively, have been introduced for melanoma treatment. These immune checkpoint inhibitors were the first treatment demonstrating a significant increase in the survival of stage IV melanoma patients[41,42]. As both drugs rescue T cell activation at different levels (ipilimumab mainly by counteracting CD28 signalling and by reducing Tregs, while pembrolizumab by inhibiting exhaustion of $CD8^+$ T cells), clinical approaches of combination are currently under investigation.

Ipilimumab: from pre-clinical model to the clinical practice

Ipilimumab is a fully human IgG1 mAb approved for the treatment of metastatic melanoma at the dose of 3mg/kg. It was approved in the United States in 2011 and in Europe in 2012. Evidence from preclinical models indicated that ipilimumab monotherapy was safe and effective in rejecting a large number of transplantable tumors, in particular in mice with low tumor burden and transplanted with cell lines that were historically considered immunogenic[167]. This result is not surprising considering the pharmacodynamics of ICI; indeed, these drugs can revert suppression of an existing anti-tumor immunity but they are not able to generate an *ex-novo* immune response towards cancer. However, subsequent studies indicated that in case of poorly immunogenic tumors, the combination of ICI and vaccination is a feasible and effective option[168] to establish an effective anti-tumor response.

During clinical development, phase II studies with escalating dose and different schedule of treatment lead to the determination of the minimum effective dose characterised by acceptable

toxicity, that is 3mg/kg (reviewed in [169]). In phase III studies ipilimumab treatment produced a significant advance in terms of OS compared to a vaccination strategy with gp100 vaccine[41] and to dacarbazine, the standard chemotherapeutic regimen for melanoma[170]. Four pattern of tumor response to ipilimumab were noted: i) immediate tumor regression in baseline lesions without development of new lesions, ii)durable stable disease followed by slow reduction of tumor burden, iii) response in the presence of new lesions and iv) response following an increase in total tumor burden[171]. Histological studies revealed that edema or leukocyte infiltration accounted for the apparent increased volume of some lesion in responding patients. Given this peculiar pattern of response, that differs from that of standard chemotherapy, new immune-related criteria were defined for assessment of disease progression following ICI treatment. Treatment with ipilimumab was typically associated with the onset of irADR which were strongly related to its immune-based mechanism of action. The onset of irADR was common and an incidence of 64% was reported in a pooled analysis of 14 studies evaluating various doses of ipilimumab[172]. According to this study, the irADR were tipically manageable, but about 20% of patients developed serious toxicity which required a prompt management by expert clinical oncologists. Most of immune-mediated irADR involved mainly the skin, the gastrointestinal mucosa, the liver and the endocrine system (reviewed in[173]). While the onset of rash, diarrhea and colitis can be rapid but readily resolved with administration of glucocorticoids, endocrine irADR were more severe and, in some cases, life threatening.

Biomarkers for immune checkpoint inhibitors

The introduction of ipilimumab in the clinical practice was welcomed with enthusiasm by clinical oncologists and the subsequent approval of pembrolizumab has open new options for therapeutics. In the next years the opportunity to have different ICI on the market will bring about the need for definition of which immune checkpoint pathway is more active, and thus constitute a better target, in each patient. Moreover, ipilimumab, as single agent, produces log-term benefits in about 12% to 49% of patients[39,174] and this response comes at the cost of a toxicity profile that can be serious and a consistent economic burden for the health care system[173]. Hence, there is an urgent need for predictive biomarker that can guide clinical oncologist in the selection of patients that have a higher probability to respond to ipilimumab and to direct other patients to alternative therapeutic strategies. Given the multiple cell-intrinsic and cell-extrinsic mechanisms of action of CTLA-4, the search for predictive biomarkers involves a large number of immune players and tumor-related factors in particular T lymphocytes, myeloid cells, serum proteins and the genetics of the tumor.

Only few studies identified baseline parameters predictive of survival or response to ipilimumab, here named predictive biomarker, while a larger number of factors, associated with the mechanism of action of ipilimumab, can predict patient's prognosis when assessed after treatment; these markers will be named early response marker to distinguish them from predictive biomarkers (reviewed in [175]).

An overview of the studies identifying predictive biomarkers indicate that patients with higher probability to benefit from ipilimumab treatment are those presenting an active immune system with low frequencies of MDSCs[176] and high absolute lymphocyte count[177-179]. In this context, the presence of humoral and cellular responses to NY-ESO-1 was correlated with OS in ipilimumab-treated patients, and this parameter may help the identification of patients with an existing anti-tumor immunity which in turns have more probability to respond to ipilimumab[180,181]. Recent studies performing gene expression profiling on melanoma biopsies from patients undergoing ipilimumab treatment revealed that clinical response was associated with a Th1 signature and expression of IFN-γ inducible genes at baseline[182]; in addition, the Th1-associated markers were further up-regulated after the treatment[182]. However, conflicting results came from another study in which clinical response to ipilimumab was correlated with high baseline expression of FoxP3 and IDO, genes typically associated with suppressive populations[183]. Two recent studies pointed out that immune rejection of cancer in response to ICI is antigen-specific both in humans and in mice, and the antigenic drivers of this process are mutant neo-epitopes arising from the genetic instability of tumors[99,100]. One of these studies deals with melanoma patients, and authors brilliantly demonstrated that this mutant immunologic signature was predictive of response to the treatment and the neo-epitopes, composing this signature, were homologous to known viral and bacterial antigens that T cells are likely to recognize[100].

In addition to immunological parameters, low tumor burden (indicated by levels of LDH and S100B below the upper limit of normal)[179], low serum VEGF[178] and low levels of inflammatory indexes (like C-reactive protein and erythrocyte sedimentation rate)[177,179] are also predictive of improved OS following ipilimumab treatment. Gene expression profiling of melanoma biopsies indicated that expression of melanoma-associated genes (NY-ESO-1, MAGE-A, MELAN-A, TYR) and cell signalling molecules involved in tumorigenesis are reduced in in post-treatment biopsies of responding patients[182].

A large number of studies identified immunological and tumor-related parameters as early response markers. Overall activation of T cells was seen in responding patients with increased expression of T cell activation markers (ICOS[184-186], Ki67[185], HLA-DR[187]), increased number of

circulating[177-179,188] and TIL[183], expansion of the central memory subset[187] and increased phosphorylation of effector molecules down-stream to TCR signalling[189]. Only a minority of studies confirmed T cell activation by functional studies, although Ipilimumab was shown to increase the response to melanoma antigens[181,187] and the humoral immunity following vaccination against influenza and pneumococcus[187]. Since Treg express CTLA-4 constitutively, it is reasonable to argue for Treg depletion via antibody-dependent cell-mediated cytotoxicity following administration of anti-CTLA-4 antibodies; however, conflicting results were observed concerning modulation of Treg by ipilimumab. Indeed, some authors reported that increasing frequency of circulating Treg correlates with an improved progression-free survival[181], while others observed a marked reduction of the levels of Treg in patients with longer survival or clinical response to ipilimumab[177,190]. The same controversial trend was observed also for tumor-infiltrating Treg[181,183]. In contrast, the monitoring of circulating MDSCs produced more homogeneous results with several authors indicating a strong correlation between reduced frequencies of monocytic, granulocytic and immature MDSCs and improved response or survival following ipilimumab treatment[176,181,191].

Technical issues on biomarkers development

A relevant issue related to the immunomonitoring of patients undergoing ICI therapy is the increasing need to compare parallel biomarker datasets generated in different laboratories (reviewed in[175]). Indeed, many efforts have been made to identify possible predictive biomarkers and early response markers for ipilimumab but, the results are of difficult interpretation since standardized reference interval are not available for most of immunological markers. Hence, since standardization of assays across laboratories is often difficult to achieve, an alternative approach is assay-harmonization. Organization of proficiency panels joined by a large number of experienced laboratories is a common strategy for assay harmonization. The aim of proficiency panels is to identify a set of parameters impacting on variance of a certain assay and subsequently find a consensus on a list of mandatory, harmonized parameters to be applied to single-laboratory protocols in order to generate comparable results. The Cancer Imunoguiding Program, a European network of leading scientists in the field of immunology, is the sponsor of a variety of proficiency panels to harmonize immunological assay like: ELISPOT, tetramer staining, intracellular cytokines staining or immunophenotyping of circulating MDSCs[192-194]. Another important target for immunomonitoring of cancer patients is the development of guidelines for a uniform report of the results. Indeed, this is a key step for sharing results with collaborating laboratories and, subsequently, for the integration of multiple datasets. The Minimal Information about T cell Assay (MIATA) project, developed by a large number of leading scientists[195], and the Minimum Information about a Flow Cytometry Experiment, developed by International Society for Advancement of Cytometry[196], are projects along this line of research.

AIM OF THE STUDY

Ipilimumab, an antibody that blocks the function of the immune checkpoint Cytotoxic T-Lymphocytes Antigen-4 (CTLA-4), was the first immunomodulatory antibody approved for the treatment of metastatic melanoma. Given its immune-mediated mechanism of action, it is important to monitor the immune profile of patients receiving this therapy, to understand the mechanisms set in motion by the treatment and to correlate them to the clinical responses. In particular, there is a great interest in monitoring suppressive populations able to divert T cell functions, and one of the key suppressive players expanded in cancer patients are myeloid-derived suppressor cells (MDSCs). The immunophenotyping of MDSCs is performed by multicolour flow cytometry and is characterised by a high degree of complexity given the fact that several myeloid phenotypes have been described, ranging from immature cells to more differentiated cells such as monocytes and granulocytes, and moreover a specific marker of such cells is still missing. At present, a uniform methodology for the phenotyping of MDSCs by flow cytometry is missing.

To overcome this obstacle, our group organised, under the umbrella of the Cancer Immunoguiding Program, a proficiency panel to harmonize the phenotypic definition of human MDSCs. The first purpose of the panel is to identify a robust markers combination for identification of a number of non-overlapping MDSC subsets; the panel also provides individual feedback to each participant laboratory in order to guide the harmonization of the experimental procedures used to phenotype circulating MDSCs. This combined effort will hopefully drive to a consensus on the minimal requirements for MDSC phenotyping that, in turns, will be used as a diagnostic tool to monitor MDSCs.

An interesting application of MDSC phenotyping is the monitoring of these cells in melanoma patients undergoing ipilimumab treatment, potentially linking their level to therapy outcomes. Indeed, given the relatively recent knowledge of the immunological profile of toxicity, and the high cost of ipilimumab treatment, it is of great interest to develop robust predictive biomarkers supporting a more rational use of this drug, including the selection of patients with a higher probability to respond to the drug. To this end, the present study was designed to collect a wide dataset composed of clinical information and of tumor-associated and immunological parameters that we analysed using a multivariate non-parametric statistical approach in order to identify the parameters useful for implementing the clinical management of patients treated with ipilimumab.

MATERIALS AND METHODS

HARMONIZATION OF IMMUNOPHENOTYPING OF MYELOID-DERIVED SUPPRESSOR CELLS

Selection of the donors

Immunophenotyping of 10 putative subsets of MDSCs was performed on cryopreserved samples from three healthy donors (HBC-480, HBC-514, L29_3). The donors were centrally pre-selected by the organising committee among a set of PBMC samples derived from leukapheresis of healthy donors. All donors gave their informed consent before enrolment. The organising committee analysed the PBMC samples from different leukapheresises by flow cytometry and chose the donors with the most significant expansion of the 10 putative MDSC subsets. Two vials of each donors were then shipped to the participant laboratories in dry-ice and stored in liquid nitrogen upon arrival.

Guidelines for the first step of the proficiency panel

Experiment guidelines were sent to participants in order to indicate the mandatory parameters which must be fulfilled in the first experimental step of the proficiency panel and, as indication, exemplary staining cocktails, protocol and gating strategy were provided.

The proposed staining cocktails contain 7 markers commonly used for MDSC recognition plus a dead-cell marker (DCM): HLA-DR, CD14, CD15, CD11b, CD33, Lineage cocktail (defined as CD3/14/19/56), CD124. This markers combination allows the identification of 10 myeloid subsets: MDSC1 $CD14^{+}/CD124^{+}$ [70], MDSC2 $CD15^{+}/CD124^{+}$ [70], MDSC3 $Lin^{-}/HLA\text{-}DR^{-}/CD33^{+}$ [78], MDSC4 $CD14^{+}/HLA\text{-}DR^{low/-}$ [72], MDSC5 $CD15^{+}/CD14^{-}/CD11b^{+}$ [74], MDSC6 $CD15^{+}/FSC^{low}/SSC^{high}$ [197], MDSC7 $CD15^{-}/CD14^{+}/CD33^{high}/HLA\text{-}DR^{low}$ [198], MDSC8 $CD15^{+}/CD33^{high}$, MDSC9 $CD14^{-}/CD15^{-}/CD33^{high}$ and MDSC10 $Lin^{-}/HLA\text{-}DR^{low}/CD11b^{+}$ [199]. Participants were asked to perform the staining twice, in two separate days, in order to calculate intra-laboratory variance. For each donor in each of the two experimental runs, participants were asked to determine cell viability, and test and report the number of total cells, singlets, monocytes and lymphocytes plus 10 putative MDSC phenotypes using one 8-color panel and/or three 4-color flow cytometry panels.

In addition guidelines indicated to use at least one million PBMCs for tube, not to perform a resting period after thawing the cells, and not to fix the stained cells.

Each group was asked to perform data analysis using their own gating strategy and to report it in single layouts; moreover, groups were asked to determine the absolute number of the requested cell populations with or without the presence of a DCM.

Experimental procedure used by our laboratory

In addition to panel design, our group was one of the laboratories that participated to the proficiency panel. As some parameters were free of choice in the panel's guidelines, this section describes the protocols we chose for staining, acquisition and analysis of the samples.

We identified the 10 putative MDSC subsets according to all mandatory parameters described in the panel's guidelines. The 8-color staining cocktail used in our laboratory is composed of: anti-CD11b Alexa 700 (clone ICRF44, BD Pharmingen), anti-CD14 APC-H7 (clone MϕP9, BD Bioscience), anti-CD15 V450 (clone MMA, BD Biosciences), anti-CD33 PECy7 (clone P67.6, BD Biosciences), anti-CD124 PE (clone 25463, R&D SYSTEMS), Lineage cocktail (anti-CD3-14-19-56) (clone UCHT1, M5E2, HIB19, NCAM16.2, BD Biosciences and BD Pharmingen), anti-HLA-DR APC (clone L243, BD Biosciences). The Live/Dead (L/D), an amine-reactive dye, was chosen as DCM because it resists to cell-fixation with unaltered staining capability.

PBMC were thawed at 37°C, then washed in ice-cold IMDM (Gibco) supplemented with 10% heath-inactivated fetal bovine serum (FBS) (Gibco), 1% Pen-Strep (Lonza), 1% Hepes (Lonza), 1% β- Mercaptoethanol, AAG (Asparagine 0.24mM, Arginine 0.55mM, Glutamine 1.5mM) (Sigma-Aldrich) and spinned at 1300 rpm for 6 minutes at 4°C. Cells were counted using trypan blue to exclude dead cells. The viability ranged from 94 to 77% and the yield after thawing ranged from 37 to 58%.

In each experimental run we prepared 4 tubes per donor, 3 tubes were used as controls (unstained cells, FMO control for HLA-DR and for CD124) while one tube contained the 8-color staining. FMO are fluorescence minus one controls, which contain all the antibodies of the staining cocktail with the exception of the antibody for which a control is needed[200]. For each donor, $1x10^6$ PBMCs were distributed in each tube, washed with staining buffer and subsequently centrifuged at 1300 rpm for 6 min at 4°C. The supernatant was discharged and the cells were resuspended in 25µl of Fc-Receptor blocking solution (Miltenyi Biotec) and incubated at 4°C for 15 minutes. Then, an appropriate quantity of staining buffer was added in order to reach the final volume of 100µl. Afterwards, anti-CD124 PE antibody was added and incubated at 4°C for 10 minutes. Subsequently, a mixture of diluted antibodies (plus L/D) was added to the tubes and incubated at 4°C for 20 minutes . Anti-HLA-DR was added only in FMO CD124 and MIX tubes. Cells were washed

with staining buffer and subsequently centrifuged at 1300 rpm for 6 min at 4°C. The supernatants were discharged and cells resuspended in 350µL of staining buffer for immediate acquisition using a LSRII flow cytometer (BD Biosciences) equipped with 4 lasers (405nm, 488nm, 561nm, 640nm). The staining buffer used was the Hanks' Balanced Salt Solution for Flow Cytometry supplemented of 1% FBS: 137mM NaCl (Sigma-Aldrich), 5mM KCl (Sigma-Aldrich), 0.3mM Na_2HPO_4 (Sigma-Aldrich), 0.7mM KH_2PO_4 (Sigma-Aldrich), 0.4mM $MgSO_4$ (Sigma-Aldrich), 0.3mM $MgCl_2$ (Sigma-Aldrich), 5mM Glucose (Sigma-Aldrich), 4mM $NaHCO_3$ (Sigma-Aldrich), 1mM EDTA (Sigma-Aldrich).

We set-up compensation on the base of automated compensation using BD Comp beads and the antibodies included in the staining cocktails. L/D was substituted with anti-HLA-DR V500 (BD Bioscience). The compensation matrix generated by BD FACS Diva software was manually edited to adjust overcompensation wrongly introduced by the software. Data were analyzed using FlowJo software (Three Star Inc).

To identify the 10 putative MDSC subsets, the following gating strategy was adopted: first set-up of a morphological gate based on FSC and SSC properties, then exclusion of doublets and dead cells (when required) according to the properties of each sample. After these preparative steps, the 10 putative MDSC subsets were identified as shown in Figure 1 of the Results. The gate for HLA-$DR^{low/-}$ cells was based on the FMO HLA-DR control performed for each sample. The gate for $CD14^+/CD124^+$ cells was set-up considering the fluorescence of FMO CD124 and $CD14^-$ cells for each sample. The same strategy was used for $CD15^+/CD124^+$ cells. The gating strategy for MDSC identification did not change between analyses with or without exclusion of dead cells. The absolute number of all requested populations was reported in a file with or without the dead cell marker.

Central data analysis

Results from the 23 participating laboratories were centrally collected and analysed. Absolute numbers of the 10 putative MDSC subsets were normalized on the count of lymphocytes + monocytes. Variance was measured as %CV = (standard deviation/mean)*100. Comparison between %CV or normalized frequencies of myeloid subsets of different groups was performed using the Wilcoxon Signed-Rank test. Significance level was set for $P<0.05$.

IMMUNOMONITORING OF MELANOMA PATIENTS TREATED WITH IPILIMUMAB

Patients

Thirty-seven patients with a diagnosis of stage IV melanoma were enrolled in the study. A description of the clinical characteristics of these patients is reported in Table 1. Exclusion criteria for the administration of ipilimumab were the presence of brain metastases with symptoms or requiring treatment and an history of autoimmune disease. Ipilimumab was administered after at least another line of treatment, and therefore previous use of systemic chemotherapy, radiotherapy or BRAF inhibitors was allowed. 21 age- and gender-matched healthy donors were used as controls. The study was approved by the ethical committee of Istituto Oncologico Veneto and all the patients enrolled provided a written informed consent before blood withdrawal.

Patients received four administrations of ipilimumab (3 mg/kg) every three weeks, as approved by the Italian Medicine Agency during the registration phase. In case of onset of immune-related adverse reactions to the treatment, requiring corticosteroids treatment, the therapy was discontinued and eventually resumed when patients recovered from toxicity. Disease progression was monitored by computered axial tomography (CAT) 12 weeks after the first infusion of ipilimumab and further confirmed with an additional CAT at week 16. Response to the treatment was assessed using the immune-related Response Criteria[171]. Patients presenting stable disease, partial and complete response were considered responders, while patients with progressive disease were included in the non-responder group. Follow-up of patients continued until disease progression and afterwards patients were provided with the best alternative therapeutic approach. Patients who presented, at any time within the observation period, immune-related adverse drug reactions (irADR) with severity above grade 3 were called ADR^+ while patients without irADR or with an irADR severity below grade 3 were named ADR^-.

Study design

Peripheral blood from melanoma patients was collected at baseline (W0) and 12 weeks after the first dose of ipilimumab was administered (W12). Additional blood samples were collected from patients every twelve weeks until disease progression. Blood samples were withdrawn in EDTA-treated vacutainer tubes (BD Bioscience) and processed immediately from healthy donors and from patients. A set of parameters associated with the immune system and tumor burden were monitored at W0 and W12. Circulating levels of myeloid and T cell subsets were evaluated by

multicolor flow cytometry, while hematological parameters were evaluated by the Central Laboratory of the University Hospital of Padova. All collected parameters are listed in Table 1.

CLASSIFICATION	NAME	ABBREVIATION
HEMATOLOGICAL PARAMETERS	C-reactive protein	CRP
	S100B	
	Vascular-endothelial growth factor	VEGF
	Lactate dehydrogenase	LDH
	Interleukin - 6	IL-6
SUBSETS OF MYELOID CELLS	$CD14^+/IL4R\alpha^+$	MDSC1
	$CD15^+/IL4R\alpha^+$	MDSC2
	$CD15^-/Lin^-/HLA\text{-}DR^-/CD33^+/CD11b^+$	MDSC3
	$CD14^+/HLA\text{-}DR^{low/-}$	MDSC4
	$CD15^+/V500^{high}/SSC^{high}$	Putative eosinophils
	$CD15^-/Lin^-/HLA\text{-}DR^+/CD33^{dim}$	Putative dendritic cells (DC)
T CELL SUBSETS	$CD3^+$	
	$CD3^+/CD4^+$	
	$CD3^+/CD8^+$	
	$CD3^+/CD4^+/PD\text{-}1^+$	
	$CD3^+/CD8^+/PD\text{-}1^+$	
	$PD\text{-}1^+$ within $CD3^+/CD4^+$	
	$PD\text{-}1^+$ within $CD3^+/CD8^+$	
CLINICAL DATA	Stage	
	Circulating tumor cells	CTC
	Eastern Cooperative Oncology Group performance status	ECOG PS
	Number of doses	
	Time-to-progression	TTP
	Overall survival	OS
	Immune-related adverse drug reaction	irADR

Table 1: Parameters monitored in melanoma patients
The table indicates the classification and the abbreviation of each parameter included in the dataset used in this study .

In addition, for each blood sample, plasma and PBMCs of patients and healthy donors were cryopreserved. PBMC and plasma were isolated from peripheral blood by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare-Amersham). Peripheral blood was diluted 1:3 in PBS, stratified on Ficoll-Paque PLUS and centrifuged 30 minutes at 1800 rpm at 20°C. After

centrifugation plasma was collected, centrifuged to discard possible contaminating cells, and aliquoted for cryopreservation at -80°C. PBMCs were aspirated, washed 3 times with PBS 1% human serum type AB (HS) (LONZA), and stored in liquid nitrogen.

Characterization of the immune profile by flow cytometry

The presence of circulating myeloid and T cell subsets was assessed in whole blood samples by multi-color flow-cytometry, in order to identify 4 subsets of MDSCs, putative eosinophils and dendritic cells (DC) and different subsets of T lymphocytes. The myeloid subsets were identified using the staining panel previously reported for the MDSC panel. The staining panel for identification of T cell subsets is composed of: anti-CD3 ECD (clone UCHT1, Beckman Coulter), anti-CD4 FITC (clone SK3, BD Bioscience) anti-CD8 APC-H7 (clone SK1, BD Bioscience), anti-PD-1 PE (clone PD1.3.1.3, Miltenyi Biotec).

For myeloid subsets phenotyping, 150μl of fresh blood were washed with staining buffer and subsequently incubated with Fc-Receptor Blocking reagent (Miltenyi Biotec) at 4°C for 15 minutes. Afterwards, cells were stained with anti-IL4Rα (anti-CD124) PE antibody and incubated at 4°C for 10 minutes. Later, the mixture of properly diluted antibodies (plus L/D) was added to the tubes and incubated at 4°C for 20 minutes. Cells were then washed with staining buffer and centrifuged at 1300 rpm for 6 min at 4°C. For T lymphocytes phenotyping, the procedure was the same with the exception that 50μl, instead of 150μl, of blood were used per tube and the total incubation time for antibody-staining was reduced from 30 to 20 minutes. After the washing step, red blood cells were lysed using Cal-Lyse whole blood lysing solution (Life Technologies) according to manufacturer instructions. Absolute counts of T cell subsets was determined using TruCount tubes (BD Bioscience). Data acquisition was performed using a FACSCalibur or a LSRII (Becton Dickinson) flow cytometer. Data were analyzed using FlowJo software (Three Star Inc). Autofluorescence, FMO controls for HLA-DR, CD124 and PD-1 and isotype control for CD124 were used as negative controls. Exemplary gating strategies for phenotyping of myeloid and T cell subsets are shown in Figure 5-6-7-9 of the Results section.

Standardization of the immunophenotyping assay

To standardize the staining panels for myeloid and T lymphocytes' subsets, a dilution of antibodies that maximize the signal to noise ratio was chosen on the basis of single antibodies titration.

In addition, a protocol to monitor the performance of antibodies targeted to HLA-DR and IL4Rα was set-up, by using an EBV-B cell line that constitutively expresses these markers at high expression intensity. To reduce inter-assay variance, we used a single batch of B cell-line, fixed and permeabilized before cryopreservation. For each staining, the control cell-line was run in parallel to blood staining, labelling the cell-line with the same amount of anti-HLA-DR or anti-IL4Rα antibodies used for blood staining (Fig. 1 A). We acquired the control cells before acquiring the blood sample, and we could thus determine whether the mean fluorescence intensity (MFI) of HLA-DR or IL4Rα were included in the range of tolerance. The range of tolerance was built by repeated staining of the control cells performed before the beginning of the study. To this end, we calculated the MFI of HLA-DR and IL4Rα of control cells in repeated measurements and we set the borders of tolerance within the mean ± 2x standard deviation of our measures (Fig. 1 B).

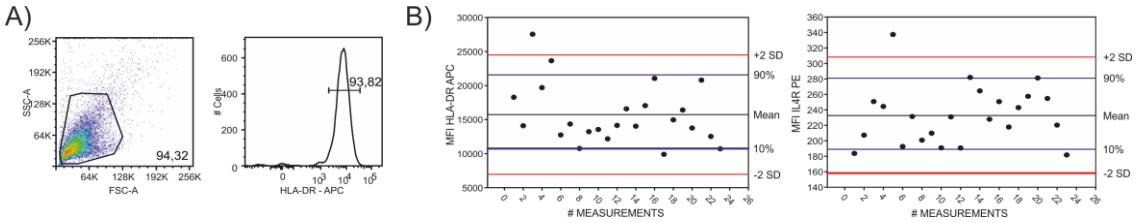


Figure 1: Standardization of antibody performance

Panel A shows a representative HLA-DR staining on reference B cell-line. Panel B reports the MFI values for reference B cell-line in independent experiments (black dot n=23), the tolerance range (red lines) and the 10th and 90th percentile of the measures (blue lines). Results describing the trend of HLA-DR and IL4Rα MFI are reported in the left and right plots of panel B, respectively.

Another source of experimental variation might be the performance of the flow-cytometer. We weekly check the performance of the flow cytometer using the automatic protocol provided by Diva software. In addition, to assess the potential variation of the performance of the flow cytometer, we monitored the performance of the instrument during every experiment using a protocol after Perfetto *et al.* [201]. A pool of commercial fluorescent particles was used and a tolerance range was defined on the basis of the MFI of the brighter peak of fluorescence emitted by the particles (Fig. 2 A). Hence, before starting the study, the multicolor particles were acquired several times using the same voltages determined for antibody staining of patients' blood. A range of tolerance was built on the basis of the fluorochrome MFI associated to the marker to monitor. The range of tolerance is included within the mean ± 2x standard deviation of the measures (Fig. 2 B). Every time a patient's sample was run, the control multicolor particles were acquired in order to determine whether the MFI of the particles lay within the tolerance range.

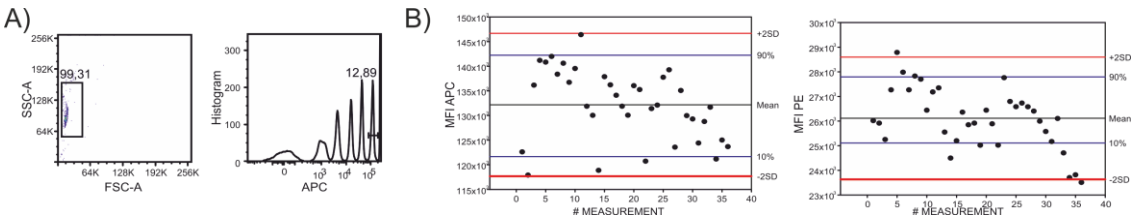


Figure 1: Standardization of flow cytometer performance

Panel A is representative of the fluorescence of multicolor particles in the APC channel. Panel B reports the MFI values of multicolor particles for independent experiments (black dot n=36), the tolerance range (red lines) and the 10^{th} and 90^{th} percentile of the measures (blue lines). The left and right plots of panel B describe the trend of MFI of APC (fluorochrome coupled to anti-HLA-DR antibody) and of PE (fluorochrome associated with anti-IL4Rα antibody), respectively.

Statistical analysis

Wilcoxon Rank Sum test was used to compare the frequencies of myeloid and T cell subsets among healthy donors and melanoma patients at baseline, while Wilcoxon Signed Rank test was used to determine the level of significance among immunological parameters measured at W0 and W12.

For identification of survival biomarkers, we defined OS as the time occurring between baseline and death or last contact with the patient.

To identify two groups of patients with homogenous characteristics in terms of hematological parameters and myeloid and T cell subsets, the Cluster-K-means algorithm was used. Wilcoxon Rank Sum test was used to compare baseline and post-treatment levels of hematological or immunological parameters between the two clusters. Difference in OS between the two clusters was tested using Log-Rank test and the survival curves of the two groups were built according to Kaplan-Meier method.

For univariate survival analysis, patients were divided in two groups according to the cohort-median value of each hematological and immunological parameter at baseline or following ipilimumab treatment. Difference in survival between patients presenting values below or above the median for each parameter was tested using the Log-Rank test and the survival of each group was reported using Kaplan-Meier method.

Correlations between hematological and immunological parameters were assessed at baseline and after ipilimumab treatment using Spearman Rank Order Correlation analysis.

To identify potential correlations between survival and onset of toxicity, patients were divided according to the development of immune-related adverse events, and the difference between their OS was evaluated using the Log-Rank test. Survival curves were reported according to

Kaplan-Meier. A 2x2 contingency table was built on the basis of patients' survival status and toxicity and the degree of correlation was tested using Fischer Exact Test.

The association between toxicity and tumor-associated or immunological parameters (TIPs) was tested dividing the cohort in two groups according to the onset of irADR with severity above grade 3. The level of significance among levels of TIPs was determined using Wilcoxon Rank Sum test or NonParametric Combination test[202] to compare inter-group differences, while Wilcoxon Signed Rank test or NonParametric Combination test were used to compare intra-group variation of TIPs between W0 and W12.

Results were considered statistically significant with $P<0.05$. All the statistical analysis were performed using SigmaPlot software v12.00 (Systat Software Inc.) and MiniTab software v17 (Minitab Ltd.).

RESULTS

HARMONIZATION OF IMMUNOPHENOTYPING OF HUMAN MYELOID-DERIVED SUPPRESSOR CELLS

At present, at least 7 myeloid cell subsets have been identified as MDSCs that encompass promyelocyte-like cells, monocytes and granulocytes. This heterogeneity, associated to the lack of a specific marker, hampered the identification of a simple and robust method of identification of these cells by flow cytometry. To overcome this obstacle, in collaboration with Dr. Walter (Immatics Biotechnologies, Tubingen) and Prof. Bronte (University of Verona) our group designed a proficiency panel to harmonize the phenotype of human MDSCs, under the umbrella of the Cancer Immunoguiding Program (CIP).

Design of the MDSC-proficiency panel

As preparative steps, we sent out two questionnaires to all the groups that published at least one paper on mouse and human MDSCs, exploring their interest in participating to the panel along with a number of questions referring to the procedures used for MDSCs identification. Participants were required to be experienced in multicolour flow cytometry. The panel design was based on a consensus of opinions from the preparatory process and it envisions a two steps approach. In the first step participants were asked to quantify 10 predefined phenotypes of putative MDSCs by multicolour flow-cytometry on centrally preselected cryopreserved PBMCs of three healthy donors (HBC-480, HBC-514, L29_3) using their own staining protocol, antibody clones, fluorochromes and gating strategy. We provided individual feedback for the results of each laboratory as compared to the entire group and we determined the inter- and intra-laboratory variance of results, indentifying a set of critical parameters influencing these indexes of variation. In the second step, laboratories will again perform quantification of 10 predefined MDSC phenotypes on blinded PBMC samples, but a number of mandatory harmonization guidelines deduced from the first step will be given.

Guidelines for the first phase of MDSC proficiency panel

The staining cocktail, proposed in the panel's guidelines, contains 7 markers commonly used for MDSC recognition plus a DCM: HLA-DR, CD14, CD15, CD11b, CD33, Lineage cocktail (defined as CD3/14/19/56), CD124. This marker combination allows the identification of 10 myeloid subsets: MDSC1 $CD14^{+}/CD124^{+}$ [70], MDSC2 $CD15^{+}/CD124^{+}$ [70], MDSC3 $Lin^{-}/HLA\text{-}DR^{-}/CD33^{+}$ [78], MDSC4 $CD14^{+}/HLA\text{-}DR^{low/-}$ [72], MDSC5 $CD15^{+}/CD14^{-}/CD11b^{+}$ [74], MDSC6 $CD15^{+}/FSC^{low}/SSC^{high}$ [197], MDSC7

$CD15^-/CD14^+/CD33^{high}/HLA\text{-}DR^{low}$ [198], MDSC8 $CD15^+/CD33^{high}$, MDSC9 $CD14^-/CD15^-/CD33^{high}$ and MDSC10 $Lin^-/HLA\text{-}DR^{low}/CD11b^+$ [199](Fig.1). The list of the 10 putative MDSC subsets to be reported and the minimal composition of the staining cocktail are listed in Table 1.

OBLIGATORY MYELOID PHENOTYPES TO BE DETERMINED	MINIMAL COMPOSITION OF THE STAINING COCKTAIL
MDSC1 = $CD14^+$ $CD124^+$	Anti-CD11b
MDSC2 = $CD15^+$ $CD124^+$	Anti CD14
MDSC3 = Lin^- $CD33^+$ HLA-DR^-	Anti-CD15
MDSC4 = $CD14^+$ HLA-DR^{low}	Anti-CD33
MDSC5 = $CD15^+$ $CD14^-$ $CD11b^+$	Anti-HLA-DR
MDSC6 = $CD15^+$ FSC^{low} SSC^{high}	Anti-CD124 (IL4Rα)
MDSC7 = $CD15^-$ $CD14^+$ $CD33^{hi}$ HLA-DR^{low}	Lineage cocktail as defined here by anti-CD14/CD19/CD3/CD56
MDSC8 = $CD15^+$ $CD33^{hi}$	Dead cell maker
MDSC9 = $CD14^-$ $CD15^-$ $CD33^{hi}$	
MDSC10 = Lin^- HLA-DR^{low} $CD11b^+$	

Table 1: Mandatory parameters for MDSC proficiency panel
Table shows the 10 phenotypes to be determined (left column), and the minimal number of markers to be used (right column) for identification of the 10 putative MDSC subsets.

Each participating laboratory was asked to perform the staining twice, in two separate experimental runs, and to report the number of total events, singlets, monocytes, lymphocytes and myeloid subsets in the presence or absence of the DCM. The reason of the repetition of the staining is to calculate intra-laboratory variance that gives an estimate of the reproducibility of results in each laboratory. Besides, as some MDSC subsets may be damaged by thawing procedures, we asked participants to perform analysis with or without the presence of a DCM, to prove whether some MDSC subsets were underestimated by the exclusion of dead cells. We also asked to use one 8-colors staining panel and/or three 4-colors panel staining, in order to include in the panel also the laboratories with flow cytometers equipped only for 4-colors analyses. Protocol of thawing and staining, fluorochrome and clone of antibodies, gating strategy and choice of negative controls were left free of choice. A detailed description of the mandatory instructions and the protocol used in our laboratory is reported in Material and Methods section. Twenty-three laboratories from Europe and the United States of America participated to this first step of the proficiency panel, results were centrally collected and analysed in our laboratory and in the laboratory of Dr. Walter at Immatics Biotechnologies.

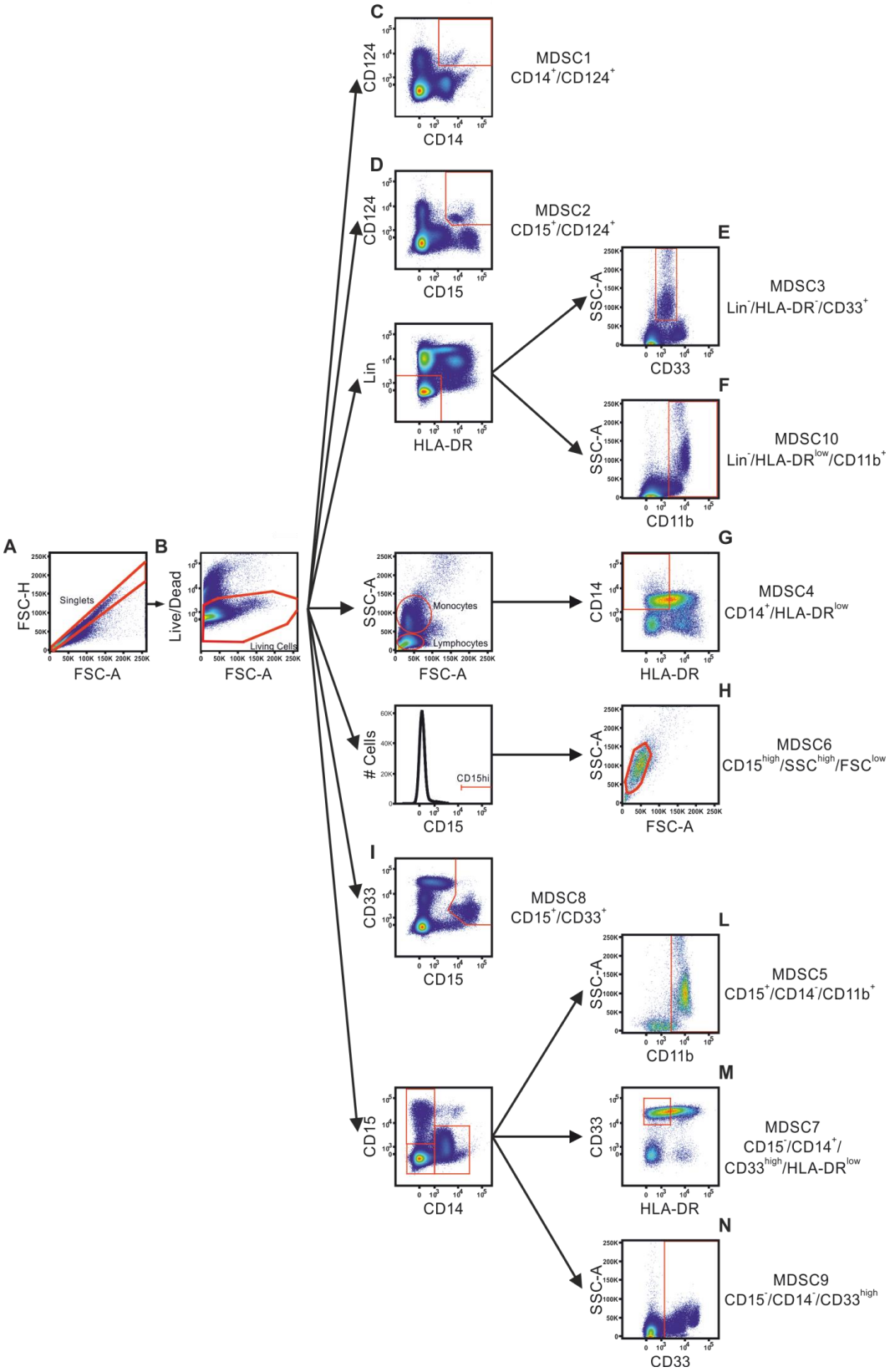


Figure 1: Exemplary gating strategy for the identification of 10 putative MDSCs subsets
Exemplary gating strategy included in the panel's guidelines is reported. Preliminary doublets exclusion (FSC-H vs FSC-A)(A) followed, when required, by dead cell exclusion (B) was suggested before proceeding with the identification of 10 myeloid subsets indicated in the figure with letters from C to N.

Determination of intra- and inter-laboratory variance

To ascertain the variance among results, we calculated the % coefficient of variation (standard deviation/mean x 100). Intra-laboratory variance was defined as the variance of results between the first and second experimental runs in each laboratory, while inter-laboratory variance was defined as the variance occurring in results obtained by the 23 different laboratories in the two experimental runs.

Intra-laboratory variance ranged from 24 to 52% (Fig. 2 A), and was almost uniform among the different subsets, but inter-laboratory variance was higher, ranging from 64 to 310%, Fig. 2 C, and with high variation in the different subsets. In fact, variance was higher when assessing granulocytic subsets (MDSC2-5-6-8, range 200-310%), intermediate for immature MDSCs (MDSC3-10, range 100-170%), and presented the lower coefficient of variation with monocytic MDSCs (MDSC 4-7, range 64-75%). A different matter is related to MDSC1 cell subset estimation, that shows a very high variance (range 180-210%), but this was likely due to other parameters used for $CD124^{+}$ cell identification (see later). We also observed that the use of DCM significantly increased intra-laboratory variance (P<0.001 on overall MDSC populations according to Wilcoxon Signed Rank test) (Fig. 2A), while inter-laboratory variance does not change significantly (Fig. 2C). We reported individual results for three MDSC subsets, representative of the three main classes of MDSCs, as an example of the great variation in the quantification of these cells (Fig. 2 B).

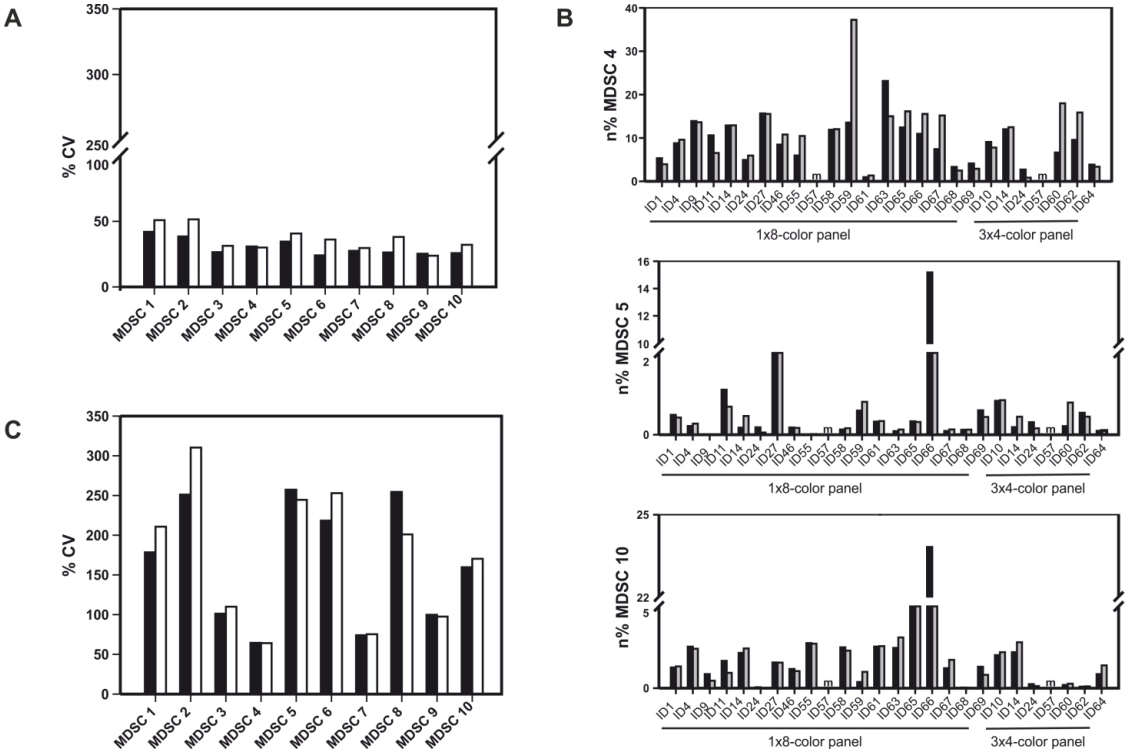


Figure 2: Intra-laboratory and inter-laboratories variance

Immunophenotyping of 10 putative MDSCs subsets was performed on PBMC of 3 healthy donors (HBC-480, HBC-514, L29_3) in two independent experimental runs. Intra-laboratory and inter-laboratory variance are shown in panel A) and C), respectively. Black bars showed average %CV from analyses performed without exclusion of dead cells, while white bars refers to analyses considering only live cells. ($P<0.001$ on overall MDSC populations according to Wilcoxon Signed Rank test for difference in intra-laboratory variance). n=18 evaluable labs reporting data for the 8-color panel. Laboratories that did not report all requested data were not considered evaluable, while laboratories performing 4-color panels were excluded in order to perform analysis on a more homogeneous cohort. Panel B) showed an example of the frequencies of monocytic (MDSC4 – upper panel), granulocytic (MDSC5 – central panel) and immature (MDSC10 – lower panel) MDSCs, normalized on the count of lymphocytes+monocytes, reported by each laboratory performing either one 8-color panel or three 4-color panels; the two paired-histograms refer to the two independent experimental runs performed by each laboratory (black bars = first run, grey bars = second run). Missing values "m".

In order to reduce variance, we normalized data by diving the number of each MDSC subset on the total count of lymphocytes plus monocytes and by reporting the value to 100. This normalization reduced the variance between laboratories for 7 out of 10 subsets (Fig. 3 A) but heterogeneity of the results was still very high even after normalization, and thus we set out to identify potential parameters responsible for this high spread. As shown in Figure 3 B, the use of DCM affects the quantification of the myeloid subsets, as it brings about a significant reduction of granulocytic subsets and, to a minor extent, also of immature subsets. On the contrary, the quantification of monocytic MDSCs (MDSC 4-7) is unaffected by the use of DCM. A possible explanation of these results lies in the fragile nature of granulocytes that are more prone to death during the thawing procedure, as compared to monocytic cells.

Identification of critical parameters influencing the variance of results

By analysing a number of parameters potentially affecting the variance, we found that an important source of variation was the gating strategy adopted by the groups to identify MDSCs subsets. To dissect the influence of the gating strategy on inter-laboratory variance, we compared the variance of 9/18 groups adherent to a homogeneous gating strategy, which corresponds to the strategy exemplified in the panel's guidelines, to those who were using a number of different gating strategies, distinct from that proposed in the guideline (9/18). Indeed, we detected a significant difference in the overall coefficient of variation when we compared these two groups (Fig. 3 C, P=0.0012 on overall MDSC populations according to Wilcoxon Signed Rank test). To minimise the interference on variance analysis given by other covariates, we considered for this analysis 18/23 laboratories that identify the 10 putative MDSC subsets using an 8-color panel and analysing data without DCM.

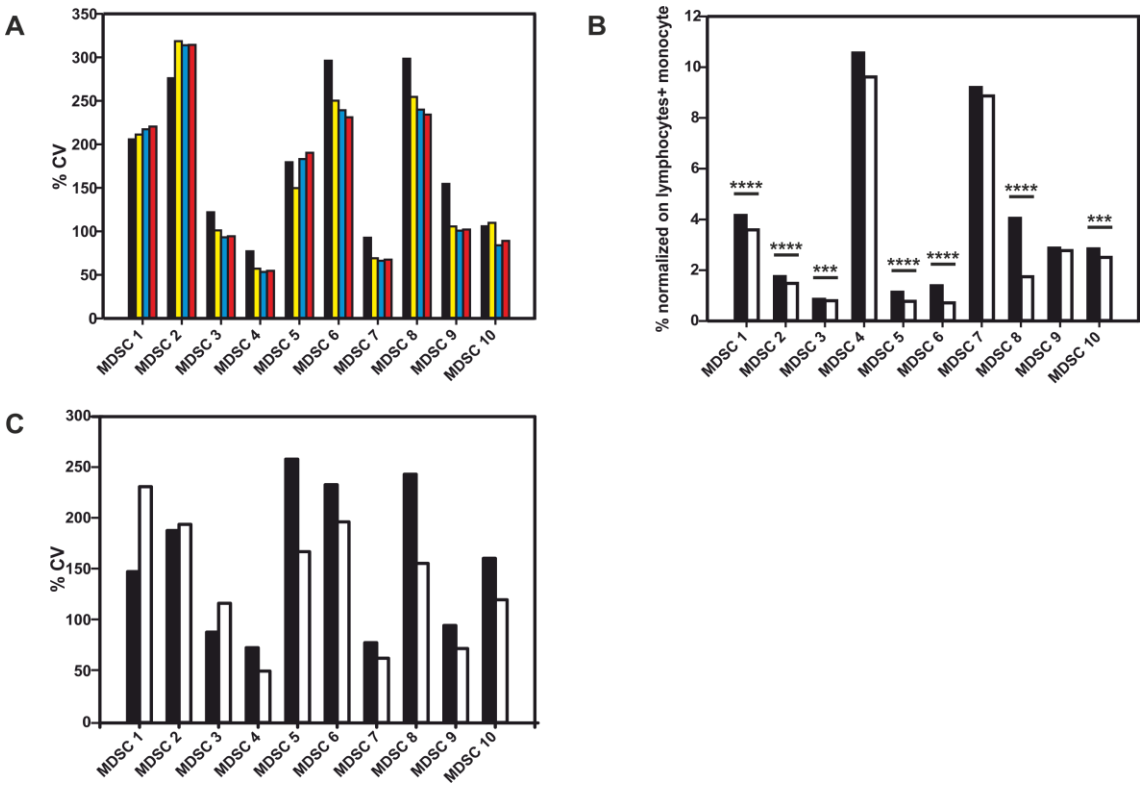


Figure 3: Candidate parameters affecting inter-laboratory variance

Immunophenotyping of 10 putative MDSCs subsets was performed by staining the PBMCs of 3 healthy donors (HBC-480, HBC-514, L29_3) in two independent experimental runs. Panel A shows average %CV (n=14 evaluable labs reporting data for the 8-color panel) of the frequencies of the ten MDSC subsets normalized according to different strategies. Results are reported without normalization (Black bars) and normalized on the number of singlets (yellow bars), lymphocytes (blue bars) and lymphocytes + monocytes (red bars) for each MDSC subset. Panel B shows the average normalized frequencies of MDSC subsets (n=18 evaluable labs reporting data for the 8-color panel), normalized on lymphocytes + monocytes identified either excluding (white bar) or not (black bars) the dead cells. C) Average %CV (n=18 evaluable labs reporting data for the 8-color panel) of the frequencies of MDSC subsets identified either using an homogenous gating strategy (white bar) or not (black bars). Differences between data have been tested using Wilcoxon Signed Rank test and labelled as *** when $P<0.001$ or as **** when $P<0.0001$.

As previously stated, the inter-laboratory variance for MDSC subsets identified by CD124 marker (MDSC1 and MDSC2) was high, ranging from 178 to 210% for MDSC1 and from 251 to 310% for MDSC2. To identify the possible sources of high variance for these subsets, we harmonized CD124-independent parameters which may otherwise influence the analysis: to this end, we considered only 16/23 laboratories that used an 8-color panel, analysed data without dead cell marker and chose one of the two most used anti-CD124 clones for identification of $CD124^+$ cells; in addition, we based our analysis on MDSC1 because the low frequencies of MDSC2 would lead to high %CV even in the presence of low relative difference between measurements. We identified two possible parameters responsible for the high variance of MDSC1: the gating strategy and the anti-CD124 clone. Indeed, CD124 is a marker characterized by a dim intensity and a unimodal distribution of the signal, and identification of positive cells brings about a certain degree of

complexity. Indeed, participants identified CD124^{+} cells following two distinct gating strategy: 9/16 groups set the gate for CD124^{+} cells only considering cells with high intensity of this marker (Fig. 4 Ai), while 7/16 included also cells with a dim expression (Fig. 4 Aii). This decision determined a very high difference in MDSC1 quantification, given equal clones used for staining (Fig. 4 B), and thus the overall variance for MDSC1 raised, but the %CV was similarly reduced when we separated the results on the base of the gating strategy (Fig. 4 C). The second main source of variation between data lies on the choice of anti-CD124 clone. 20/23 laboratories used two clones for CD124 identification (n=13 clone hIL4R-M57, n=7 clone 25463) and we recorded a discrepancy between their staining potential: on equal gating strategy, clone 25463 identified higher frequencies of MDSC1 than clone hIL4R-M57 (Fig. 4 D).

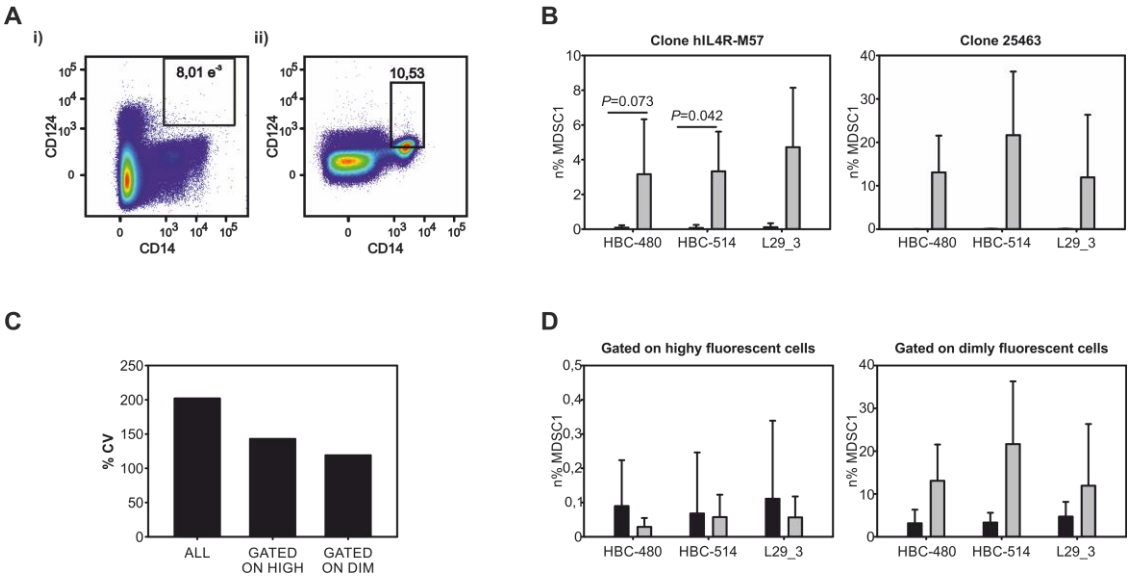


Figure 4: Parameters affecting inter-laboratory variance for MDSC1

Quantification of MDSC1 was included in the immunophenotyping of the 10 putative MDSCs subsets performed on PBMC of 3 healthy donors (HBC-480, HBC-514, L29_3) in two independent experimental runs. Figure shows results relative only to MDSC1. In this analysis 16 laboratories out of 23 were included as they performed an 8-color panel staining that included either anti-CD124 clones hIL4R-M57 or 25463, and data were analysed without exclusion of dead cells. A) Two different exemplary gating strategies for determination of CD124^{+} cells. CD124^{+} cells determined as those expressing a high fluorescence intensity for this marker (i) or as those dimly fluorescent (ii). Panel B shows the average normalized frequencies of MDSC 1 normalized on lymphocytes + monocytes identified either including cells with high (black bars) or high + dim (grey bars) fluorescence. Comparison between different gating strategies was performed on equal clones (either hIL4R-M57 or 25463) as indicated on the top of the plot. C) Average %CV of the frequencies of MDSC1 determined as those expressing a high fluorescence intensity for CD124 (gated on high) or as those dimly fluorescent (gated on dim) or any gating strategy (all). Panel D depicts the average frequencies of MDSC 1 normalized on lymphocytes + monocytes identified either using anti-CD124 clone hIL4R-M57 (black bars) or 25463 (grey bars). Comparison between different staining potential was performed on equal gating strategy as indicated on the top of the plot.

Immature MDSCs are defined as myeloid cells with low or negative expression of HLA-DR and negative for the staining of Lineage cocktail. In the panel's guidelines we proposed to use a Lineage

cocktail properly excluding T, B and NK cells plus monocytes. However, only 12/23 laboratories used the proposed Lineage cocktail. 6/23 used a cocktail including markers only for T, B lymphocytes and NK cells while 5/23 used a Lineage cocktail with a richer composition including markers for T, B lymphocytes and NK cells, monocytes and granulocytes. This discrepancy probably increased the variance of the results (range 100-170% Fig. 2 C) and the addition of a granulocytic marker in the Lineage cocktail led to identification of more defined, albeit lower, percentage of immature subsets (data not shown).

Future perspectives for the second experimental step

Results from the first phase of the proficiency panel demonstrate that participants identified the 10 putative MDSC subsets with good intra-laboratory reproducibility but with a very high inter-laboratory variance, thus supporting the need of an effort for the harmonization of human MDSC determination. Such large variance may be partially corrected by normalizing the results on an internal control (number of lymphocytes plus monocytes) and using a homogenous gating strategy for MDSC identification. To this end, we are currently setting up an *in-silico* panel to clearly assess the influence of the gating strategy on the spread of the results and to identify a robust gating strategy that will be used in the second step of the proficiency panel. Other aspects that require attention are the use of DCM and the identification of $CD124^{+}$ cells. Indeed, as the presence of DCM significantly impacted on intra-laboratory variance and on quantification of granulocytic subsets, we will discuss with the participants whether it is recommendable to include the DCM in the staining. Other issues open for discussion are the harmonization of CD124 staining, and the composition of the Lineage cocktail.

IMMUNOMONITORING OF MELANOMA PATIENTS TREATED WITH IPILIMUMAB

Clinical characteristics of the cohorts

We enrolled 37 patients affected by metastatic melanoma and selected to receive ipilimumab. The mean age of the cohort was 63 years (range 33-83) and it was composed of 25 males (68%) and 12 females (32%). In parallel, we enrolled also 21 healthy donors matched for age and gender: the mean age of this cohort was 58 years (range 37-87) and it was composed of 14 males (67%) and 7 females (33%). Clinical characteristics of melanoma patients and healthy controls are detailed in Table 2.

At baseline, all melanoma patients were affected by metastatic disease (stage IV) and the majority of them (n=25, 67%) presented with IV M1c stage, which is characterized by the presence of visceral metastasis and/or elevated LDH levels, eight patients (22%) had stage IV M1b with lung metastasis and normal LDH levels, and only a minority of patients had distant metastasis restricted in the skin and normal LDH levels (stage IV M1a, n=4, 11%). Albeit the high prevalence of late stage patients, the Eastern Cooperative Oncology Performance Status (ECOG PS) was good for most of the patients (n=27, 73% ECOG PS ≤ 1).

Most of the patients were previously genotyped for B-RAF and N-RAS mutations (n=35, 99,3%). 59% of the patients did not carry any mutations in these genes while 31% presented B-RAF mutations and were pre-treated with B-RAF inhibitors. Only one patient have a mutation on N-RAS.

Baseline levels of circulating tumor cells (CTC) was also available from 11 patients (36%) who presented elevated or stable CTC post-treatment. However, this low number of patients reduced the power of any statistical association with immune parameters and therefore we did not include this parameter in our statistical analysis.

Ipilimumab treatment was successfully completed for 28 patients (75%), while one fourth of the patients discontinued the treatment because of toxicity or death (n=9, 25%). The median time to progression was 12 weeks and partial response was achieved by 19% of the patients (n=7), 11% had a stable disease (n=4), and 70% of the patients did not control the disease (n=25). Complete responses were not achieved in this group of patients. OS was evaluated: one-year survival rate was 43% while 62% of the patients were still alive when survival was measured at the common minimum time of follow-up, that is 29 weeks for this study (W29).

	PATIENTS			HEALTHY DONORS
N	37			21
Age (mean, range)	63 (33-83)			58 (37-87)
Gender M/F (n, %)	25/12 (68/32%)			14/7 (67/33%)
		n	**%**	
ECOG PS	≤ 1	27	73	
	2	10	27	
Stage	M1a	4	11	
	M1b	8	22	
	M1c	25	67	
Mutations (n=32)	WT	19	59	
	B-RAF	10	31	
	N-RAS	1	0,3	
	ND	2	0,7	
CTC (n=11)	W0	4	36	
Variation of CTC (W12-W0)	up	4	36	
	down	2	18	
	stable	5	46	
Ipilimumab therapy	completed	28	75	
	not completed	9	25	
Response W12	Median TTP	12 weeks		
	CR	0	0	
	PR	7	19	
	SD	4	11	
	PD	25	70	
Survival	Median OS	32 weeks		
	1-year OS n=28	11	43%	
	Alive W29	23	62	
	Deceased W29	14	38	

Table 2: Baseline characteristics of melanoma patients and healthy donors

Response was assessed 12 weeks upon enrollment according to immune-related response criteria and classified as follows: complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD). Eastern Cooperative Oncology Performance Status (ECOG PS), Circulating Tumor Cells (CTC), week 0 (W0), week 12 (W12).

Evaluation of the immune profile of melanoma patients at baseline

The first step of this study compared the immune profile of melanoma patients with healthy donors to evaluate the modifications induced by melanoma in the immune system of these patients. To this end, we investigated different populations of circulating myeloid cells, T cells and serum proteins named haematological parameters herein.

Among the myeloid cells analysed, we considered four subsets of MDSCs, eosinophils and putative dendritic cells (DCs). Given the high heterogeneity of phenotypes attributed to MDSCs, we chose to monitor the presence of two monocytic, one immature ad one granulocytic subsets because they are the most common MDSC subsets found in the whole blood of cancer patients[57]. Since we phenotyped MDSCs on whole blood, it was not possible to monitor also the levels of low-density polimorphonuclear cells that often co-purify with PBMCs in cancer patients and constitute a subsets of granulocytic MDSCs[75]. The phenotypes of MDSCs subsets assayed in this study are reported in Figure 5: MDSC 1 ($CD14^+/IL4R\alpha^+$, panel B), MDSC 2 ($CD15^+/IL4R\alpha^+$, panel D), MDSC 3 ($CD15^-/Lin^-/HLA\text{-}DR^-/CD33^+/CD11b^+$, panel E), MDSC 4 ($CD14^+$/ HLA-DR $^{low/-}$, panel C).

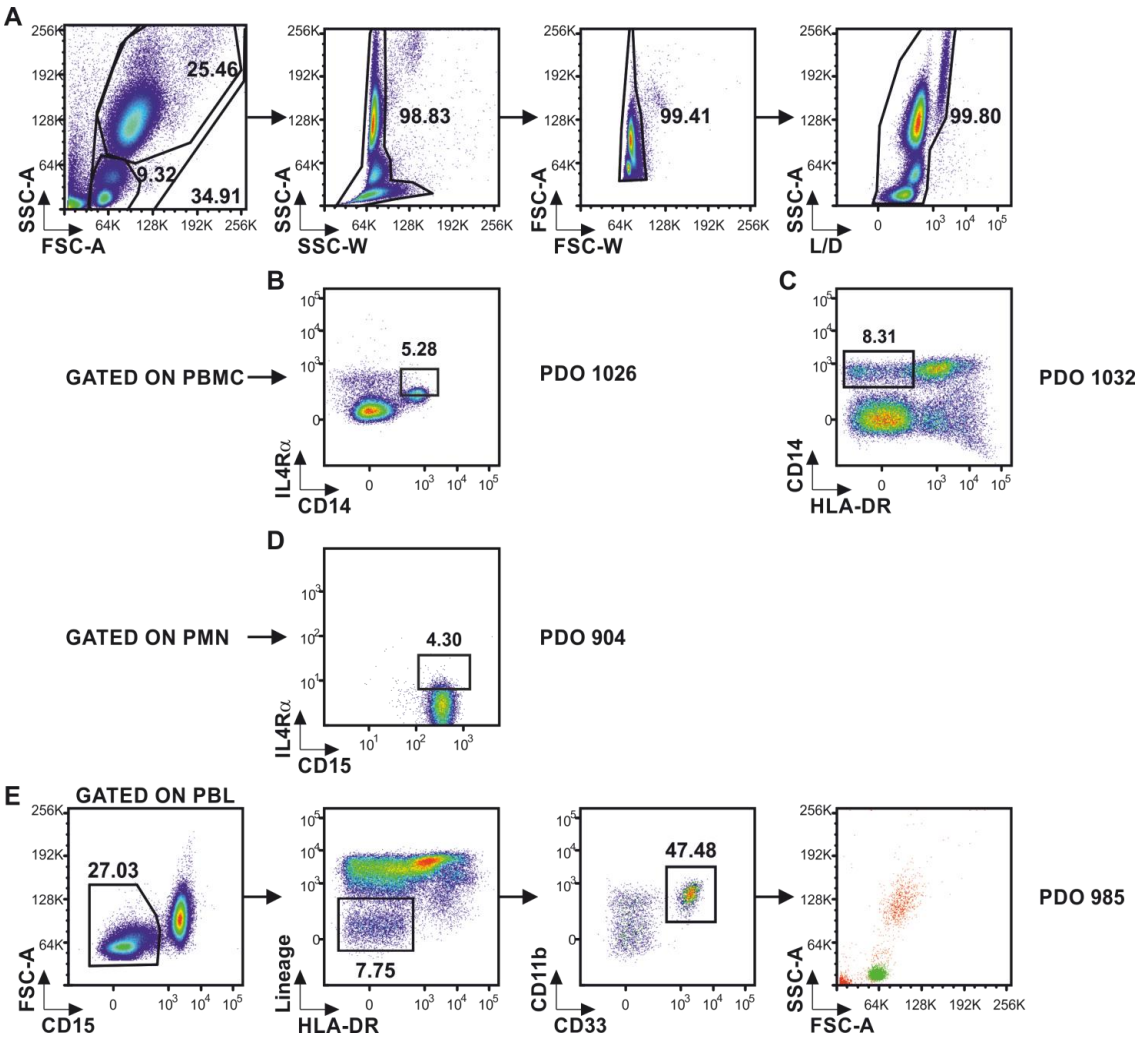


Figure 5: Identification of four MDSC subsets in whole blood by multicolour flow cytometry

A) Gating strategy to identify MDSCs consists in the definition of a morphological gate, set on FSC vs SSC parameters, followed by exclusion of doublets and dead cells and further definition of MDSC subsets as illustrated in panels B-E. B) and C) illustrate the gating strategy to identify monocytic MDSC: MDSC1 ($CD14^+/IL4R\alpha^+$, panel B) and MDSC4 ($CD14^+$/ HLA-DR $^{low/-}$, panel C); panel D exemplified the gating strategy for granulocytic MDSC2 ($CD15^+/IL4R\alpha^+$) while panel E shows the gating strategy for immature MDSC3 ($CD15^-/Lin^-/HLA\text{-}DR^-/CD33^+/CD11b^+$). The MDSC subsets reported belong to different patients indicated in the figure with the acronym PDO plus the serial number of the sample. FMO controls were used for HLA-DR and IL4Rα electronic gating.

Eosinophils were defined using the pan-granulocytes marker CD15 and considering their peculiar intense autofluorescence at 520nm, when excited with the 405nm violet laser. The use of autofluorescence to identify eosinophils was previously reported by other authors[203,204] and it is primary due to flavins contained in their granules. The gating strategy to identify eosinophils is depicted in Figure 6.

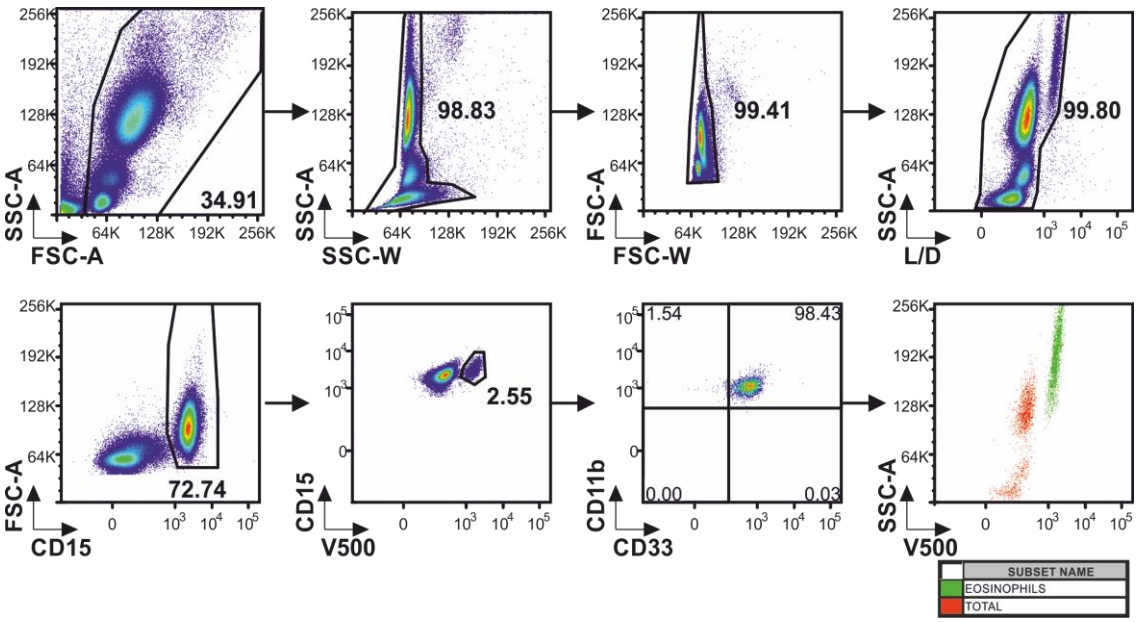


Figure 6: Identification of eosinophils in whole blood by multicolour flow cytometry

Peripheral blood leukocytes were analysed using a morphological gate set on FSC vs SSC parameters. After exclusion of doubles and dead cells, the eosinophils' population was defined on the basis of CD15 expression and of high autofluorescence in the V500 channel. To confirm that gated cells belong to the myeloid lineage, the pan-myeloid markers CD33 and CD11b were also used. As shown in the overlay representation, eosinophils have high SSC and a characteristic autofluorescence in the V500 channel.

Finally we identified a subset of myeloid cells which did not express markers of mature lymphocytes, granulocytes and monocytes and expressed high levels of HLA-DR ($CD15^-/Lin^-/HLA\text{-}DR^+/CD33^{dim}$). This phenotype is compatible with the phenotypic definition of DCs. The gating strategy for this putative DCs subset is depicted in Figure 7.

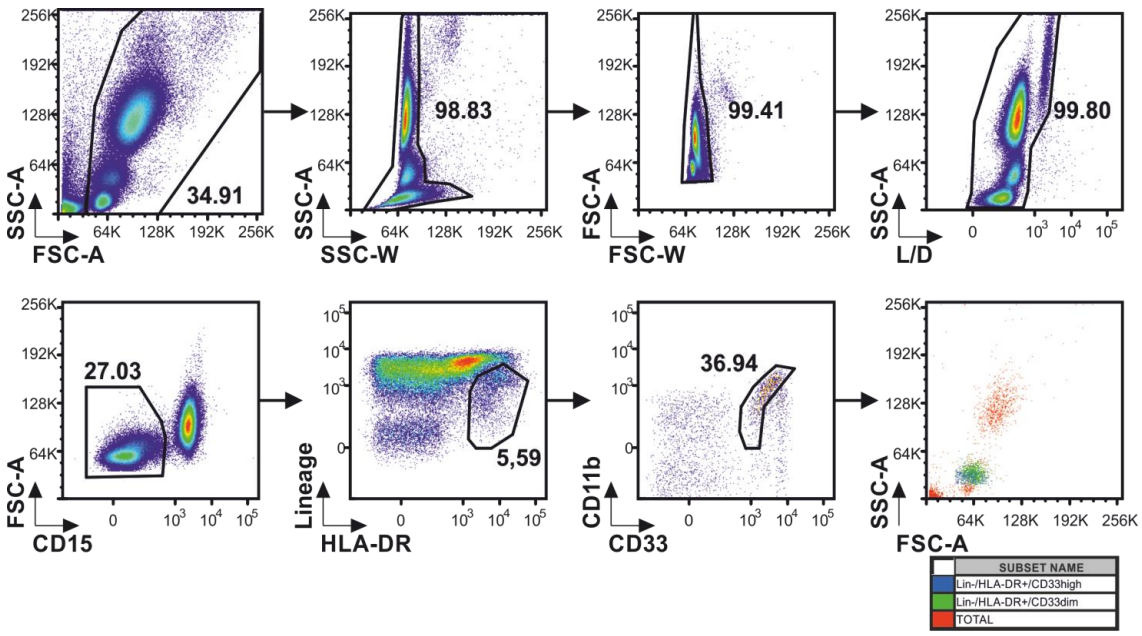


Figure 7: Identification of putative dendritic cells in whole blood by multicolour flow cytometry

Putative DCs were identified among blood leukocytes by using a morphological gate set on FSC vs SSC parameters. After exclusion of doubles, dead cells and $CD15^+$ granulocytes, mature monocytes and lymphocytes were excluded by a progressive gating on $Lineage^-$ cells and putative DCs were identified on the basis of the high expression of HLA-DR and of a dim expression of CD33. In the overlay representation, these cells are characterized by peculiar SSC properties, i.e. lower than monocytes but higher than lymphocytes.

Melanoma patients showed a significant expansion of MDSC-1, -2 and -4 subsets in the blood, and had reduced frequencies of eosinophils compared to healthy donors, while putative DCs and MDSC 3 levels did not differ significantly from the control group of healthy donors, matched by age and gender (Fig. 8 C-F). Both subsets of MDSCs expressing IL4Rα had a significant expansion (MDSC1 *P*<0.001, Fig. 8 A, and MDSC2 *P*=0.009, Fig. 8 B), along with MDSC4 (*P*=0.004, Fig. 8 D). In addition, melanoma patients presented significantly lower levels of circulating eosinophils compared to healthy donors (*P*= 0.001, Fig 8 E).

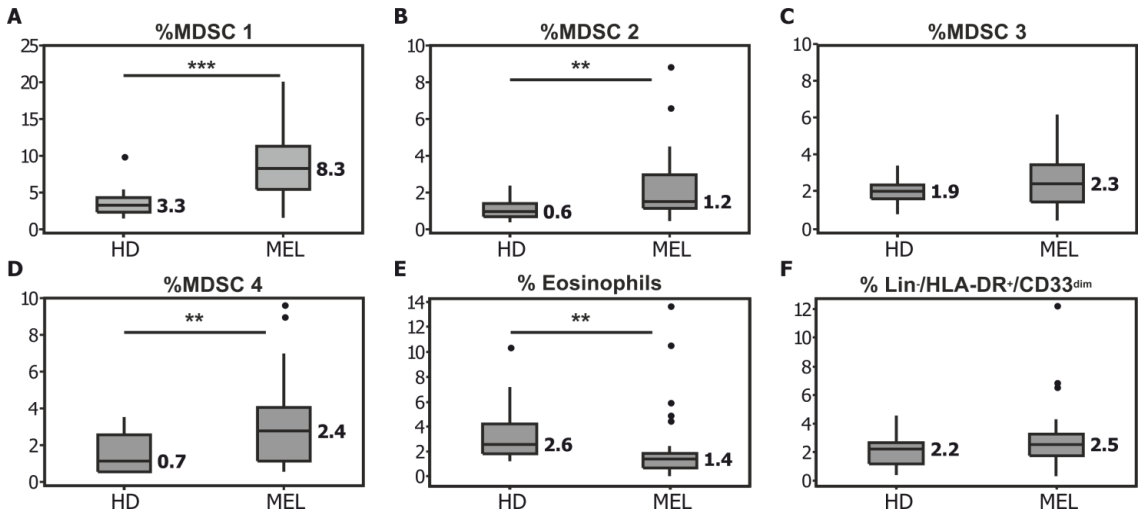


Figure 8: Baseline levels of myeloid cells in stage IV melanoma patients and healthy donors.

Baseline levels of myeloid subsets in melanoma patients (MEL, n=27 to 33 depending on the myeloid subset considered) and age- and gender-matched healthy donors (HD n=21) are shown. Each box plot show first and third quartiles and median values of the myeloid subset indicated at the top of the plot. Outlier are plotted as individual points. Wilcoxon Rank Sum test was used to compare the frequencies of myeloid subsets between the two groups (** P<0.01, *** P<0.001). Values were considered statistically significant for P<0.05.

Frequencies of T cell subsets were assessed on whole blood by restricting the analysis among PBMCs (Fig. 10 A), and absolute counts of these cells were obtained using Trucount tubes (Fig. 10 C). We considered both total $CD3^+$ T cells (Fig. 9 B) and their major sub-population: $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells (Fig. 9 C-D). In addition, we decided to monitor PD-1 expression on both T cell subsets. The expression of PD-1 was evaluated as frequency and as absolute numbers of $CD3^+/CD4^+/PD\text{-}1^+$ and $CD3^+/CD8^+/PD\text{-}1^+$ cells; we also quantified PD-1 expression within each T cell subsets (Fig. 9 E-F). Gating strategy is reported in Figure 9. Cumulative results are depicted in Figure 10.

At baseline melanoma patients did not show an altered frequency of total $CD3^+$ T cells and T cell subsets compared to healthy donors (Fig. 10 A), but the absolute count of total T lymphocytes was significantly reduced (Fig. 10 C, P<0.001). This was primary due to a reduction in the number of $CD3^+/CD4^+$ cells while the number of $CD3^+/CD8^+$ cells was unaffected (Fig. 10 C, P<0.001 for $CD3^+/CD4^+$ cells). Of note, we observed a significant up-regulation of PD-1 expression on $CD4^+$ T cells compared to healthy donors (Fig. 10 B) and indeed, the count of $PD\text{-}1^+$ cells was elevated in both lineages of T cells compared to controls (Fig 10 C P=0.007 for $CD3^+/CD4^+/PD\text{-}1^+$ cells and P<0.001 for $CD3^+/CD8^+/PD\text{-}1^+$ cells).

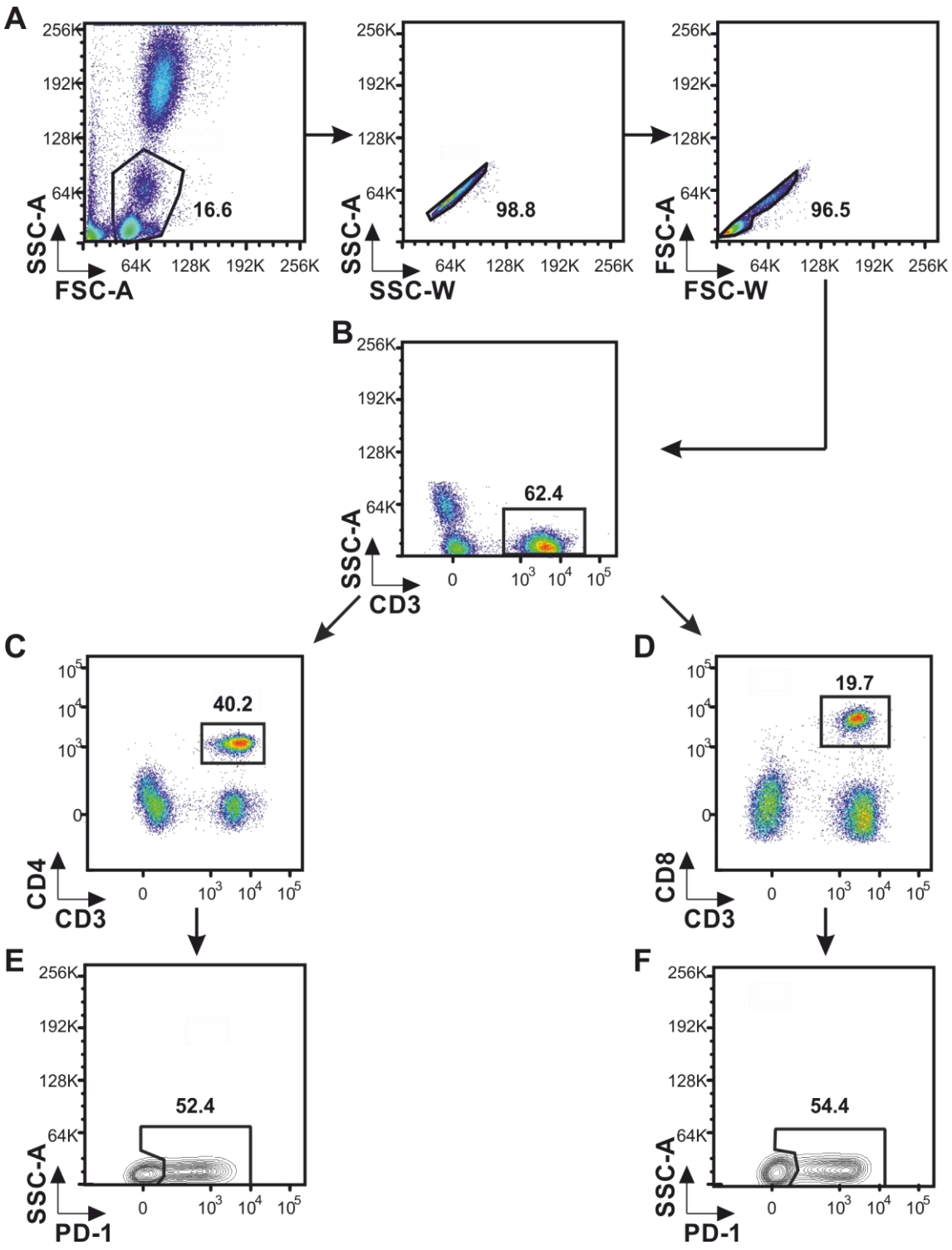


Figure 9: T cell subsets identification in whole blood by flow cytometry

PBMCs were included in the analysis using a morphological gate set on FSC vs SSC parameters (A). After exclusion of doublets, $CD3^+$ T cells were considered (panel B). $CD4^+$ and $CD8^+$ T cells were identified as reported in panels C and D, respectively. PD-1^+ cells were quantified within each T cell subset (panels E-F), setting the gate on FMO control.

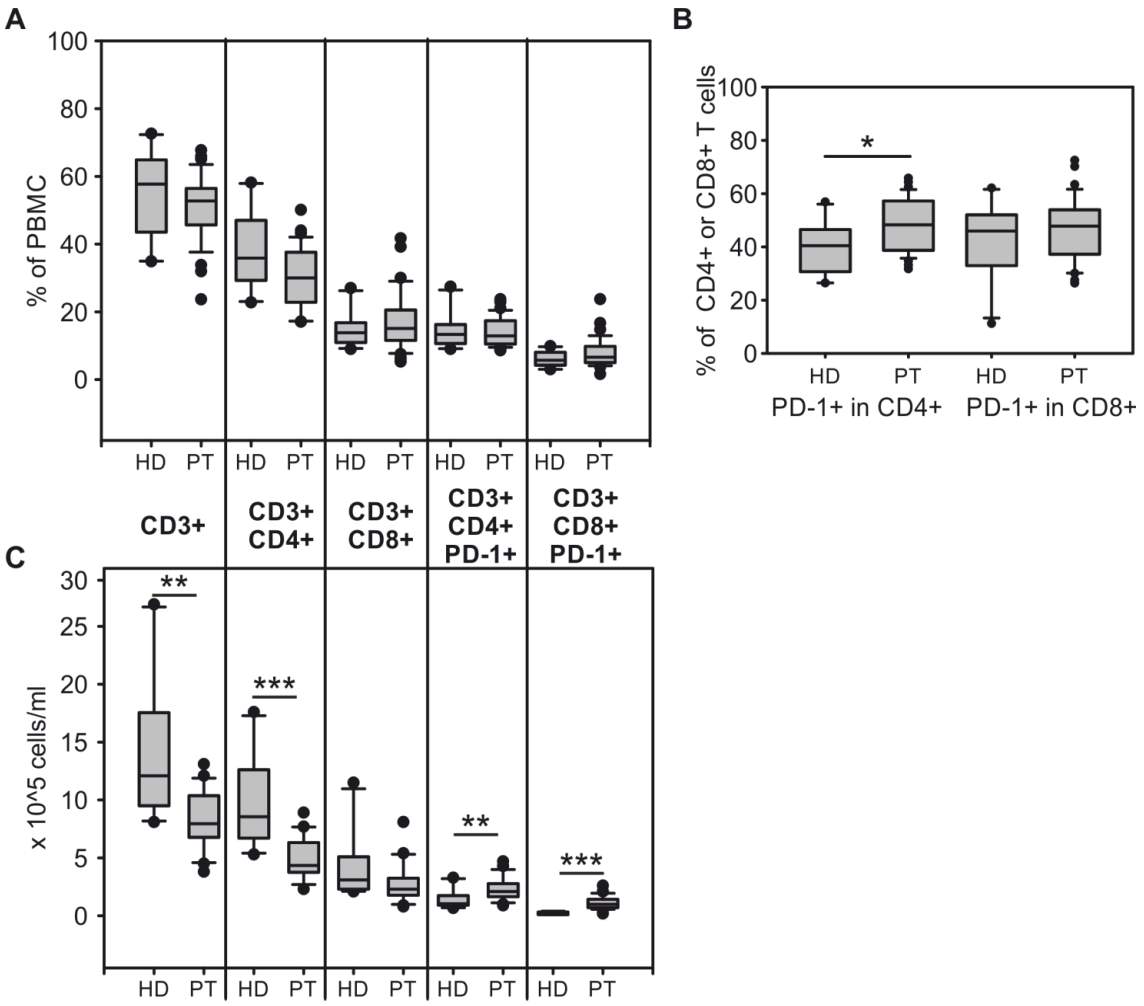


Figure 10: Baseline frequencies and absolute numbers of T cell subsets in melanoma patients and healthy donors

(A) Baseline frequencies of T cell subsets in melanoma patients (PT, n=37) and age- and gender-matched healthy donors (HD n=21). (B) frequencies of PD-1$^+$ cells within the CD4$^+$ or CD8$^+$ subsets of T lymphocytes. (C) absolute count of T cell subsets in a group of patients (n=22) and healthy donors (n=10). Each box plot shows first and third quartiles and median values of the T cell subset indicated at the top of the plot. Outlier are plotted as individual points. Wilcoxon Rank Sum test was used to compare the frequencies of T cell subsets between melanoma patients and healthy donors (* P<0.05, ** P<0.01, *** P<0.001). Values were considered statistically significant for P<0.05.

We also assessed the levels of serum factors of melanoma patients that are associated either with the immunological status of patients or with tumor-burden. In detail, we considered circulating levels of: i) C-reactive protein (CRP) and interleukin-6 (IL-6), which are proteins associated with inflammation and MDSCs expansion[32,107,108], and ii) vascular endothelial growth factor (VEGF), lactate dehydrogenase (LDH) and S100B, which are related to angiogenesis, tumor burden and contribute to staging of metastatic melanoma (LDH)[57,179]. As shown in Table 3, about half of the patients presented consistent alteration of CRP and S100B levels at baseline, while a lower proportions of patients had altered VEGF, IL6 and LDH levels compared to the upper limit of normal (ULN).

	Baseline		Post-treatment	
	%Below ULN	*%Above ULN*	*%Below ULN*	*%Above ULN*
C-reactive protein (CRP)	50	50	53	47
Interleukin 6 (IL6)	79	21	65	35
Vascular endothelial growth factor (VEGF)	70	30	65	35
S100B	47	53	29	71
Lactate dehydrogenase (LDH)	65	35	40	60

Table 3: Haematological parameters in melanoma patients.
Percentage of patients with values of serum factors below or above the ULN at baseline and post-treatment.

In conclusion, melanoma patients significantly expanded three subsets of MDSCs (MDSC 1-2-4) and had lower frequencies of eosinophils compared to healthy donors. Besides, patients underwent significant alterations in the T cell compartment showing lower numbers of T lymphocytes, especially $CD4^+$ T cells, and an up-regulation of PD-1 on $CD4^+$ T cells. Finally, patients presented altered levels of serum proteins associated with inflammation and tumor burden such as CRP and S100B.

Immune profile of melanoma patients during ipilimumab treatment

Since ipilimumab exerts its major functions through modulation of the T cell response, we investigated whether the administration of the drug was able to induce also a modification in the immune profile of patients. To this end, we compared the parameters evaluated at baseline (W0) also after 12 weeks (W12), when patients underwent to their first clinical evaluation of response via CAT.

In the myeloid compartment we observed a significant reduction of immature MDSC3 in post-treatment samples compared to baseline (Fig. 11 C, P=0.024) and a significant expansion in the putative DC subset $Lin^-/HLA\text{-}DR^+/CD33^{dim}$ (Fig. 11 F, P=0.017). On the other hand, MDSC 1-2-4 and eosinophils, which were significantly altered at baseline compared with healthy donors, did not change significantly upon treatment with ipilimumab (Fig. 11 A-B-D-E).

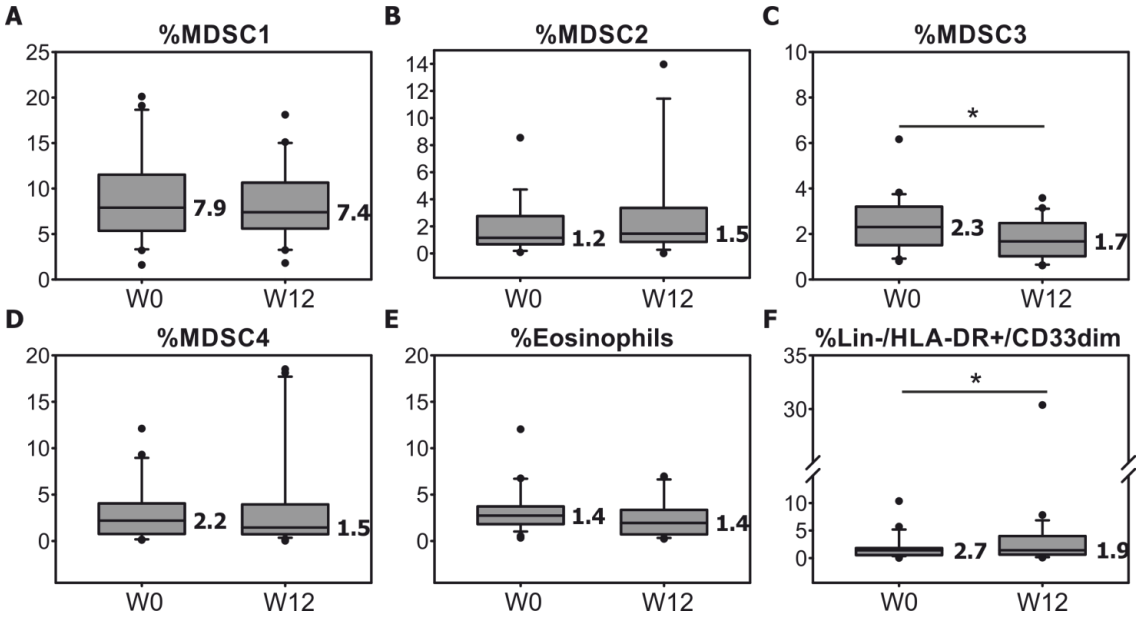


Figure 11: Evaluation of myeloid subsets during ipilimumab treatment

Levels of myeloid subsets monitored in melanoma patients at W0 (n=20 to 24 depending on the myeloid subset considered) and at W12 (n=20 to 24 depending on the myeloid subset considered). Each box plot shows first and third quartiles and median values of the myeloid subset indicated at the top of the plot. Outliers are plotted as individual points. Wilcoxon Signed Rank test was used to compare the frequencies of myeloid subsets in melanoma patients at different time-points (**P*<0.05). Values were considered statistically significant for *P*<0.05.

Ipilimumab treatment did not change total percentage of $CD3^+$ T cells, nor their absolute number (Fig. 12 A-B). On the contrary, following treatment PD-1 expression was significantly up-regulated on both $CD4^+$ and $CD8^+$ T cell subsets (Fig. 12 A, *P*=0.003 for $CD3^+/CD4^+/PD\text{-}1^+$ cells and *P*=0.016 for $CD3^+/CD8^+/PD\text{-}1^+$ cells). This effect was not due to an expansion of $CD4^+$ or $CD8^+$ T cells, whose frequencies and absolute numbers remained stable (Fig. 12 A-C), and it is even more significant when considering the percentages of $PD\text{-}1^+$ cells within the $CD4^+$ or $CD8^+$ gate (Fig.12 B, *P*<0.001 within $CD4^+$ T cells and *P*<0.001 in $CD8^+$ T cells).

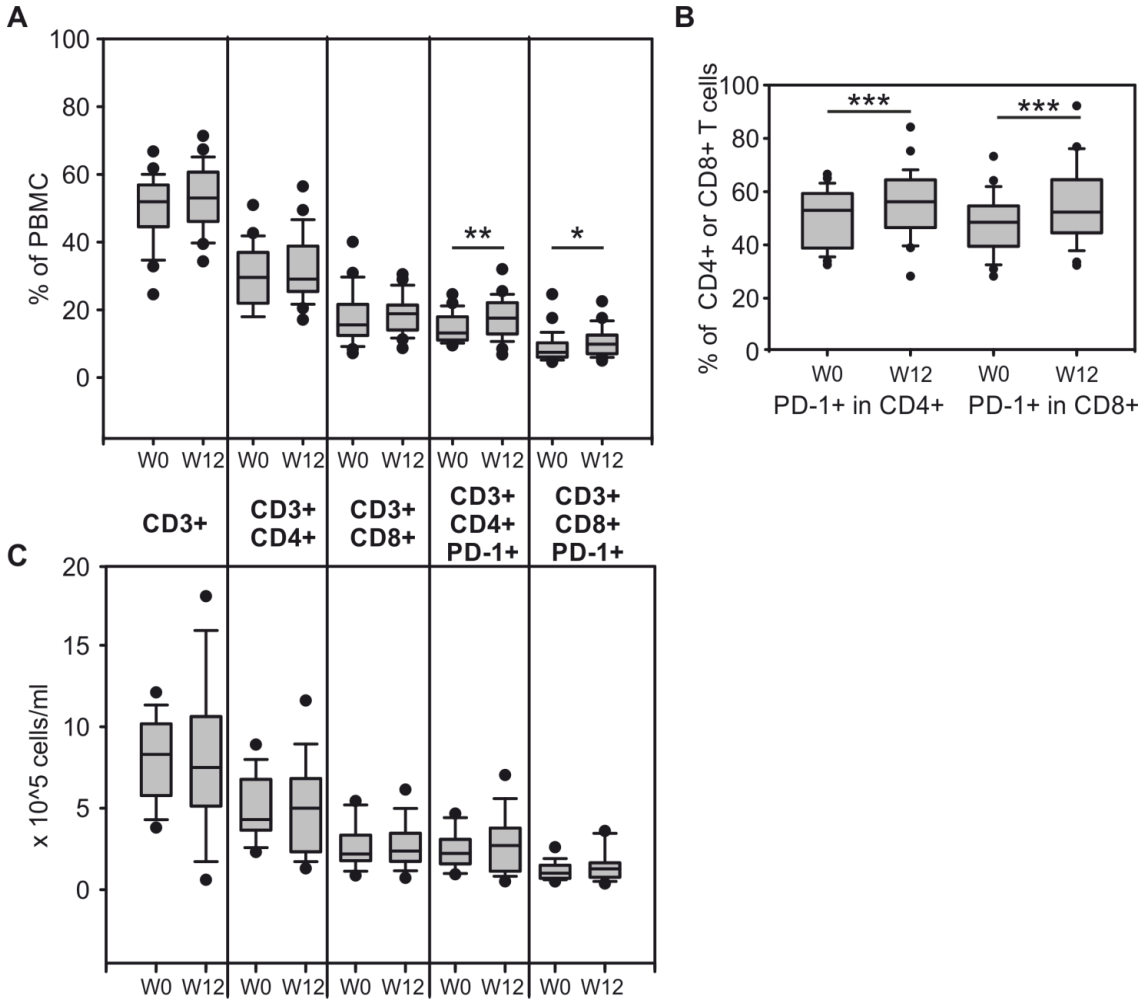


Figure 12: Changes of T cell subsets following ipilimumab treatment

Frequencies and absolute numbers of T cell subsets monitored in melanoma patients at W0 (n=29 for percentages and n=16 for absolute count) and at W12 (n=29 for percentages and n=16 for absolute count). Panel A shows the frequencies of T cell subsets referred to PBMC. Panel B shows the frequency of PD-1$^+$ cells referred to CD4$^+$ or CD8$^+$ subsets while the absolute count of T cell subsets are reported in panel C. Each box plot shows first and third quartiles and median values of the T cell subset indicated at the top of the plot. Outlier are plotted as individual points. Wilcoxon Signed Rank test was used to compare the frequencies of T cell subsets in melanoma patients at different time-points (* P<0.05, ** P<0.01, *** P<0.001). Values were considered statistically significant for P<0.05.

We monitored also the potential effect of ipilimumab on hematological parameters, but the levels of these serum proteins remained stable upon treatment with the exception of a significant increase in circulating S100B (Fig 13, P=0.036).

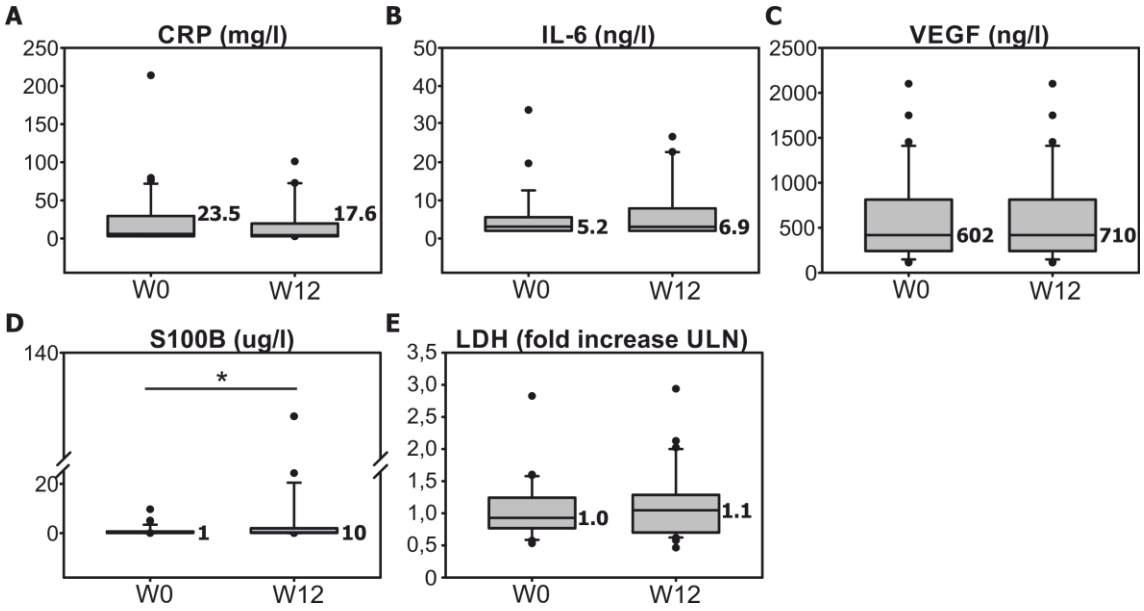


Figure 13: Evaluation of hematological parameter during ipilimumab treatment
Serum levels of proteins at W0 (n=37 to 34 depending on the considered parameter) and at W12 (n= 31 to 29 depending on the considered parameter). Each box plot shows first and third quartiles and median values of the hematological parameter indicated at the top of the plot. Outlier are plotted as individual points. Wilcoxon Signed Rank test was used to compare levels of hematological parameter in melanoma patients at different time-points (* P<0.05). Values were considered statistically significant for P<0.05.

Overall, ipilimumab treatment did not alter significantly the immune profile of the patients in this study with the exception of a significant up-regulation of PD-1 on both $CD4^+$ and $CD8^+$ T cell subsets and an increase in serum levels of S100B.

Identification of early predictors of toxicity

It has been demonstrated that ipilimumab treatment is associated with a toxicity profile mainly due to irADR consequent to an impairment of tolerance towards self-antigens[173]. We investigated whether toxicity was correlated with tumor-associated and immunological parameters (TIPs), like myeloid subsets, T cell subsets and hematological parameters. To this end, we grouped the patients on the basis of development or absence of grade 3 immune-related adverse reactions (ADR+ and ADR-) and we compared W0, W12 frequencies and absolute (W12/W0) or relative (W12 minus W0) variations of TIPs among the groups.

We observed that patients with lower incidence of irADR expressed higher levels of PD-1 on $CD4^+$ T (Fig. 14 B P=0.011), however, the presence of irADR was mainly reflected by post-treatment measures of TIPs. In fact, upon treatment, we observed a further up-regulation of PD-1 expression on $CD4^+$ and $CD8^+$ T cells of ADR- patients compared to baseline (Fig. 14 B-D P<0.001 and Fig. 14 C-E P=0.003). Besides, after ipilimumab treatment patients without grade 3 toxicity were

characterised by a significant expansion of $CD3^+$ T cells (Fig. 14 A P=0.006) and by a significant decrease of immature MDSC3 (Fig. 14 F P=0.04). On the contrary, ADR+ patients failed to up-regulate PD-1 expression on T cells, that, in turns, were significantly reduced compared to ADR- patients (Fig. 14 A P=0.05) at W12. The presence of toxicity was associated also to a trend toward lower frequency of eosinophils (Fig. 14 H) and wider variance in MDSC4 (Fig. 14 G) at W12.

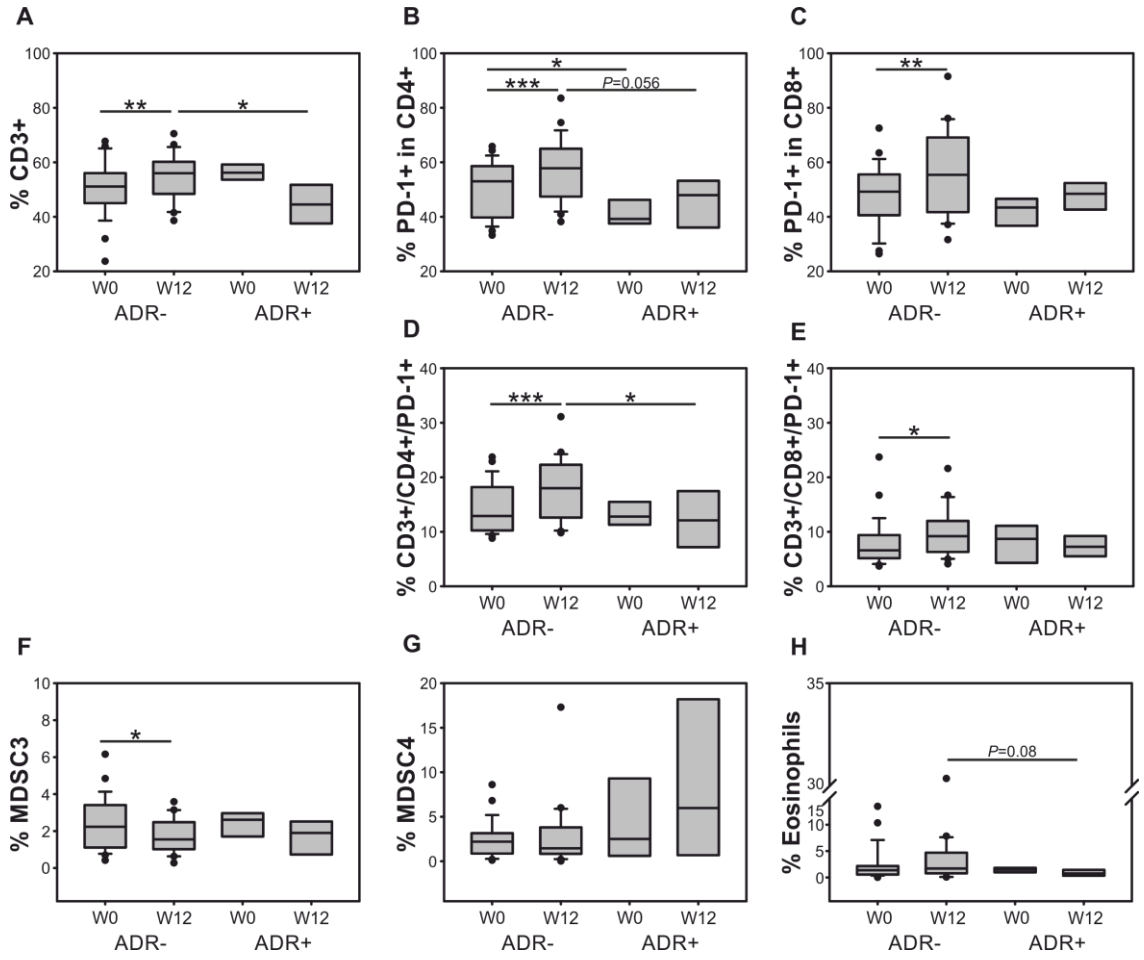


Figure 14: Early predictors of toxicity

Box plots report median, first and third quartiles of TIPs measured at W0 and W12 in patients without ADR- or with ADR+. Outlier are plotted as individual points. Significance of intra-group variations between W0 and W12 values were assed using Wilcoxon Signed Rank Test while Wilcoxon Rank Sum Test was used to compare inter-group variations (* P<0.05, ** P<0.01, *** P<0.001)). Values were considered statistically significant for P<0.05.

Since high grade toxicity is a sign of hyper-activation of the immune system, some authors correlated the development of irADR to efficacy of ipiliumab treatment[205,206]. However, we did not find evidence of association between toxicity and OS (P=1,00 using Fischer Exact Test, P=0.950 using Log-Rank test Fig. 15), in line with a recent report[179].

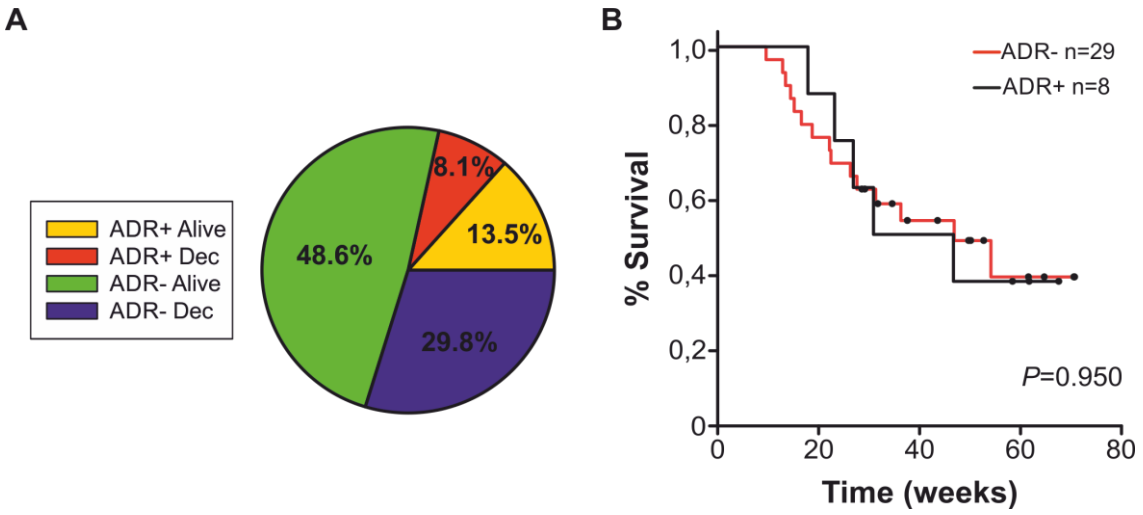


Figure 15: Correlation between immune-mediated toxicity and clinical efficacy of ipilimumab

A) Pie plot represents proportions of ADR+ and ADR- patients according to their survival status at W29 (Dec= deceased). B) Kaplan-Meier estimates of survival for ADR+ and ADR- patients. Values were considered statistically significant for $P<0.05$.

In conclusion, our results indicate that the development of toxicity was not predictive for longer OS in response to ipilimumab, but it is associated with reduced $CD3^+$ T cells, increased variance in MDSC4 and lower frequencies of eosinophils at W12. Interestingly, we observed an also an association between lower toxicity and reduced baseline expression of PD-1 on $CD4^+$ T cells. Besides, ADR- patients expanded T lymphocytes, and in particular PD-1 expressing T cells, upon treatment.

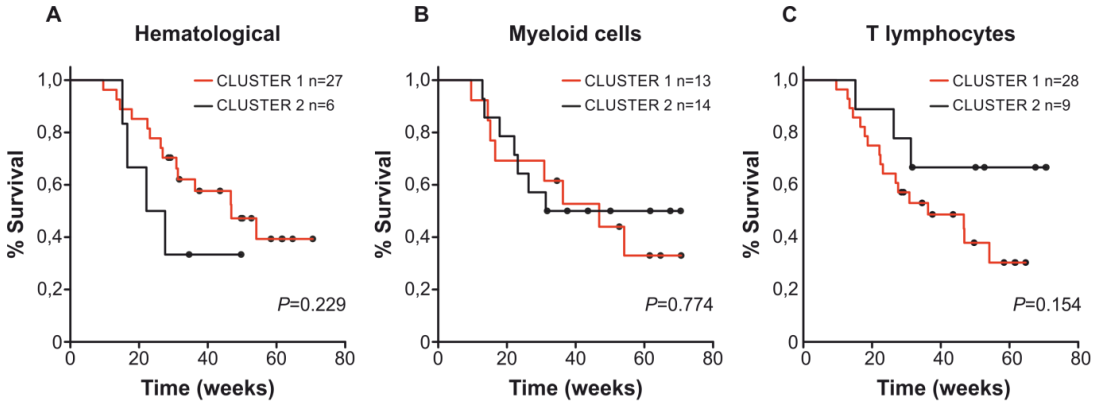
Identification of biomarkers associated with overall survival

Ipilimumab treatment has demonstrated survival advantages in pre-treated metastatic melanoma patients, when compared to gp-100 peptide vaccine[41], but validated predictors of OS are still missing. We exploited our dataset of TIPs, to identify potential biomarkers of OS. We defined OS as the time occurring between baseline and death or last contact with the patient and we planned three different statistical strategies for survival analysis. We first performed an exploratory unsupervised multivariate analysis, dividing the cohort of patients in two clusters, characterized by homogeneous levels of TIPs, and subsequently testing for a difference in OS between the two clusters. The second step was to divide the patients on the basis of their survival status (alive vs deceased) at a homogeneous time-point and test whether there was a difference in the levels of TIPs in the group of alive and deceased patients. In the third step, results of this analysis were further confirmed using univariate survival analysis in which patients were stratified on the basis of the cohort-median value of the TIP under investigation and testing whether a significant difference in OS was present between the two groups.

Cluster analysis of tumor-associated and immunological parameters

In this analysis we considered the frequencies and absolute variations of TIPs at W0 and at W12. Parameters were analysed in three homogeneous groups: hematological values (CRP, IL6, VEGF, S100B), myeloid cells (MDSC1-2-3-4, eosinophils, putative DCs), and T lymphocytes (L1=%CD3$^+$/CD4$^+$, L2=%CD3$^+$/CD8$^+$, L3=%CD3$^+$/CD4$^+$/PD-1$^+$, L4=%CD3$^+$/CD8$^+$/PD-1$^+$, L5=%PD-1$^+$ in CD3$^+$/CD4$^+$, L6=%PD-1$^+$ in CD3$^+$/CD8$^+$, L7=%CD4/%CD8, L8=%CD3$^+$). In each group, two clusters with homogeneous baseline values were identified using Cluster K means algorithm and difference in OS between clusters was tested using the Log-Rank test.

Results of this analysis do not demonstrate significant correlations between clusters of baseline TIPs and OS, a part from a trend towards better prognosis in patients with lower baseline levels of hematological parameters IL-6, CRP, VEGF and S100B and higher levels of T lymphocytes (Fig. 16).



D

	cluster 1	cluster 2	*P*
CRP	4.6	19.5	0.079
IL6	2.6	5.6	0.089
VEGF	329	1427	<0.001
S100B	0.1	1.9	0.027

E

	cluster 1	cluster 2	*P*
MDSC1	6.2	11.4	<0.001
MDSC2	0.8	1.5	0.076
MDSC3	2.3	2.3	0.752
MDSC4	1.4	3.3	0.012
Eosinophils	1.4	1.4	0.423
DC	2.8	2.0	0.085

F

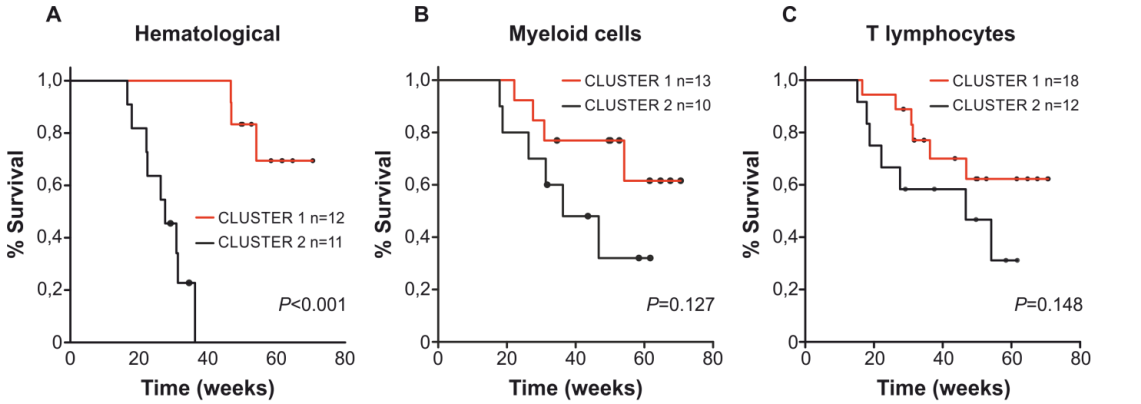
	cluster 1	cluster 2	*P*
L1	32.8	21.8	0.003
L2	18	11.7	0.042
L3	6.7	10.3	0.023
L4	6.7	5.7	0.330
L5	47.3	58.4	0.272
L6	45.9	52	0.096
L7	2.0	1.5	0.873
L8	55	40.3	<0.001

Figure 16: Kaplan-Meier estimates for overall survival

Survival of cluster 1 and 2 using Kaplan-Meier curves in panel A,B,C. Median baseline levels of the indicated TIPs are shown in panels D,E,F. Difference between baseline values of TIPs in the two clusters was tested using Wilcoxon Rank Sum test. Values were considered statistically significant for P<0.05.

On the contrary, when we analysed post-treatment levels, or the variation of TIPs following treatment, we observed significant associations with OS. Indeed, higher absolute variations in hematological parameters, and in particular significantly higher absolute variations of VEGF (Fig. 17 C, *P*=0.013), conveyed a survival advantage (Fig. 17 A, *P*<0.001).

Analogously, lower frequency of putative DCs and MDSC subsets, (significant for MDSC 1-2-4), coupled to an increased presence of eosinophils in the blood of patients following ipilimumab treatment described an immune profile of patients with better prognosis (Fig. 17 B). In particular, the main difference between the two clusters consisted in a significant reduction of the MDSC subsets expanded at baseline (MDSC1-2-4) in patients with longer OS (Fig. 17 E, P<0.001 for MDSC1, P=0.004 for MDSC2, P=0.03 for MDSC4). On the contrary, greater relative variations of T cell subsets upon treatment were associated with prolonged OS (Fig. 17 C). Indeed, the group of patients with better prognosis presented significantly higher $CD3^+$, $CD3^+/CD4^+$, $CD3^+/CD8^+$ T cells and a major up-regulation of $CD4^+$ T lymphocytes expressing PD-1 (Fig. 17 F, P<0.001 for $CD3^+$, P<0.001 for $CD3^+/CD4^+$, P=0.019 for $CD3^+/CD8^+$, P<0.001 for $CD3^+/CD4^+/PD\text{-}1^+$ cells).



D

	cluster 1	cluster 2	*P*
CRP	1	1	0.187
IL6	1.1	1	0.456
VEGF	1.5	0.8	0.013
S100B	2.2	1.3	0.667

E

	cluster 1	cluster 2	*P*
MDSC1	5.9	11.3	<0.001
MDSC2	0.9	4.8	0.004
MDSC3	1.2	1.8	0.163
MDSC4	1.1	5.4	0.030
Eosinophils	1.4	0.9	0.515
DC	0.8	1.9	0.598

F

	cluster 1	cluster 2	*P*
L1	5.1	-3.9	<0.001
L2	3.2	-2.3	0.019
L3	5.7	-0.2	<0.001
L4	2.3	-0.3	0.09
L5	6.2	6.1	0.916
L6	5.6	8.7	0.472
L7	0.1	-0.1	0.409
L8	7.5	-5.1	<0.001

Figure 17: Kaplan-Meier estimates for overall survival

Absolute variations of hematological parameters (A-D), post-treatment levels for myeloid subsets (B-E) and relative variations of T lymphocytes (C-F) were considered for cluster analysis. Kaplan-Meier curves are referred to OS of the two clusters shown in panels A-B-C, while panels D-E-F reported median variations of the indicated TIPs. Difference between TIPs in the two clusters was tested using Wilcoxon Rank Sum test. Values were considered statistically significant for P<0.05.

Univariate survival analysis

To identify potential TIPs representing early predictors of OS, we performed a univariate analysis. We thus divided the patients in two groups, alive or deceased at W29; we considered this time interval as homogenous because it represents the minimum period of follow-up of the cohort following ipilimumab treatment. For each group, we compared W0, W12, the relative or absolute

variations of TIPs in the two groups. When a trend toward association between a parameter and the survival status was identified, we confirmed the result by testing the difference between OS of patients characterized by values below or above the median of the group for that parameter.

This strategy revealed some interesting associations between TIPs and OS and the description of results will be divided in three parts accounting for the three main classes of TIPs: haematological parameters, T cell subsets and myeloid subsets.

Haematological parameters

When we considered parameters involved in inflammatory conditions like CRP and IL-6, we found that patients deceased at W29 presented significantly higher levels of CRP post-treatment (Fig. 18 A, *P*=0.023). Albeit not statistically significant, the same tendency was present also considering baseline CRP values (Fig. 18 A, *P*=0.058). Indeed, survival analysis confirmed the tendency towards a worse prognosis for patients presenting with CRP levels above the median of the cohort at baseline (Fig. 18 B). In the same way, we detected a significant association between both W0 and W12 levels of IL-6 and prognosis (Fig. 18 D, *P*= 0.015 and *P*= 0.011, respectively). The survival analysis confirmed that lower levels of IL-6 in the serum are strongly predictive of longer OS in patients treated with ipilimumab (Fig. 18 E-F, *P*=0.006 baseline, *P*=0.041 post-treatment).

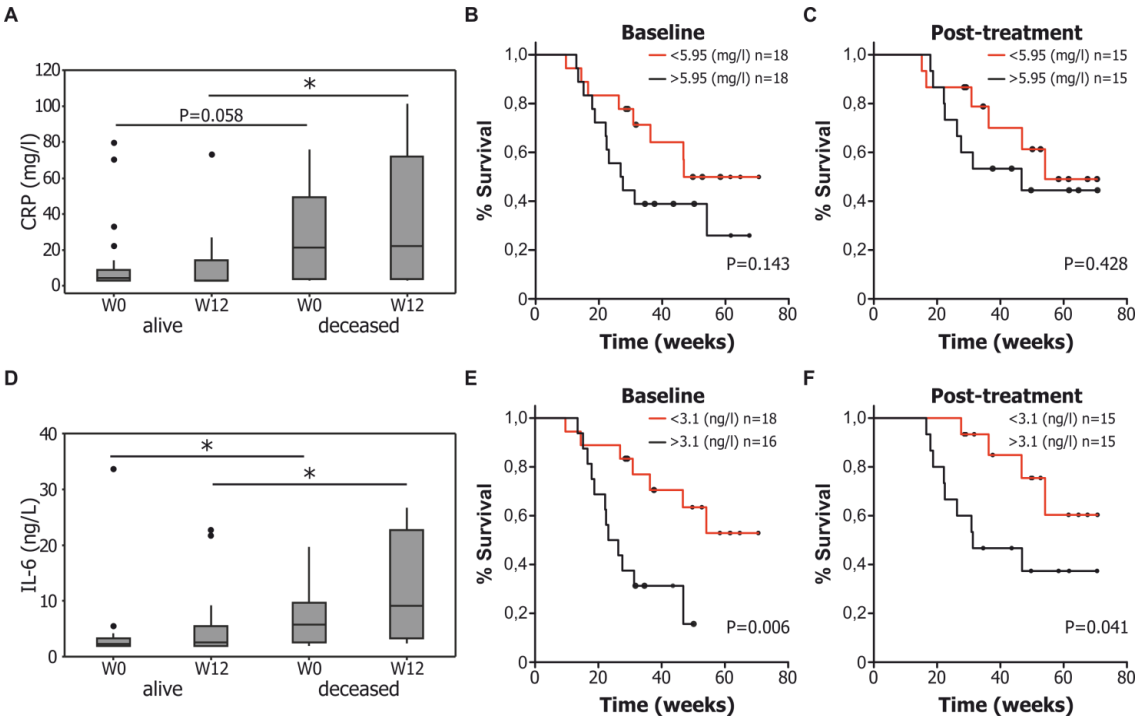


Figure 18: Survival estimates according to levels of CRP and IL-6
W0 and W12 values of CRP and IL-6 are reported in panels A and D, dividing the cohort of patients in two groups on the basis of the survival status at W29. Box plot report median, first and third quartiles. Outlier are plotted as individual points. Wilcoxon Rank Sum Test was used to compare inter-group

variations (* *P*<0.05). Kaplan-Meier curves referred to survival analysis are reported in panels B-C-E-F. Panels A-B-C show association between OS and CRP levels while panels C-D-E depict results about IL-6. Values were considered statistically significant for *P*<0.05.

We then correlated OS with B-RAF mutations, LDH, S100B and VEGF. Patients were divided in two groups on the basis of: i) presence (B-RAF mut) or absence (WT) of mutations in V600 codon of the B-RAF gene; ii) LDH above or below the median value of the cohort at W0; iii) S100B above or below the median value of the cohort at W12; iii) ECOG PS below or above 1. Results indicate that the presence of B-RAF mutations or elevated levels of LDH do not affect OS (Fig. 19 A, B), while patients with reduced levels of S100B after ipilimumab treatment have a trend toward longer OS (Fig. 19 C). As expected, the ECOG performance status is a good predictor of OS (Fig.19 F, *P*=0.01).

Besides, we tested whether a significant difference was present between W0 and W12 levels or relative variations of VEGF between patients alive or deceases at week 29. The pattern of association between VEGF and survival is peculiar; in fact patients with lower baseline levels of this protein have a tendency toward a better prognosis (Fig. 19 D), but when we considered the relative variation of this parameter, we found that increasing levels of VEGF at W12 were associated with longer survival (Fig. 19 E). Hence, the involvement of serum VEGF levels in prediction of survival must be further investigated.

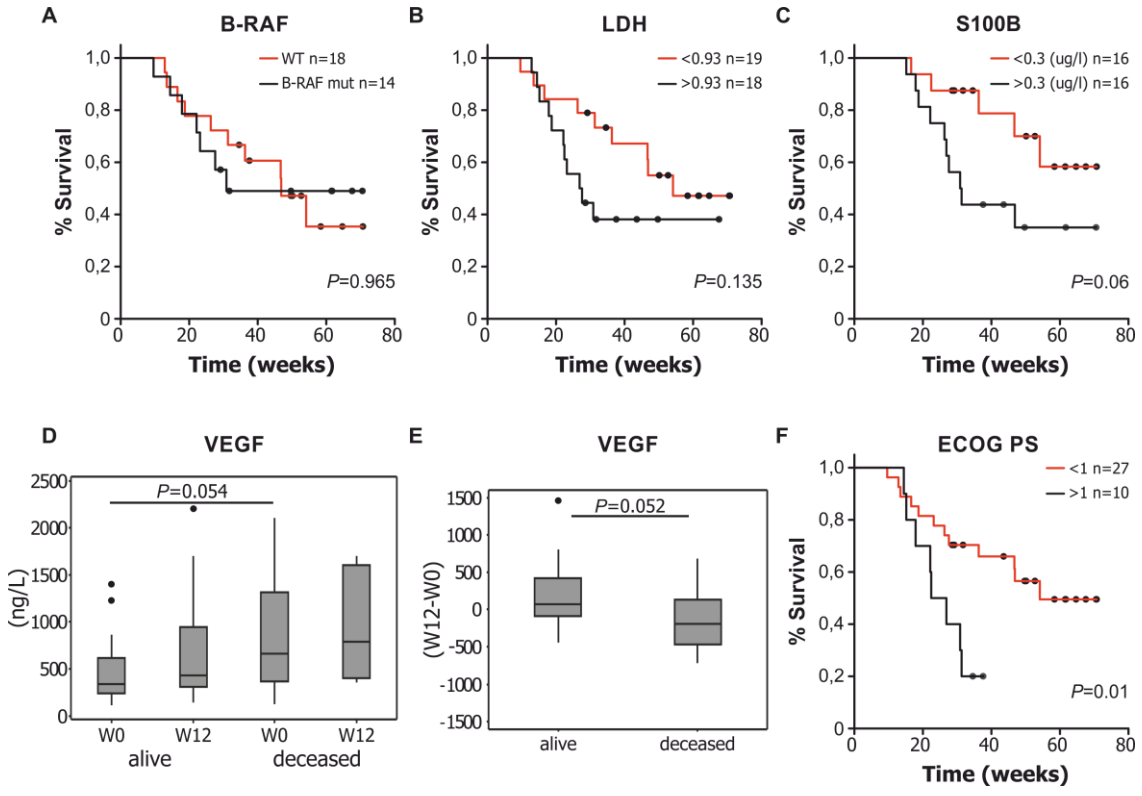


Figure 19: Survival estimates according to the clinical characteristics of the patient
A-B-C-F) Kaplan-Meier estimates for OS according to mutational status of BRAF, LDH at W0, S100B at W12 and ECOG PS are shown. D-E) W0, W12 levels and relative variations of VEGF in patients alive or

deceased at W29 are shown. Box plots report median, first and third quartiles. Outlier are plotted as individual points. Wilcoxon Rank Sum Test was used to compare inter-group variations. Values were considered statistically significant for $P<0.05$.

T cell subsets

Ipilimumab releases the inhibitory signals triggered by CTLA-4 on T cells and it is currently believed that it activates tumor-specific T cells, whenever they are pre-existing (Snyder NEJM 2014). We divided the cohort in two groups according to the survival status at W29. According to this analysis, we observed a significant up-regulation of $CD3^+$ T cells frequency in patients alive at W29 (Fig. 20 A, $P=0.023$) while survival was not associated with a preferential expansion of $CD4^+$ or $CD8^+$ T cells (data not shown), but rather to PD-1 frequencies on the two T cell subsets. Indeed, after treatment, PD-1 expression was significantly up-regulated on $CD4^+$ and $CD8^+$ T cells of patients alive at W29 (Fig. 20 C $P=0.003$, Fig 20 E $P=0.013$) while patients deceased at same time-point had stable PD-1 expression on $CD4^+$ cells but statistically increased frequencies of $PD\text{-}1^+$ cells within $CD8^+$ T lymphocytes (Fig. 20 E, $P=0.031$). We further confirmed these results using Log-rank survival analysis comparing the difference in terms of OS in patients presenting levels of T cell subsets below or above the median value of the cohort. Univariate survival analysis confirmed the association between increased number of $CD3^+$ T cells and OS and it also demonstrated that an increased number of $CD3^+/CD4^+/PD\text{-}1^+$ cells is associated with longer OS (Fig. 20 D P=0.05), while a higher absolute variation of PD-1 expression in the $CD8^+$ counterpart predicted a worse prognosis (Fig. 20 F $P=0.026$).

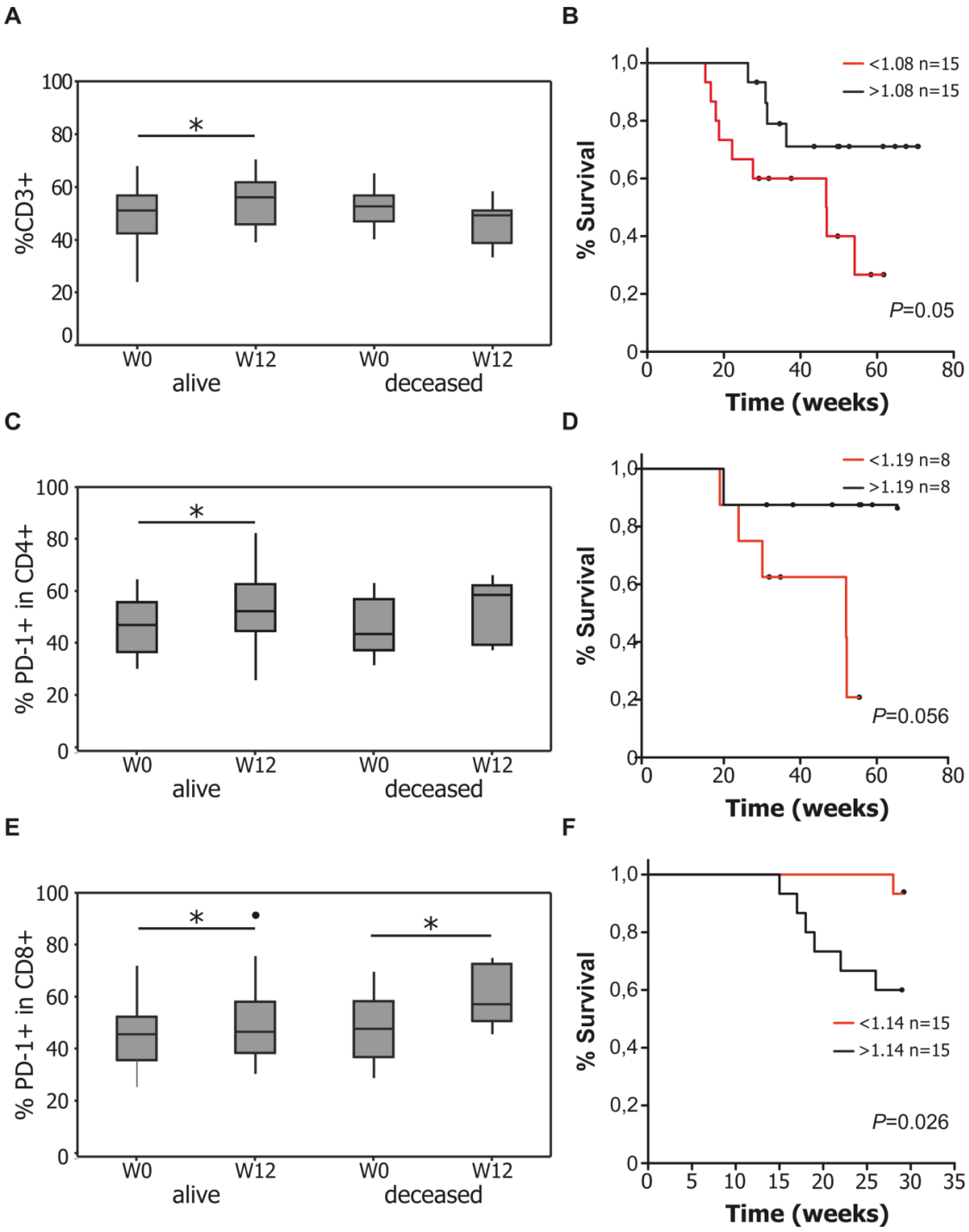


Figure 20: Changes in T cell subsets during ipilimumab treatment and association with OS

W0 and W12 frequencies of T cell subsets are reported in panels A, C and E according to the survival status of patients at W29. Box plot report median, first and third quartiles. Outlier are plotted as individual points. Significance of intra-group variations between baseline and post-treatment values were assed using Wilcoxon Signed Rank Test (* P<0.05). Panels B-C-E) Kaplan-Meier curves estimating OS on the base of absolute variation of: $CD3^+$ cell number (B), $CD3^+/CD4^+/PD\text{-}1^+$ cell number (D), and frequency of $PD\text{-}1^+$ cells within $CD8^+$ T lymphocytes (E). Values were considered statistically significant for $P<0.05$.

Myeloid-Derived Suppressor Cells

We and others demonstrated that MDSC levels correlate with OS in cancer patients[78,94,207] (reviewed in [63]); We thus explored the potential association between levels of myeloid subsets and OS in ipilimumab-treated patients. To this end we considered W0, W12, absolute and relative variation of myeloid subsets and we divided the cohort of patients in two groups according to the median value of the parameter under investigation. Difference in OS between the two groups was tested using the Log-Rank test and reported using Kaplan-Meier curves (Fig. 21)

Two out of 3 MDSC subsets expanded at baseline were significantly associated with OS (Fig. 21 A-B, *P*=0.024 for MDSC1, *P*=0.049 for MDSC2). On the contrary, the association between MDSC4 and survival stemming from cluster analysis (Fig. 17) was not confirmed by univariate survival analysis (*P*=0.445, data not shown).

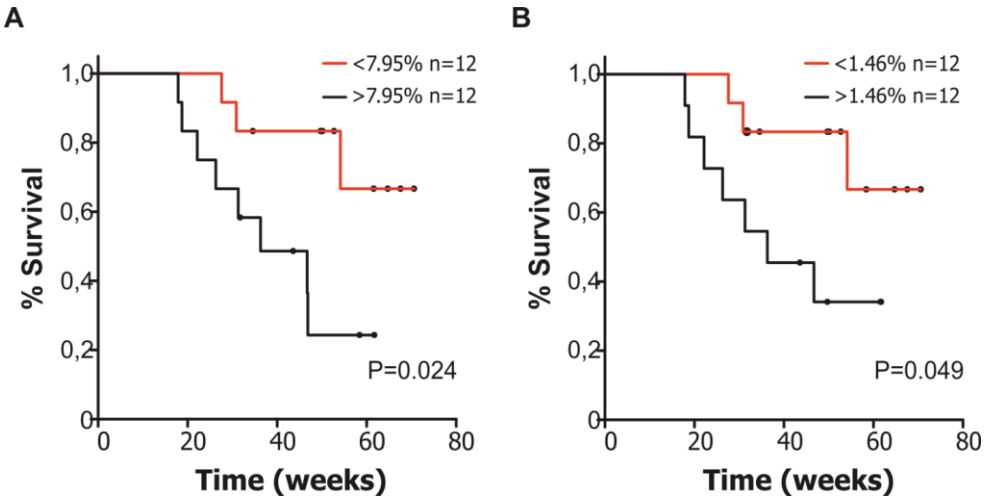


Figure 21: Kaplan Meier estimates for overall survival according to post-treatment frequencies of MDSCs

Survival curves of patients with levels of MDSC1 (A) and MDSC2 (B) below or above the median of the cohort at W12. Values were considered statistically significant for *P*<0.05.

In conclusion, our results indicate that OS is associated with some immunological parameters of melanoma-treated patients. In fact, multivariate clustering analysis revealed multiple associations between survival and hematological parameters, myeloid and T cell subsets. These results were confirmed by univariate analysis and we identified two immunological profiles with opposite prognosis: lower baseline levels of CRP, IL-6, a better performance status and an expansion of $CD3^+$ and $CD4^+$/$PD\text{-}1^+$ T cells post-treatment characterize patients with prolonged OS. On the other hand, patients with higher frequencies of MDSC1, MDSC2, and $CD8^+$/$PD\text{-}1^+$ T cells at W12 had significantly reduced OS.

Correlation between immune parameters predictors of survival.

To assess whether early predictors of survival are inter-connected, we correlated baseline and post-treatment levels of such TIPs using the Spearman test.

We found significant correlations within each class of TIPs, and results are shown in Table 4 and Figure 22. For example, IL-6 and CRP, two proteins associated with inflammation, were tightly connected both at baseline and post-treatment (P<0.001 W0, P=0.007 W12). CRP and IL-6 levels significantly correlated also with VEGF levels (P= 0.001 and P=0.002, respectively). We also observed a strong correlation between MDSC1 and MDSC2 (P=0.027 W0, P=0.0006 W12), which similarly express IL4Rα.

Interestingly, significant correlations were present also between different classes of TIPs. For example, there was a striking correlation between baseline levels of CRP and the three subsets of MDSCs expanded at baseline in melanoma patients, i.e. MDSC1-2-4 (Fig. 22 A-B-C, P<0.001 for MDSC1-2-4). This association was not statistically significant for other myeloid subsets, and for MDSC3, which were not expanded by these patients at baseline (Table 4). Analogously, baseline levels of IL-6 are significantly correlated with MDSCs expansion, in particular with MDSC1 and MDSC4 (Fig. 22 D-E, P<0.001 for both MDSC subsets) and the association with MDSC1 was maintained also post-treatment (Fig. 22 F P=0.013). Finally, the T cell compartment was not significantly linked to serum proteins, but we observed a significant inverse correlation between MDSC4 and $CD8^+$ T cells which was present both at baseline and post-treatment (Table 3, P=0.009 W0 and P=0.026 W12).

TIME-POINT	1ST VARIABLE	2ND VARIABLE	R	*P*
BASELINE	CRP	IL-6	0,753	0,0000002
	CRP	VEGF	0.525	0.0011
	CRP	MDSC1	0.661	0.000114
	CRP	MDSC2	0.614	0.000871
	CRP	MDSC3	-0.212	0.241
	CRP	MDSC4	0.566	0.000785
	CRP	Eosinophils	-0.269	0.135
	CRP	Lin-/HLA-DR-/CD33dim	-0.267	0.138
	IL-6	VEGF	0.512	0.00211
	IL-6	MDSC1	0.690	0.000067
	IL-6	MDSC2	0.318	0.128
	IL-6	MDSC3	-0.020	0.912
	IL-6	MDSC4	0.589	0.00066
	IL-6	Eosinophils	-0.212	0.257
	IL-6	Lin-/HLA-DR-/CD33dim	-0.125	0.506
	MDSC1	MDSC2	0.425	0.027
	MDSC4	CD3+/CD8+ T cells	-0.449	0.00904
POST-TREATMENT	CRP	IL-6	0.488	0.00745
	IL-6	MDSC1	0.498	0.013
	MDSC1	MDSC2	0.648	0.000608
	MDSC4	CD3+/CD8+ T cells	-0.436	0.0261

Table 4: Correlation between levels of tumor-associated and immunological parameters
Baseline and post-treatment levels of TIPs were correlated using Spearman Correlation. The two variable undergoing the correlation test are indicated as "1st variable" and 2nd variable". Values were considered statistically significant for $P<0.05$.

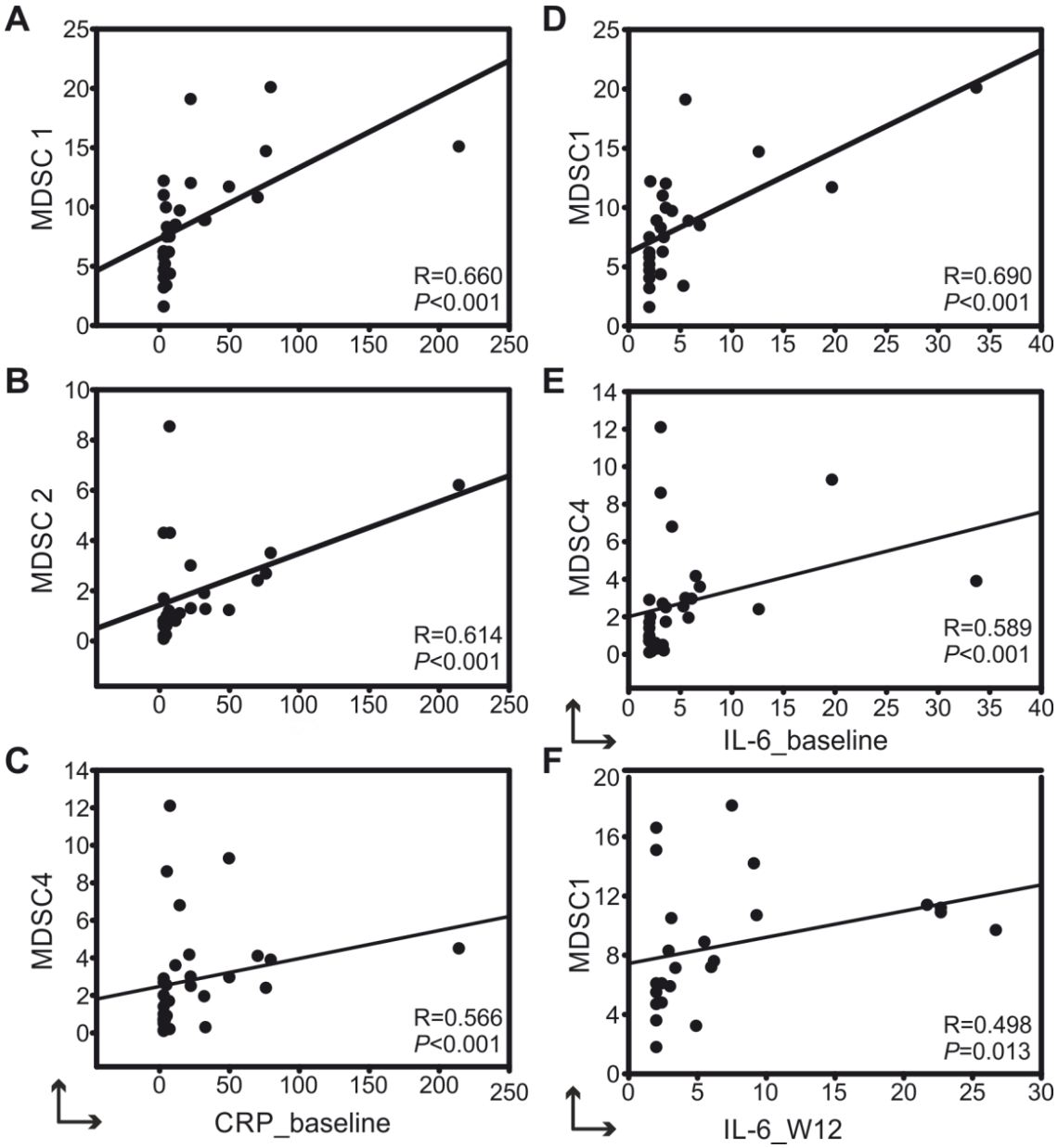


Figure 22: Correlation between serum proteins and MDSCs

Spearman Correlation was used to assess correlation between CRP levels and frequencies of MDSC1 (A), MDSC2 (B) and MDSC4 (C) at W0; IL-6 levels and frequencies of MDSC1 (D) or MDSC4 (E) at W0; IL-6 levels and MDSC1 frequencies at W12. R, coefficient of correlation. Values were considered statistically significant for P<0.05.

The results from this correlation analysis revealed a net of connections that could open new working hypothesis. For example, the correlations between IL-6, CRP, MDSC1 and MDSC2 suggest a possible connection between cancer-associated inflammation and expansion of MDSCs and it is of particular interest if we consider that these four parameters are among the strongest predictors of OS identified by this study.

DISCUSSION

Ipilimumab was the first ICI showing an OS benefit in metastatic melanoma and a number of studies demonstrated the predictive role of immunological parameters as surrogate biomarkers of clinical response to this treatment (reviewed in [175]). Besides, the identification of predictive and pharmacodynamic biomarkers is fundamental to select patients with higher probability of response to this expensive treatment, also in the view of the promising effect of combined approaches using PD-1 and CTLA-4 blockade. Circulating levels of MDSCs have been correlated to tumor burden and OS in different types of cancer, and some studies demonstrated their prognostic role for the outcomes of different chemotherapeutic regimens (reviewed in [63]), thus becoming a promising biomarker also for response to immunotherapy. However, the validation of the predictive significance of MDSCs in multicenter studies is complicated by the phenotypic complexity of human MDSCs, thus creating a challenge in finding a consensus on the minimal requirements for MDSC monitoring. To meet this request, we organised the first proficiency panel to harmonize human MDSC phenotyping. Compared to other proficiency panels, the MDSC panel was challenging in terms of number of participants, complexity of the staining panel and number of subsets to be identified. Given these premises, it is not surprising that the quantification of the 10 requested MDSC subsets was characterised by a high inter-laboratory variance, increasing from monocytic to immature and granulocytic subsets. The number and the international origin of the participating laboratories indicate that the high inter-laboratory variance observed in the panel could be similarly implied also in studies on human MDSC published. On the contrary, the intra-laboratory variance was acceptable indicating that the participating laboratories were experienced in the field of multicolour flow cytometry.

We identified three critical parameters that impacted on the quantification of MDSC phenotyping: the choice of reagents, the use of a DCM and the gating strategy. As staining reagents were relatively free of choice, a considerable number of different antibodies were used to identify MDSC subsets using an 8-color staining panel. As a result, we observed that the clone of anti-CD124 antibody and the composition of the Lineage cocktail significantly influenced the quantification of $CD124^+$ MDSCs and immature MDSCs, respectively. Indeed, this problem was also identified by previous proficiency panels in which a partial standardization of reagents was suggested[194], especially for culturing of PBMCs intended to functional T cell assays[193,208,209].

The MDSC proficiency panel introduced the use of a DCM, and we observed that the quantification of several MDSC subsets, and in particular of granulocytic ones, was significantly reduced by dead cell exclusion. Hence, these results open the discussion whether it is worth to quantify also dead

MDSCs because they were most likely present and alive in the fresh sample, or whether inclusion of dead cells could alter MDSC quantification due to unspecific binding of antibodies to other cell types.

Besides the choice of the reagents, we found that the gating strategies used for identification of MDSC subsets had a significant influence on the variance of results. Indeed, when we included in the analysis only those laboratories which applied a homogenous gating strategy, similar to that proposed in the panel's guidelines, we observed a significantly improved inter-laboratory performance. This was not unexpected since most of the proficiency panels based on flow cytometry similarly recommended to harmonize the gating strategies across laboratories. In a multimer-based proficiency panel, results were audited in order to exclude wet-laboratories which regularly used a wrong gating procedure[194]. However, since identification of MDSC subsets derive from a complex combination of signals, we believe that a more adequate approach is to train the participating laboratories to perform a properly gating pipeline through an *in-silico* panel, as done in ICS proficiency panels[193,210]. In addition, this *in-silico* approach may be further used by participants as learning module for training operators in order to reduce intra-laboratory variance.

Overall, we demonstrated that it is feasible to conduce a proficiency panel including such a large number of participants and identifying a high number of myeloid subsets using 8-color flow cytometry. In this first step, we provided individual feedback of performance for each laboratory and we identified a number of parameters that must be harmonized in the second step, hence establishing the foundation for the development of a robust assay for MDSC phenotyping.

From this experience, we developed a method to reduce inter-assay variance of MDSC phenotyping and we used this standardized approach to monitor the circulating levels of four MDSC subsets in melanoma patients receiving ipilimumab. Several non-overlapping MDSC phenotypes have been described[198], classified as immature, monocytic and granulocytic subsets, but most of the studies reduced MDSC monitoring to only one phenotype. We believe that all the subsets should be investigated in clinical studies for a complete overview of MDSC expansion, and the results from the first phase of the proficiency panel demonstrated that this is a feasible objective. To date, only the study by Walter and colleagues monitored six MDSC subsets simultaneously in renal cell carcinoma patients, and found that five out of six subsets were significantly expanded in the blood and, moreover, that the levels of two out of the six subsets were negatively associated with OS in response to a multipeptide-based vaccination protocol[94]. In line with these results, we observed that also melanoma patients significantly expanded more than one MDSC subset, thus suggesting the presence of immune suppression in these patients. In

particular, the cohort under investigation expanded three MDSC subsets (MDSC1-2-4) which were originally discovered in melanoma patients and further described also in other types of cancer (reviewed in [63]). In addition, in a previous study we demonstrated that the expression of IL4Rα on monocytes (MDSC1) of cancer patients was directly correlated with inhibition of T cell proliferation[70]. Evidence form mouse models indicated that IL4Rα is involved in MDSC function[69,71] and survival[69], but the functional role of this marker in human MDSCs in still under investigation.

Recently Kitano and colleagues addressed the problem of standardization of MDSCs, and developed a computational algorithm-based method to perform a uniform analysis of the levels of MDSC4 in melanoma patients treated with two different doses of ipilimumab (3mg/kg or 10mg/kg)[176]. This was the first study reporting a significant association between baseline levels of MDSC4 and OS, independently of LDH levels and absolute lymphocytes count, but the prognostic significance of MDSCs was restricted only to patients receiving the higher dose of ipilimumab. In our study, in which ipilimumab was administered at 3 mg/kg, we did not observed any association between baseline levels of MDSCs and OS. Nevertheless, we identified, by cluster analysis, that post-treatment levels of MDSC4 were significantly reduced in the group of patients with longer survival and that a significant inverse correlation between these cells and $CD8^+$ T cells was present at W0 as well as at W12. A similar inverse correlation was observed, in two independent studies, both in non-treated lung cancer patients[211] and in melanoma patients receiving ipilimumab[176].These findings probably reflect the balance between suppressive and effector leukocytes characterising the immunological profile of cancer patients, and ipilimumab could alter this equilibrium by boosting T cell responses which, in turns, contrast the action of immune suppression exerted by MDSCs.

This is not the first evidence of a prognostic role for MDSCs, in fact the levels of MDSCs have been associated to OS and response to different chemotherapeutic regimens both at baseline and post-treatment[77,78,88,93,207,211,212]. However, in view of the immune-mediated effect of ipilimumab, lower levels of suppressive cells could represent not only an estimator of clinical benefit but also a pharmacodynamic biomarker, reflecting the shift from immune escape to immune-response. In fact, using a multivariate non-parametric statistical approach, we demonstrated that a survival benefit can be estimated on the base of decreasing levels of monocytic and granulocytic MDSCs, expressing IL4Rα (MDSC1 and MDSC2) and a parallel increase in the number of T cells, which indeed may reflect the conversion from escape to rejection phase. The role of MDSCs as a biomarker for ipilimumab treatment was demonstrated in a number of studies, mostly reporting correlations between MDSC4 and clinical response[181,191,213]. Only one study monitored

granulocytic MDSC in ipilimumab-treated patients describing a significant reduction of the granulocytic subset coupled with down-regulation of Arginase-1 expression on the whole myeloid compartment[190].

Given the reliability of MDSCs as biomarker of clinical outcomes to ipilimumab treatment, it remains to be clarified whether ipilimumab targets MDSCs or, conversely, if the decreasing levels of MDSCs observed following ipilimumab treatment simply reflect the shrinkage of the tumor in response to immune rejection. Even if a direct evidence of CTLA-4 expression by MDSCs is still missing, we believe that it would be important to understand the possible direct targeting of MDSCs by anti-CTLA-4, and different intriguing hypothesis could be derived by the experience matured on DCs. Indeed, from one side, CTLA-4 expressing DCs showed an impaired maturation profile, reduced stimulatory function[214] and produced IL-10 [215], as MDSCs. On the other side, CTLA-4 could act on DCs also by reverse signalling through CD80/CD86. Grohmann and colleagues demonstrated that CD80/CD86 ligation by CTLA-4-Ig induced IDO expression in DCs and a consequent functional impairment of T cells[216]; Since IDO has a role in the mechanism of MDSC suppression[85], the reverse signalling described for DCs could be active also in these cells and, according to this hypothesis, ipilimumab would relieve T cell activation both following a T-cell intrinsic pathway and through reduction of the suppressive function of MDSCs.

Another strategy to track the onset of immune rejection triggered by ipilimumab is to monitor the activation and functional status of T cells. In fact, absolute lymphocyte count is one of the first parameter identified as strongly associated to clinical effect of ipilimumab[176-179,183,188], and many studies characterized the immune correlates of this drug. As expected, T cells of responding patients displayed an activated phenotype, expressing ICOS or HLA-DR[186,187,217], increased antigen specific responses[180,187] and boosted IFN-γ production which was correlated to a Th1-associated signature at the tumor site[182]. However, less is known about the expression of other immune checkpoint molecules in response to the blockade of CTLA-4. We also monitored the levels of PD-1 expression on T cells in response to ipilimumab and we found that melanoma patients expressed significantly higher levels of PD-1 in the $CD4^+$ compartment at baseline. Moreover, T cells further up-regulated PD1 on both $CD4^+$ and $CD8^+$ T cell subsets upon treatment. Of note, post-treatment variation of PD-1 expression on the two subsets was differently associated with OS: in fact, patients with longer OS increased the absolute count of $CD4^+/PD\text{-}1^+$ T cells during the treatment, while those with higher expression of PD-1 on the $CD8^+$ counterpart had a reduced OS.

Only one study investigated the expression of PD-1 on T cells during ipilimumab, and it showed an up-regulation of this marker on T cells after the first dose, followed by a sudden down-regulation

of PD-1 expression at the end of the treatment. Interestingly, also the relative frequencies of Tregs were evaluated in this study, and the variation of these cells had the same trend of expression of PD-1 on T lymphocytes[190].

Since in our cohort of patients the expansion of $CD4^+/PD\text{-}1^+$ T cells was associated with reduced toxicity, we speculated that increasing frequencies of these cells was due to an expansion of Tregs. This hypothesis is based on the fact that PD-1 and CTLA-4 play an important role in Treg induction and functional activity[163,218]. In addition, the constitutive expression of CTLA-4 on Tregs suggests a potential effect of ipilimumab on this cell subset[145]. However, this hypothesis is only partially supported by evidence from the literature because the presence of Tregs was monitored in ipilimumab-treated patients with conflicting results which make the predictive power of Treg not extensively reliable[177,181,183,190].

On the contrary, the association between higher PD-1 expression on $CD8^+$ T cells and reduced OS suggests the induction of an exhausted phenotype on cytotoxic T cells. However, in a recent report PD-1 and Lag-3 expression were significantly down-regulated following blockade of PD-1 and/or CTLA-4 on the surface of $CD8^+$ T cells specific for antigenic mutant epitopes driving the immune-rejection of the tumor[99]. Hence, further characterisation of $CD8^+/PD\text{-}1^+$ cells is needed because PD-1 expression alone does not permit to define unambiguously an exhausted cell, which is indeed characterised by the expression of multiple inhibitory checkpoint molecules like Tim-3, Lag-3, BTLA-4[21].

In addition to circulating MDSC and T cell subsets, we observed that the pro-inflammatory cytokine IL-6 was a strong estimator of OS in response to ipilimumab, while a trend toward association was present between survival and other markers of inflammation like VEGF and CRP. Given the predictive and prognostic power of inflammatory proteins and MDSCs described so far, it is important to consider that baseline levels of these parameters were highly interconnected, thus supporting the existence of an immunological loop associated with OS in response to ipilimumab. In fact, CRP and IL-6 significantly correlated with the circulating levels of the MDSC subsets expanded at baseline (MDSC1/MDSC2/MDSC4). These correlations suggest the presence of a suppressive net at the tumor site, spreading to the circulation, in which inflammatory proteins induce MDSCs and regulate their suppressive functions as previously demonstrated in murine models[219] (reviewed in [25]) and in human cells *in-vitro* [32,220]. In addition, the down-stream mediator of IL-6 signalling, signal transducer and activator of transcription 3 (STAT-3), regulated the suppressive activity of tumor-infiltrating MDSC from head and neck squamous cell carcinoma patients through modulation of arginase 1 activity[221]. The connection observed in our study

between IL-6, MDSC levels and OS suggests the hypothesis of a combination therapy of anti-IL-6 mAb and immune-checkpoint inhibitors. From one side, IL-6 blockade could enhance the efficacy of immune checkpoint inhibitors by disrupting the mechanisms of immune escape at the tumor site, and from the other side, it could also reduce the toxicity of this treatment, which is characterised by autoimmune responses. Indeed, blockade of IL-6 pathway is currently approved for the treatment of autoimmune diseases, like rheumatoid arthritis, and combinations of anti-IL-6 and chemotherapy are already under development in phase I studies for cancer treatment (reviewed in [222]).

In addition to prediction of survival, the present study was also designed to identify an immune profile associated with the development of irADR due to ipilimumab treatment. Albeit early reports indicated that the onset of irADR was prognostic of response to ipilimumab[205,206], the potential link between irADR and clinical benefit awaits further confirmation from large perspective studies. Our results indicate that the development of toxicity was not predictive for longer OS in response to ipilimumab. However in our study, toxicity was associated with a defined immune profile characterised by reduced $CD3^+$ T cells, increased variance in MDSC4 and lower frequencies of eosinophils. In addition, patients with lower toxicity significantly expanded $PD\text{-}1^+$ T lymphocytes compared to those experiencing grade 3 irADR. As the prediction of toxicity was less investigated in clinical studies, our results constitute a first attempt to characterise the immune profile of patients prone to toxicity and are supported only by the study of Wang and colleagues. In this study, Authors observed that the onset of irADR was associated with lower frequencies of proliferating, $Ki67^+$, CD4 and CD8 T lymphocytes[185]. In particular, patients free from toxicity expanded a subset of $CD4^+$ T cells expressing eomesodermin, a transcription factor that regulates the generation of memory T cells[223] and, similarly to our results, was associated to increased PD-1 expression[224].

In conclusion, this study explored different aspects of immunomonitoring of ipilimumab-treated melanoma patients and it constitutes from one side a preparatory step for the development of a robust assay for MDSC identification, and from the other side it defines the immune profile of patients that most likely benefit from ipilimumab treatment. Our results should be further validated in an independent cohort of ipilimumab-treated patients, nevertheless they constitute an important first step towards identification of biomarkers that improve the clinical use of immune checkpoint inhibitors.

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APPENDIX 1

PUBLICATIONS AS FIRST AUTHOR

Damuzzo V., Solito S., Pinton L., Pigozzo J., Chiarion-Sileni V., Bronte V., Mandruzzato S.; Decreasing levels of IL4Rα+ CD14+ myeloid derived suppressor cells are associated with clinical response to Ipilimumab in melanoma patients, 11th CIMT Annual meeting 2013; (poster).

Damuzzo V., Solito S., Pinton L., Pigozzo J., Chiarion-Sileni V., Bronte V., Mandruzzato S.; Decreasing levels of IL4Rα+ CD14+ myeloid derived suppressor cells are associated with clinical response to Ipilimumab in melanoma patients, 2nd International Graduate Student Immunology Conference 2013; (poster).

Damuzzo V., Solito S., Pinton L., Valpione S., Pigozzo J., Chiarion-Sileni V., Bronte V., Mandruzzato S, Immunomonitoring of circulating Myeloid-Derived Suppressor Cells as a possible predictive tool for response to Ipilimumab treatment in melanoma patients, 12th CIMT Annual meeting 2014; (poster).

Damuzzo V, Pinton L, Desantis G, Solito S, Marigo I, Bronte V, Mandruzzato S., Complexity and challenges in defining myeloid-derived suppressor cells., Cytometry B Clin Cytom. 2014 Nov 26. (peer-reviewed review article).

PUBLICATIONS AS CO-AUTHOR

Solito, S.; Pinton, L.; Damuzzo, V.; Basso, G.; Zanovello, P.; Bronte, V.; Mandruzzato, S.; Analyzing the molecular regulatory pathways at work in human myeloid-derived suppressor cells; SIC Congress 2012; (poster).

Pinton, L.; Solito, S.; Perilli, L.; Sonda, N.; Damuzzo, V.; Oro, D.; Anselmi, A.; Francescato, S.; Zanovello, P.; Bronte, V.; Mandruzzato, S.; Dissecting the role of transcription factors C/EBPβ and STAT3 in MDSC-mediated immune suppression; SIC Congress 2012; (oral presentation).

Solito, S.; Pinton, L.; Damuzzo, V.; Perilli, L.; Basso, G.; Zanovello, P.; Bronte, V.; Mandruzzato, S., Dissecting the molecular regulatory networks in human myeloid-derived suppressor cells; EFIS Congress 2012; (oral presentation).

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Solito S.; Pinton L.; Damuzzo V.; Perilli L.; Basso G.; Zanovello P.; Bronte V.; Mandruzzato S.; Characterization of the molecular regulatory pathways involved in MDSC-mediated tolerance; 15th International Congress of Immunology 2013; (poster)

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Mandruzzato S.; Brandau S.; Britten C.M.; Bronte V.; Damuzzo V.; Gouttefangeas C.; Maurer D.; Ottensmeier C.H.; Van der Burg S.; Welters M.; Walter S.; Towards harmonized phenotyping of human myeloid-derived suppressor cells by flow cytometry: results from an interim study, (under review as peer-reviewed brief contribution article to Cytometry part B)