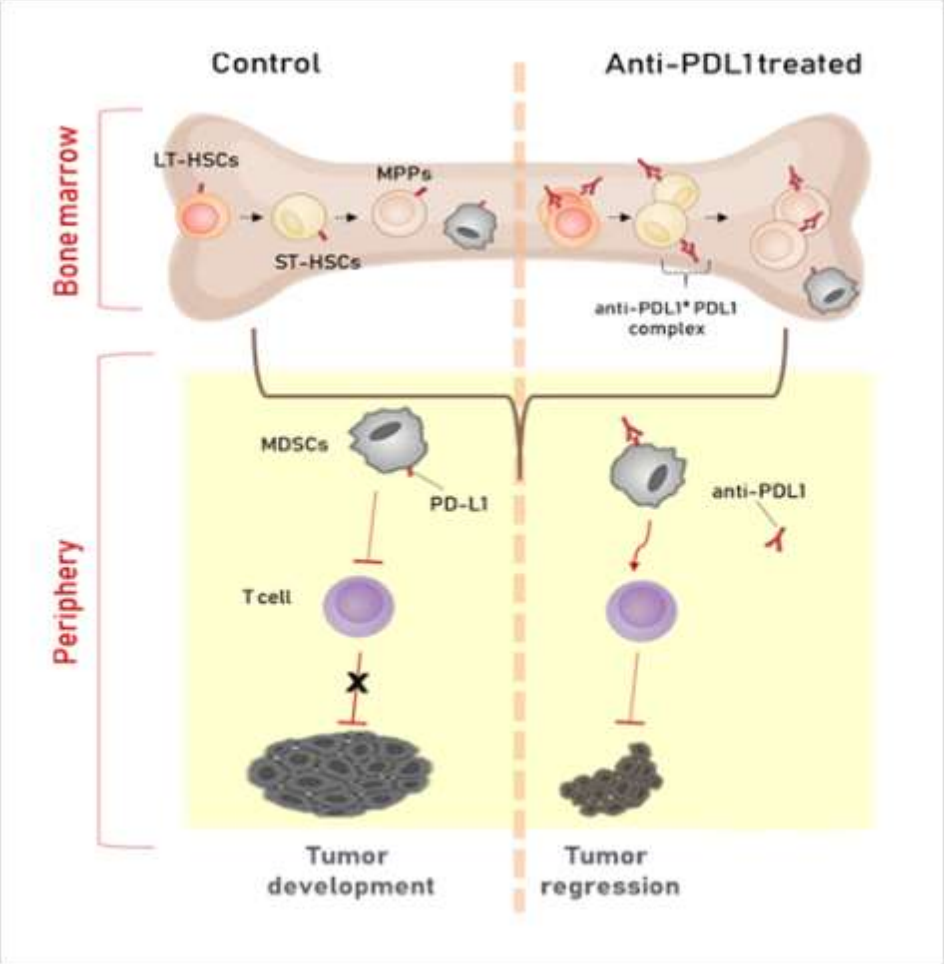
HELLENIC REPUBLIC
National and Kapodistrian University of Athens
"ALEXANDER FLEMING" Biomedical Sciences Research Center

International MSc
Molecular Biomedicine

BRFAA
BIOMEDICAL RESEARCH FOUNDATION ACADEMY OF ATHENS



Investigation of the role of Bone Marrow microenvironment in response to immunotherapy

Antonis Stylianos Papaioannou | MSc Thesis | Athens 2020

The following Master thesis was carried out in the Laboratory of Immune Regulation and Tolerance, under the supervision of Panayotis Verginis, PhD. The laboratory of Dr. Verginis is located at the Center of Clinical, Experimental Surgery and Translational Research at the Biomedical Research Foundation of the Academy of Athens, This thesis was undertaken for fulfillment of the requirements for the degree of Master of Science in “Molecular Biomedicine: Mechanisms of Disease, Molecular and Cellular Therapies and Bioinnovation”, National and Kapodistrian University of Athens, Medical School.

Results from the following thesis, that will not be shown here, were included in a handbook chapter publication from Elsevier, currently in Press

Supervisor: Panayotis Verginis, PhD, Researcher C’ (BRFAA)

Thesis Committee members

- D. Boumpas, Professor at the National Kapodistrian University of Athens (NKUA), Medical School
- M. G. Roubelakis, Assistant Professor at the National Kapodistrian University of Athens (NKUA), Medical School
- P. Verginis, Researcher C’ (BRFAA

Table of Content

Abstract

Immune checkpoint inhibitors (ICI) has revolutionized cancer therapeutics, yet a sizable portion of patients is associated with low response rates or side effects (immune related Adverse Events, irAE). A major impediment in the effectiveness of ICI therapy is the generation of cells with immunosuppressive capacity that home to tumor microenvironment (TME). These cells are generated via an altered program of hematopoiesis. Even though most studies have focused on the role of ICI therapy in tumor cells and immune cells in the periphery, the effect of this therapy in the generation of these cells, has remained elusive. Hematopoiesis primarily occurs in the bone marrow, where hematopoietic stem and progenitor cells (HSPC) are responsible for the generation of all blood cell types. Although their function in tumor progression is acknowledged, their capacity to be targeted by ICI and reshape their differentiation potential is yet to be defined.

Herein, we demonstrate that C57BL/6 melanoma bearing mice that receive anti-PD-L1 therapy, have a marked increase in the frequency of their HSPC population in the bone marrow, during early stages of the disease. We determine that anti-PD-L1 immunotherapy targets those cells, shown as decrease in the mean fluorescent intensity (MFI) compared to the control, untreated mice, group. Also, this immunotherapy scheme is associated with shifts in the frequency of immune cell population, such as total Dendritic cells (DC), Immature myeloid cells (IMC), B cells and T cells, in the bone marrow. We also report a marked increase in the DC population in peripheral blood of anti-PD-L1 melanoma bearing mice, concurrent with a decrease in the frequency of MDSC. In the spleen DC and MDSC frequencies are decreased associated with an increase in the MHCII expression of the MDSC population.

To sum up our results indicate that anti-PD-L1 might act in a previously neglected manner, influencing the HSPC compartment in the bone marrow, pushing them to proliferate and alter their output. Investigation of the mechanisms responsible could improve ICI therapy efficacy and possibly limit the extent of irAE developed after treatment.

Περίληψη

Οι αναστολείς ανοσιακού ελέγχου (ΑΑΕ) έχουν φέρει επαναστατική πρόοδο στην θεραπευτική του καρκίνου, παρόλα αυτά ένα μεγάλο μέρος τον ασθενών παρουσιάζει μικρή απόκριση σε αυτούς ή σοβαρές παρενέργειες. Μια μεγάλο εμπόδιο στην δράση των ΑΑΕ είναι η δημιουργία ανοσοκατασταλτικών κυττάρων που εισχωρούν στο μικροπεριβάλλον του όγκου. Τέτοια κύτταρα προέρχονται από ένα αλλαγμένο πρόγραμμα αιμοποίησης. Παρόλο που υπάρχει εκτενής έρευνα σχετικά με την δράση των ΑΑΕ στα καρκινικά κύτταρα αλλά και στα κύτταρα του ανοσοποιητικού στην περιφέρεια, ο ρόλος των ΑΑΕ στην δημιουργία αυτών των κυττάρων παραμένει άγνωστος. Τα αιμοποιητικά βλαστοκύτταρα είναι υπεύθυνα για την δημιουργία όλων των ώριμων κυτταρικών τύπων του αίματος. Αν και ο ρόλος τους στην πρόοδο του καρκίνου είναι πλέων αναγνωρισμένος, το ενδεχόμενο να στοχοποιούνται από τους ΑΑΕ έτσι ώστε να αλλάζουν την διαφοροποίησή τους παραμένει να καθοριστεί.

Στην παρούσα εργασία, περιγράφουμε μια αύξηση στην συχνότητα του πληθυσμού των αιμοποιητικών βλαστοκυττάρων σε ποντικούς του στελέχους C57BL/6 οι οποίοι φέρουν μελάνωμα και στα οποία χορηγείτε ανοσοθεραπεία αντι-PD-L1 ομάδα θεραπείας). Ακόμα, προσδιορίσαμε ότι η ανοσοθεραπεία προσδένετε σε αυτά τα κύτταρα γεγονός που αποδεικνύετε από την μείωση της μέσης έντασης φθορισμού για το συγκεκριμένο επιφανειακό αντιγόνο, σε σχέση με την ομάδα αναφοράς. Επίσης, το συγκεκριμένο σχήμα ανοσοθεραπείας σχετίζεται με αλλαγές στις συχνότητες των ώριμων δενδριτικών κυττάρων, των κυττάρων με φαινότυπο CD11b+ Gr1dim, καθώς και κυττάρων τις επίκτητης ανοσίας. Τέλος αναφέρουμε μια αύξηση της συχνότητας των δενδριτικών κυττάρων στο περιφερικό αίμα, με ταυτόχρονη μείωση της στον σπλήνα, των ποντικών της ομάδας θεραπείας, και μια συστημική μείωση των κατασταλτικών κυττάρων της μυελικής σειράς σε συνδυασμό με αύξηση της έκφρασης του μορίου ιστοσυμβατότητας τύπου δύο.

Εν κατακλείδι τα αποτελέσματά μας υποδεικνύουν ότι η ανοσοθεραπεία αντι-PD-L1 μπορεί να δρα επηρεάζοντας τα αιμοποιητικά βλαστοκύτταρα οδηγώντας τα σε πολλαπλασιασμό και αλλαγμένη διαφοροποίηση. Αποσαφήνιση του μηχανισμού θα

μπορούσε να βελτιώσει την αποτελεσματικότητα τον ΑΑΕ και πιθανά τον περιορισμό των παρενεργειών οι οποίες οφείλονται σε αυτούς.

Introduction

Cancer remains one of the leading cause of death globally and is responsible for an estimated 9.6 million deaths in 2018. Thought a lot of effort has been put to advance cancer therapeutics (e.g. Immune Checkpoint Inhibition, cancer vaccination, etc.)[1] a significant portion of patients does not achieve complete remission and also develops several side-effects (e.g. irAE)[2–4]. It is well established that immune surveillance mechanism exist to combat tumor cells[5], nonetheless tumors have evolved ways to circumvent immune recognition and destruction, one such mechanism is the expression of membrane molecules (Immune Checkpoint molecules) capable of inducing an immunosuppression, restricting effector function or enforcing anergy on effector immune cell (e.g. T cells, NK cells etc.) [6,7]. An equally important mechanism is the influence tumors exert on the hematopoietic system to skew hematopoiesis to their advantage, by generating immunosuppressive cells that impede the establishment of potent anti-tumor immune responses[8,9] and impair the success of immunotherapy. As a result, a better understanding of cancer biology, and specifically cancer hematopoiesis is necessary for improving these dismal outcomes.

Hematopoiesis refers to the prosses by which all mature cell types of the blood system are replenished in a tightly regulated, hierarchical manner by the differentiation of the hematopoietic stem cells (HSC)[10]. This prosses is often presented in a tree like figure in which the HSC is at the apex and give rise to progenitors with gradually restricted differentiation potential and absence of self-renewal capacity. Eventually leading to progenitors capable of generating a distinct type of mature cell.

To sustain the homeostatic production of mature blood cell types throughout life, HSC must keep a balance between self-renewal and differentiation. This is mainly achieved by the slow cycling rate of the HSC. Under physiological conditions the HSC are usually locked in the G_0 state. This is often imposed in the HSC by other neighboring cells. The spatial structure in which the HSC reside and are able to self-renew is termed the hematopoietic stem cell niche. The cells comprising the stem cell niche can be of hematopoietic (e.g. megakaryocytes, macrophages T cells, monocytes, osteoclast etc.)

as well as of non-hematopoietic (e.g. mesenchymal stem cells, endothelial cells, perivascular cells etc.) origin.

In recent years, the role of the hematopoietic compartment in promoting cancer progression and resistance to therapy has gathered great attention[11]. Tumor progression and metastasis formation do not only depend on cancer cell genetic and epigenetic defects but are also facilitated by the TME[10,12–21]. Among the constituents of the tumor microenvironment is the stroma but also cells of hematopoietic origin (e.g. Neutrophils, Macrophages, Myeloid Derived Suppressor Cells MDSC, T cell and Dendritic Cells DC) which are termed the tumor immune microenvironment (TIME)[22,23].

The tumors have evolved multiple ways to attract/ recruit cells of immune origin[24–32]. Various tumors have been found to secrete cytokines and chemokines of hematopoietic origin[33–36], and even if cancer cells themselves do not, they manipulate other cells, such as adaptive-, innate-immune and stromal cells, to secrete them[37–41]. In turn those cytokines (tumor derived factors, TDFs) establish the immunosuppressive microenvironment often found at the tumor site, and through the vascularization of tumor enter the blood stream to influence distant organs such as the spleen, the bone marrow and the metastatic site. Among the functions that TDF exert is induction of emergency myelopoiesis a prosses in which the myeloid progenitors (MyP) expand in the bone marrow and differentiate towards immunosuppressive population such as immature myeloid cells (IMC), and myeloid derived suppressor cells (MDSC). Bone marrow stimulation with TDF can also result in the mobilization of resident monocytes and the HSPC compartment. Stimulated HSPCs exit the bone marrow and home to peripheral organs such as the liver or the spleen, where extramedullary hematopoiesis takes place, and in some cases the premetastatic niche itself were they generate cells of immunosuppressive capacity[12].

Hematopoiesis

Hematopoiesis under physiological conditions

Hematopoiesis is the lifelong process by which all the cell compartments of the blood system (red blood cells and wight blood cells) are produced in a hierarchical and stepwise manner from a restricted rare pool of HSC[42]. The stem-cell concept has been reviewed extensively and it is often visualized as a tree-like model, in which multipotent stem cells give rise to their progeny through an ordered series of branching steps figure1.

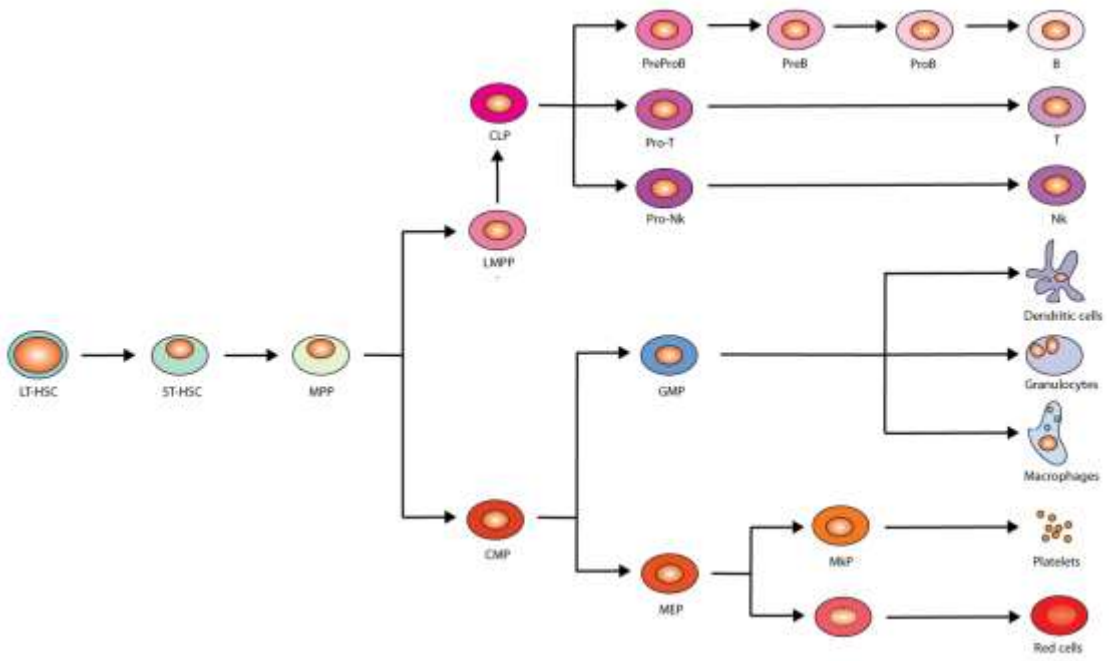


Figure 1 THE HEMATOPOIETIC TREE. HSC are categorized into long-term (LT)-reconstituting hematopoietic stem cells (HSC) with extensive self-renewal capacity and short-term HSC (ST-HSC) with limited self-renewal. Multipotent progenitors (MPP) differentiate to mature blood cells via several developmental stages where lymphoid and myeloid lineage restricted progenitors (CLP, CMP) are well defined

Initially the HSC compartment can be found in the aorta- gonad- mesonephros region during embryonic life and subsequently after gestation HSC migrate to the primary organ tasked with hematopoiesis, the bone marrow, in the trabecular regions of long bones in which they reside during adult life. This journey is accompanied by a change in the transcriptome as well as cell surface markers of these cells with the upregulation of adhesion molecules[43–45]. A study conducted by Tober *et al* also showed

that Program death ligand 1 (PD-L1) is also upregulated during the transition from embryonic to mature/adult HSC [46]

Hematopoietic stem and progenitor cells have often been characterized by two essential properties they possess, self-renewal and multipotency. Through asymmetric division they differentiate to progenitor cells that become increasingly lineage restricted cells and eventually progress into all mature blood cell types. As HSPCs are called to respond to stress signals and continuously replenish cells that are lost, they must self-renew to maintain their number over the lifetime of an organism[47]. Homeostasis within the hematopoietic system depends on the replacement of the immune effector cells by hematopoietic precursors[48]. By contrast, progenitors are defined by the absence of extended self-renewal and a restricted lineage differentiation capacity (most often bi- or unipotent).

Hematopoietic Stem Cell

With the development of techniques such as fluorescent activated cell sorting (FACS), the identification of markers that enrich for HSC became possible. With a combination of positive and negative selection criteria the HSC were isolated from total bone marrow cells[49]. In adult mice a compartment enriched for HSC can be isolated, this cells are negative for a cocktail of markers characteristic of mature cell types (Lineage) and positive for the markers cKit (the receptor of Stem cell Factor 1, SCF1R) and Sca1 (Stem cell antigen1, marker Ly6A/E). LSK cells represent approximately 0.05% of the adult BM cells[50] during steady state, and expand in regenerative or inflammatory conditions. However this population is heterogeneous and bona fide HSC can be isolated by using more advanced criteria such as the expression of the CD150 marker and the lack of CD48 expression[51].

The molecular mechanisms underlying this transition from HSC to progenitor cells with defined potential have taken center stage in recent years with the development of single cell transcriptomic techniques leading to the rearrangement of hematopoietic tree into a more plastic structure were HSC might skip multiple steps of the classical differentiation and lead to the generation of progenitors restricted to a single linage.

One such example was recently published by Upadhaya et al. were they demonstrate that HSC can also contribute directly to the megakaryocytic lineage, previously thought to be branching from a common progenitor between the megakaryocyte/erythrocyte and the myeloid lineages[52].

To date, two major theories have arisen to explain the events leading to the generation of committed progenitors from the pool of HSC. The first theory (intrinsic theory) postulates that there is an autonomous and stochastic upregulation of transcription factor programs that dictates the differentiation potential of individual HSC, while the second one (extrinsic theory) supports that, whether an intrinsic mechanism for the upregulation of specific programs exists on not, the HSC are responsive to extrinsic stimuli from neighboring cells (e.g. cytokines signals).

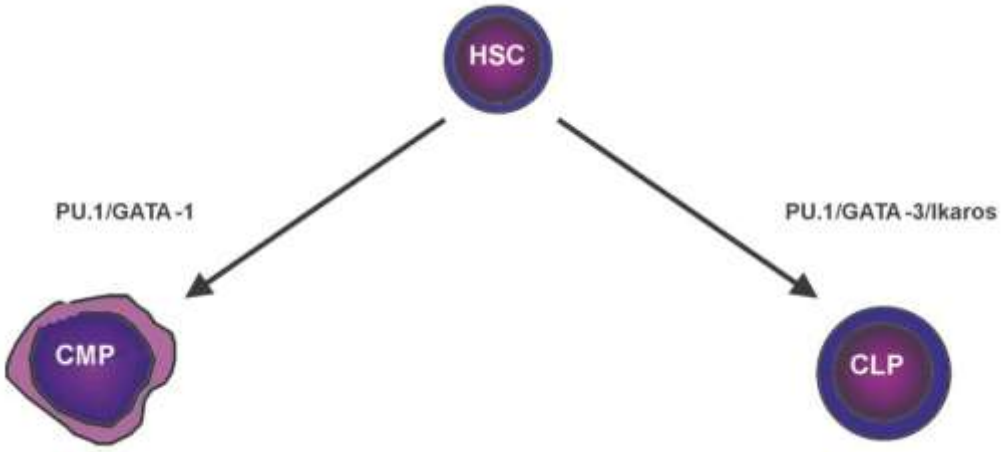


Figure 2 Transcriptional regulation of early stages of HSC differentiation, adapted from Zhu et all 2002

Of the transcription factor governing the commitment of HSC to the specific lineages the clearest example is PU.1. The effect of PU.1 levels in lineage potential seems to be dose dependent with higher levels of the transcription factor leading, through the upregulation of other transcription factors (e.g. GATA1) and cell surface receptors such as M-CSFR, to the commitment to myeloid lineage[53,54]. The commitment to the lymphoid lineage is regulated by the PU1 transcription factor, but also the upregulation of specific transcription factors dictating the transcription of lymphoid specific genes, such transcription factors include GATA3, members of the Ikaros/Aiolos family and

Pax5, also the expression of lymphoid specific surface receptors such as IL7 receptor[54,55].

Hematopoietic stem cell Niches

In 1978 the concept of the hematopoietic niche was introduced by R. Schofield. It refers to complex microenvironment responsible for the maintenance and regulation of the HSC population[56]. Multiple cell types are responsible for the organization of the stem cell niche structure and microenvironment, such as different types of stromal cells, as well as cell of hematopoietic origin[57].

The first morphological evidence demonstrated that HSC are present in close proximity to the lining of the trabecular regions of long bones and in close association with osteoblastic cells[58]. Based on the location as well as the cell types participating in it, the niche was called the osteoblastic niche. Recent evidence, however, suggests the existence of another form of hematopoietic stem cell niche. This niche is identified in a different location in the bone marrow shaft, close to the bone marrow capillaries and sinusoidal membranes[51] this structure in turn was named the vascular niche. Because of the existence of this two distinct niche modes it is safe to assume that each niche has a different function in terms of the HSC maintenance and differentiation/ mobilization.

Osteoblastic niche

HSC that reside on the endosteal niche tend to be in a more quiescent state and highly enriched for long term repopulating HSC[59,60]. Specific factors that are released as well as other conditions in this niche such as Osteopontin[61], angiopoietin[59], CXCL12[62] as well as the limited oxygen concentration[63,64] are primarily responsible for enforcing HSC self-renewal and quiescence.

Vascular niche

On the other hand, HSC residing close to the vascular niche tend to have a higher proliferation rate and are more prone to respond to signals from the periphery leading ether to mobilization or differentiation[65]. HSC might home to this type of niche because

of endothelium derived FGF-4 and CXCL12 as well as the oxygen concentration gradient that exist in the bone marrow[66]. Also, during stress sSCF1 can be released from its membrane bound form by MMP9, leading to HSC proliferation and differentiation[60].

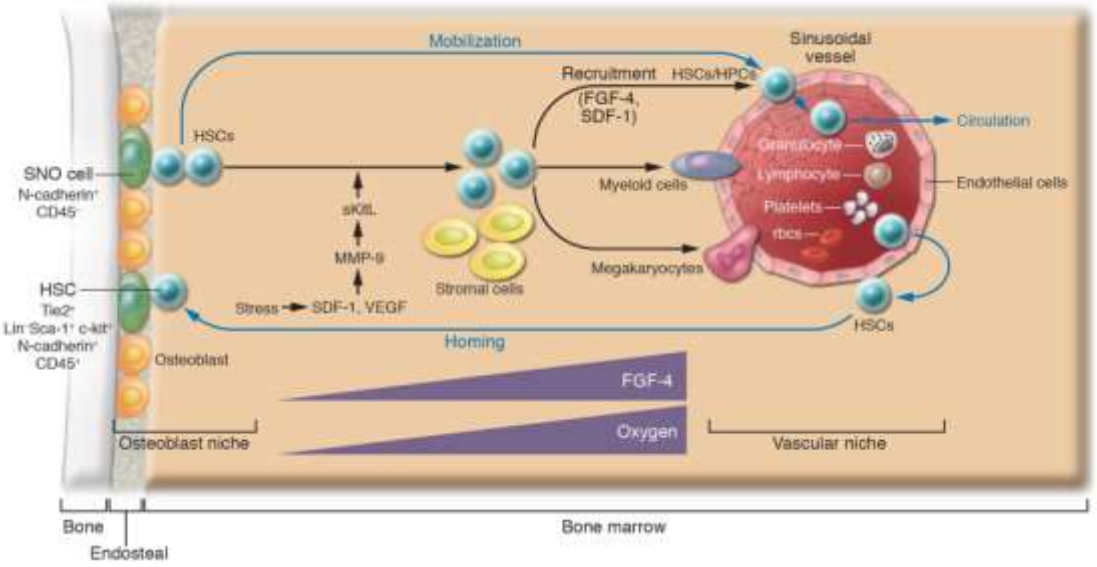


Figure 3 The osteoblastic and vascular niches in the bone marrow. Adapted from Yin et al 2006

Recently a study identified a subset of $CD4^+$ T regulatory cells, that express high levels of the Hematopoietic stem cell marker CD150, to be in close proximity to the HSC indicating possibly a direct interaction of this two cell types possibly adding them as niche constituents. This interaction between Tregs and HSC increased the engraftment of HSC in allo-HSC transplantation setting possibly through the inactivation of adenosine (adenosine is cleaved by CD39 expressing Tregs)[67]. Another effect of the Treg on the bone marrow is the secretion of IL10 to retain the immune-privileged state of the bone marrow.

Same of the cell types participating in niche formation as well as their function are presented in the *Table1* and *Figure3*.

Table 1 Cells paricipating in niche formation and their function

Cell type	Function	Reference
Osteoblastic Cells	Osteoblast secreted Osteopontin mediates HSC quiescence	61,68
Mesenchymal Stromal/Stem Cell	Major contributor of many currently known niche factors, such as CXCL12, SCF and Interleukin-7 (IL7)	69–72
Adipocytes	Under regenerating conditions produce SCF to maintain the HSC pool	73
Endothelial Cells	contribution to niche factors, such as SCF, CXCL12 and JAGGED-1, results in HSC maintenance	70–72,74
Megakaryocytes	Megakaryocyte-derived CXCL4, TPO and TGFβ1 have been identified as the molecular signals that mediate quiescence in HSCs	75–77
Macrophages	Macrophage derived TGFβ and DARC contribute to HSC quiescence	78

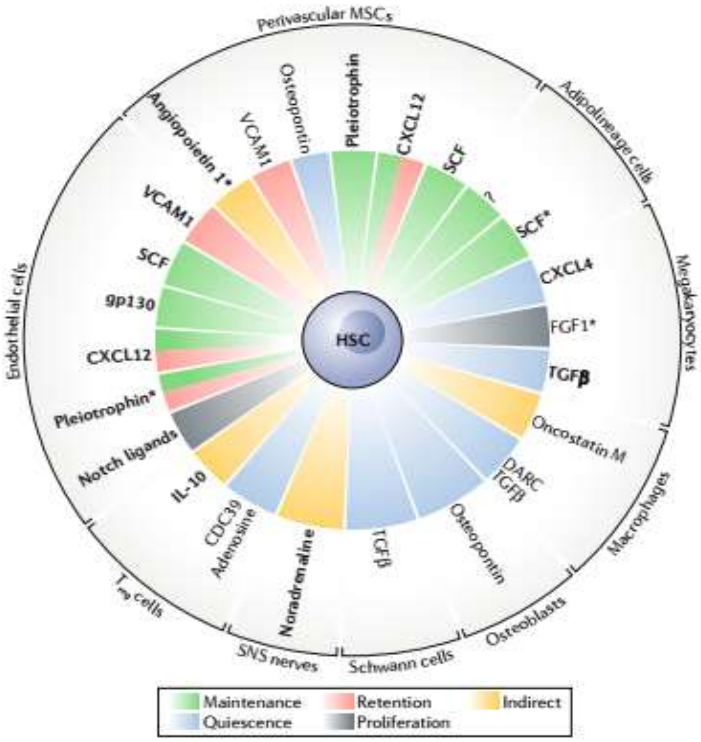


Figure 4 Cellular and molecular constituents of the HSC niche. Adapted from Pinho et al 2019

In summary, a complex and coordinated combination of migration, adhesion, proteolysis and signaling occurs at the interface between HSC and the different components of BM niche. In addition, signals originating from the periphery can influence HSC homing, retention, and mobilization, therefore determining whether a niche is silent or whether HSC exit the niche in response to stress.

Cancer Hematopoiesis

It is well established that tumors hijack the immune system in order to avoid immune destruction and also to support tumor growth and metastasis[79]. For that reason, it is generally accepted that Immune evasion is one of the hallmarks of cancer[11]. Tumors have evolved multiple ways of avoiding the recognition and destruction by immune cells, one of those ways is the induction and accumulation of immunosuppressive cell populations (such as the MDSC, T regulatory, M_2 like Macrophages etc.) at the tumor site[80–85]. Deregulation of myelopoiesis (increased neutrophil to lymphocyte ratio) has been found to be a major contributing factor in cancer progression and metastasis formation[86]. Therefor in recent years the influence of that cancer exerts on the hematopoietic system has taken center stage.

Inflammatory conditions, such as injury, infection and cancer, increase proliferation and self-renewal among HSC compartment. HSC can also be “pulled” towards cell division following the depletion of committed progenitor populations from the BM[48,87]. HSPCs adapt to inflammation by a combination of cell-intrinsic mechanisms (transcriptional, epigenetic and metabolic) and cell-extrinsic mechanisms (soluble growth factors, cytokines, microbial ligands and adhesive interactions)[88]. However, chronic activation of HSPCs, could cause impairment of their function and exhaustion, or lead to their contribution to the chronicity of inflammatory pathologies[89].

The mechanisms that cancer cells exploit to alter hematopoiesis are direct, influencing the HSC, or indirect, influencing different aspects of the hematopoietic niche constituents leading to changes in the microenvironment in which the HSC reside. Cancer cells orchestrate this hematopoietic response through expression of surface proteins, secretion of chemokines and growth factors *Figure4*, and exosome shedding.

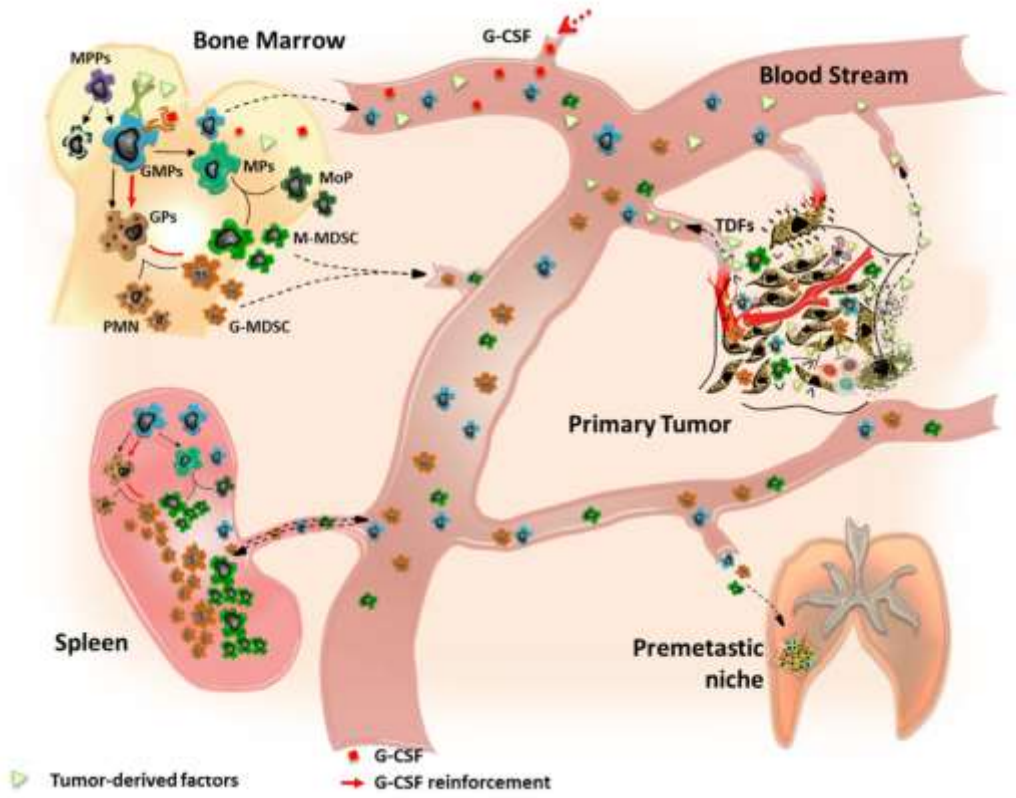


Figure 5 Induction of abnormal hematopoiesis in response to TDFs. Tumors produce and secrete a variety of soluble TDFs that influence both the TME and the Hematopoietic system (bone marrow and spleen). Adapted from Alvarez et al 2018.

Cytokine and growth factor secretion

It has been found that cancer patients have increased levels of GM-CSF in their serum[90], this cytokine has been proven to influence hematopoiesis and lead to increased myeloid cell differentiation[91]. In a mouse model of glioblastoma it was shown that GM-CSF as well as G-CSF that can be produced by the cancer cells as well as the surrounding tissue because of local inflammation can shift the hematopoietic program towards immunosuppressive neutrophil generation[92]. The same growth factors (G-CSF, GM-CSF) can promote HSC proliferation as well as affect the monocytic compartment

of the nice leading to HSC mobilization[93–95].This phenomenon has been observed in a vast array of other tumor models such as breast, colon and lung cancer to name few[96–103].

Renal cell carcinoma tumors and multiple myeloma have been identified as tumors that produce both IL6, M-CSF and VEGF among other cytokines[104]. These cytokines stimulate the bone marrow, specifically the HSPC compartment, leading to increase myelopoiesis in favor of the monocytic lineage, at the expense of DC differentiation which is inhibited by IL6[105,106] using an in vitro system of bone marrow cell culture with conditioned media. Rescue of these phenotype was achieved upon neutralization of IL6, M-CSF, or in the case of multiple myeloma IL6 and VEGF, in the condition medium[104,107]. Also, addition of IL4 in the culture media was able to rescue this effect[108].

TNF-a is another cytokine that has been shown to influence hematopoiesis and the levels of TNF-a are elevated in cancer patient serum[109]. Using different models of cancer in mice it was found that, TNF-a secreted specifically by T cells in the periphery can act directly on HSC to induce proliferation and generation of Myeloid progenitors and MDSCs[110].

In another study condition media from B16F10 cancer cell culture was identified to contain high levels of IL3 and increase myelopoiesis in total bone marrow cultures by directly influencing the LSK compartment[111]. This effect could be recapitulated in vivo as IL3 has been found to be increased in cancer patient sera[112].

Osteopontin (OPN) has been found with increased concentration in the circulation of patients with various cancer types [113,114]. OPN influences the distant metastasis site, rather than the primary tumor, by activating the bone marrow[115,116], inducing the mobilization, activation and survival of BM-macrophage[117,118]. This could possibly lead to altered cytokine production in the microenvironment of the HSC niche. Also OPN has been identified to induce HSC migration[68].

VEGF is responsible for tumor vasculature formation, but VEGF secretion has also been linked to reduced sinusoidal area in the bone marrow[119]. Possibly leading to reduced niche availability, and as a result increased mobilization and proliferation of HSC. Additionally, VEGFR+ HSC have been implicated in the formation of the premetastatic niche, by their accumulation and production of immunosuppressive cell types such as MDSC[120]. This accumulation can possibly point to a role of VEGF as a chemoattracting agent.

In addition proteins secreted by tumor cells that can function as matrix metalloproteinases such as MMP12 have been found to induce myelopoiesis in the bone marrow an effect most likely caused by altering the properties of the niche rather than acting on progenitor cells directly[121]. Another example of MMP that modulates the niche biochemistry is MMP9. Multiple types of cancer have been found to secrete IL8[122], BM neutrophils are activated by, and respond to IL8 by producing MM9[123,124], which as mentioned previously can lead to the release of sSCF1, resulting in HSC proliferation and myeloid progenitor cell production.

Tumor-derived exosomes

Tumor-derived exosomes have been shown to stimulate the production of inflammatory cytokines (e.g. IL-6, IL-8, and MCP-1) from MSC in vitro[125]. This suggests a role for exosomes in indirectly influencing the HSC by acting upon MSCs. In another study melanoma exosomes were found to educate bone marrow HSPC leading to increased tumor burden, mobilization and homing of HSPC in tumor as well as increased tumor vasculature[126–128].

Cancer immunotherapy

Immunotherapy has only recently been evaluated as a viable alternative treatment option for cancer patients. Cancer immunotherapy is an artificial stimulation of the immune system to treat cancer, improving the organisms, natural capacity to fight cancer. It exploits the machinery of the immune system that halted the immune system to restore or induce of effector cells function to target and destroy tumor cells[129,130].

Immune checkpoints are a set of transmembrane molecules capable of inducing or silencing the elicitation of an immune response. Under normal conditions, they are involved in initiating the immune response, regulating its intensity and duration. They also operate to reduce tissue damage during antimicrobial immune responses. More importantly, they maintain self-tolerance and play a crucial role in preventing autoimmune diseases. During tumor growth, cancer benefits from the function of these receptors to escape immune surveillance[131]. Some of these receptors have recently been targets to improve cancer therapy with the development of monoclonal antibodies against them[132].

Anti PD-1/ anti PD-L1 axis immunotherapy in cancer

PD-1 is a cell surface receptor that recognizes and binds to the endogenous ligands PD- L1 and PD- L2. It is expressed primarily on T cells and B cells, but also cells involved in innate immunity, like natural killer cells and myeloid cells. PD-L1 is primarily expressed by antigen-presenting cells (APC), MDSCs, and tumor cells that induce T-cell anergy and apoptosis by engaging its PD-1 receptor[129,130]. In the absence of malignancy, activation of either or both of these receptors has an inhibitory effect on the T cell response, thus inducing immune tolerance and preventing autoimmunity[133]. However, during tumor development and metastasis immune checkpoint molecules are abused by cancer cells to promote immunosuppression and tolerant aiding in tumor growth (Figure 4). Immune checkpoint inhibitors, of the PD-1 and PD-L1 molecules have shown beneficial clinical effects against many different solid and hematologic malignancies [134]. Monoclonal antibodies that target either PD-1 or PD-L1 block this binding and boost the immune response against cancer cells, by releasing the brakes of T cell priming from APC, reverses T cell suppression and enhances endogenous antitumor immune response to achieve long-term antitumor responses for patients with a wide range of cancers[135]. In addition to binding PD-1, PD-L1 also interacts with cis: B7 (CD80, CD86) creating negative signals on T cells and dampens antitumor immunity[134–136]. reverses T cell suppression and enhances endogenous antitumor immune response to achieve long-term antitumor responses for patients with a wide range of cancers[135]. In addition

to binding PD-1, PD-L1 also interacts with cis: B7 (CD80, CD86) creating negative signals on T cells and dampens antitumor immunity[134–136].

Currently, there are three approved PD-L1 inhibitors by the US Food and Drug Administration (FDA) for cancer treatment ranging from non-small cell lung cancer to Merkel cell carcinoma.

Malignant melanoma, a fatal form of skin cancer with high rates of genomic mutations, has shown an effective response to immunotherapy options compared to other conventional treatments, like chemotherapy. Melanoma cancer cells represent upregulated expression of PD-L1 compared to inflammatory signaling activators, such as IFN-γ, which, in turn, upregulate PD-1 on T cells and mediate inhibition of antitumor action by cells of the immune system[137,138]. As a result, checkpoint protein inhibition directed against the programmed death-1 axis (e.g., nivolumab, pembrolizumab) on tumor cells has emerged as an effective therapeutic option for melanoma[129,138–144], resulting in dramatic improvements in the prognosis of patients[145].

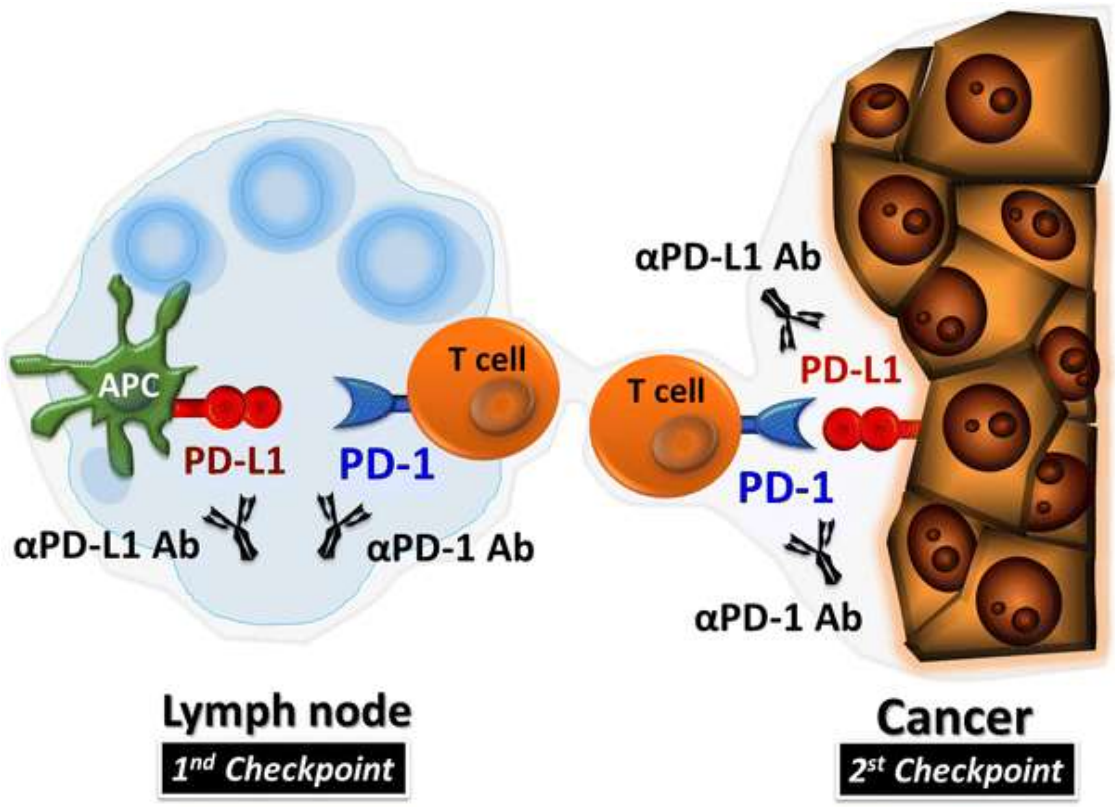


Figure 6 Function of Immune checkpoint inhibitor therapy of the aPD-1 or aPD-L1 scheme. Adapted from Hamanishi et al 2016

Although PD-L1 therapy schemes for melanoma are rear. Combination therapy of PD-L1 coupled with BRAF inhibitors have been showing promising results for the treatment of BRAF mutant, late stage melanoma in the clinical trial setting (Trail ID number: NCT02303951, NCT02908672)[146]*Figure7*. Another report of anti-PD-L1 monotherapy with promising results in the clinical trial setting comes from Hamid et all were they treated patients with advanced or metastatic melanoma, with an engineered PD-L1 antibody, favorable results in term of progression free survival and overall remission[147].

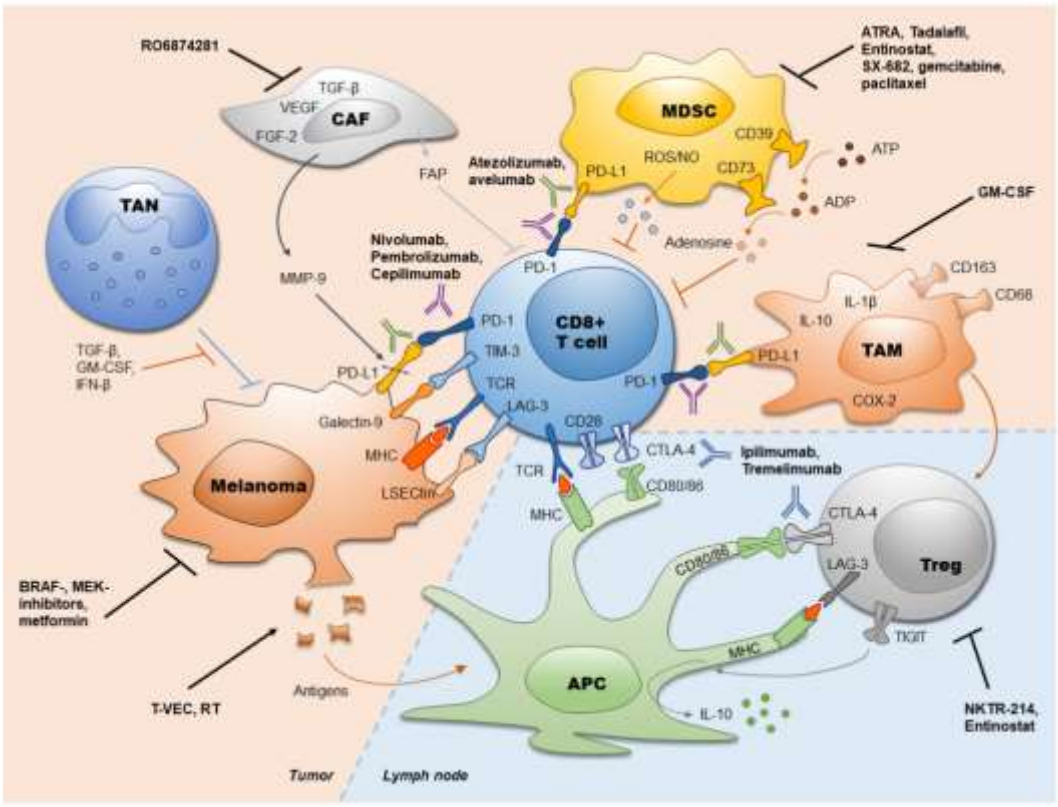


Figure 7 Immune checkpoint inhibitor therapy in melanoma. Combination treatment with other agents also presented. Adapted from Petrova et al 2020

Aim

The hematopoietic system has been proven to aid tumor progression and metastasis. Many treatment schemes, such as ICI treatment, target the hematopoietic system, especially the immune system, in order to activate it to fight the tumor. Different ICI treatment regimens has been proven to induce changes in the activity of an array of immune cells such as T cells, macrophages, DCs and other cells types. These functional changes have been associated with alterations in the frequencies of those cells in peripheral tissues. That has led as to theorize that such changes might stem form an alteration of hematopoiesis at its core in the bone marrow, where those cells are generated

Extending on the above-mentioned information we formed the hypothesis that the bone marrow and especially the HSC may represent a target of ICI treatment previously neglected. In this study we sought to determine the effect of the aPD-L1 ICI therapy in the context of hematopoiesis during early stage melanoma development. To that effect we assessed the different stages of hematopoiesis in the bone marrow from HSC to mature cells and also analyzed those same mature cells in peripheral tissues as a read out of the effect of aPD-L1 immunotherapy.

Materials and Method

Animals

C57BL/6J and Rag1−/− mice (on a C57BL/6J background) were purchased from Jackson Laboratory, Foxp3-GFP KI mice (on a C57BL/6 background) were kindly provided by A. Rudensky (Memorial Sloan–Kettering Cancer Center). All mice were maintained in the animal facility of BRFAA. All procedures were in accordance with institutional guidelines and approved by the Institutional Committee of Protocol Evaluation together with the Directorate of Agriculture and Veterinary Policy, Region of Attika, Greece (1202/19 March 2018). Unless otherwise indicated, experiments used female, age-matched mice aged between 8 and 12 weeks.

Cell lines.

The cancer cell line B16.F10 was kindly provided by A. Eliopoulos (School of Medicine, University of Crete, Greece). Cells were maintained in RPMI Medium (Gibco) supplemented with 10% fetal bovine serum (FBS, StemCell Technologies Inc), β-mercaptoethanol (Gibco) and a mix of Penicillin/Streptomycin (P/S, Gibco) in a cell incubator (5% CO_2, absolute humidity), and were negative for Mycoplasma spp. tested by PCR.

Transplant tumor models.

Mice were implanted subcutaneously on the back with 3×10^5 B16.F10 melanoma cells. To study the effect of immunotherapy at the day of implantation and every 3 d after, mice were treated with anti-PD-L1 (clone MIH5, Bioceros LB) (200μg per 100μl intraperitoneally (i.p.) in each mouse), as control, i.p. injections of PBS alone were performed, mice were euthanized at day 8 and 15 past tumor implantation. Tumors were measured every day using calipers, and the tumor volume was calculated using the equation $\frac{(\boldsymbol{d1}\times\boldsymbol{d2^2})}{\mathbf{2}}$. Mice were euthanized when tumors grew larger than 1,100 mm^3. At the endpoint of each experiment, the tumor weight was also determined.

Cell isolation from tumors and lymphoid organs

Single-cell suspensions from LNs and Spleens were generated by passing them through a 40-µm cell strainer. Bone marrow cells were isolated by flushing out with, ice cold PBS, the femurs, tibiae and humeri. Red blood cells from Spleen and Bone marrow cell suspensions were lysed by incubation in 2 ml RBC for 2 minutes in RT. TILs were isolated by dissociating tumor tissue in RPMI supplemented with collagenase D (1 mg ml^{-1}, Roche) and DNase I (0.25 mg ml^{-1}, Sigma) for 45 min before passing through a 40-µm cell strainer.

Flow cytometry.

For staining of extracellular markers, cell suspensions were incubated with antibodies for 20 min at 4°C. The following antibodies were used (all antibodies were purchased from BioLegend): CD45 (clone 30-F11), CD4 (RM4-4) (GK1.5), CD8 (53-6.7), CD11c (N418), CD11b (M1/70), IAb (AF6-120.1), CD16.32(93), Gr1(RB6/8C5), Ter119(TER/119), B220((RA3-6B2), PD-L1(10F.9G2), cKit(2B8), Sca1(D7)(E13-161.7), CD150(TC15-12F12.2), CD48(HM48.1), Ly6G(1A8), Ly6C(HK1.4). For Foxp3 (150D) intracellular staining, cells were stained for the extracellular markers, and then fixed and stained using the Foxp3 Transcription Factor Staining Buffer Kit (Molecular Probes™) according to the vendor's instructions.

Bone marrow fluid collection

To collect BM extracellular fluid, two femoral bones were flushed with ice cold PBS (500 µl) in eppendorf tubes, the supernatant was harvested after pelleting cells by centrifugation at 1800rpms for 10min at 4°C. Cytokine profile was evaluated via Mass Spectrometry.

Bulk RNA-Seq

The library preparation for mRNA-seq was carried out in the Greek Genome Center (GGC) of BRFAA. RNA was collected from total LSK sorted cells from BM using the NucleoSpin RNA XS kit (Macherey-Nagel) according to manufacturer's guidelines. RNA-

seq libraries were prepared using the TruSeq RNA kit and at least 100 ng total RNA. The libraries were constructed according to Illumina's protocols and then mixed in equal amounts. Paired-end 75-basepair (bp) reads for 6 samples were generated using NextSeq500 in the GGC. RNA-seq analysis was done using an in-house-developed sub-pipeline.

Statistical analysis

All data are presented as mean ± SEM. A two-tailed unpaired Student's t test for parametric variables and a Mann-Whitney U test for non-parametric variables were used for the comparison of two groups. All statistical analysis was performed using GraphPad Prism (GraphPad Inc., La Jolla, CA). Significance was set at $p < 0.05$.

Results

To investigate the effect of anti-PD-L1 immunotherapy during early response to B16-F10 melanoma, we inoculated B16-F10 melanoma cells in C57BL/6 mice, which were then treated with anti-PD-L1 immunotherapy at the day of inoculation and subsequently every three days until the endpoint of the experiment. The experiment timeline can be found in the figure below.

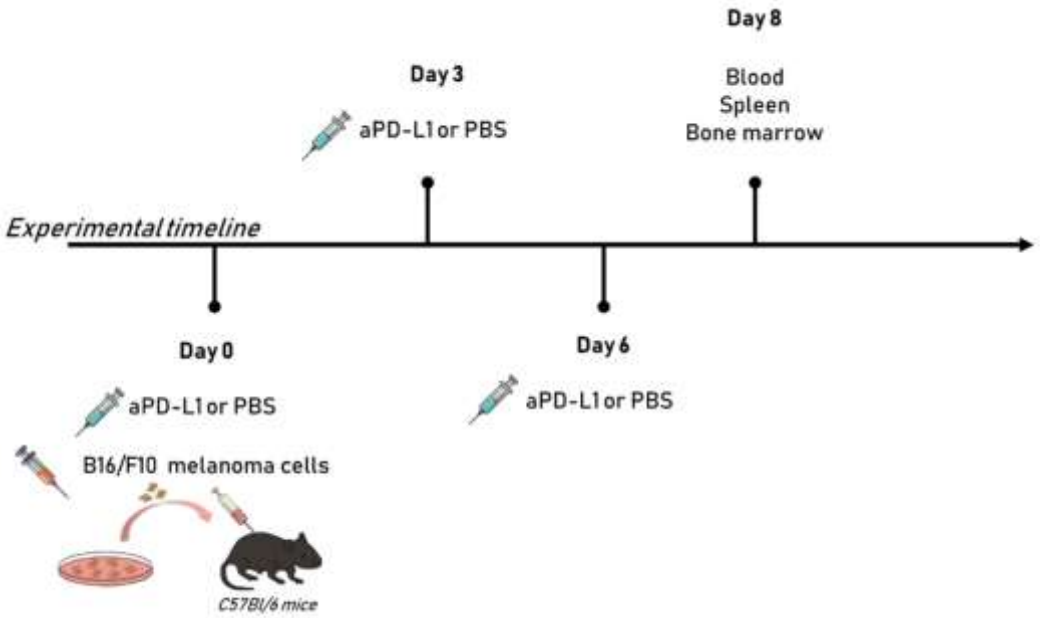


Figure 8 Graphical representation of the experimental design.

As shown in the figure above we analyzed peripheral tissues of melanoma bearing mice to gain insight to the impact of immunotherapy on key population impeding or mounting the anti-tumor immune response. Among the cell populations that were analyzed in the periphery were those of myeloid origin such as MDSC, their subpopulations[148], which have been shown to dampen the antitumor immune response[149,150]. We also investigated DC[151], as well as a subset of dendritic cells bearing the phenotype CD11c+ CD11b+ Gr1+ cells, which are essential players for the successful priming of T cells capable of mounting the anti-tumor immunity.

As mentioned, T cell play a major role in antitumor immunity with immunosuppressive populations (e.g. T regulatory cells) promoting tumor growth and effector populations (e.g. CD4+ effector T cells and cytotoxic T cells) contributing to

tumor cell eradication. For this reason, we also investigated changes in those cell populations.

Based on previous unpublished results from our lab (Athina Boumpas dissertation) that show, the PDL1 expression, on various peripheral cell population (e.g. DC moDC, and MDSC), to peak at Day 8 upon tumor inoculation we chose to investigate the response to anti-PD-L1 immunotherapy on that time point, were we presume it will have the most prominent effect.

PD-L1 immunotherapy leads to changes in myeloid cell population composition in the periphery

Anti-PD-L1 treatment decreases splenic and blood PD-L1-expressing MDSCs in tumor bearing mice

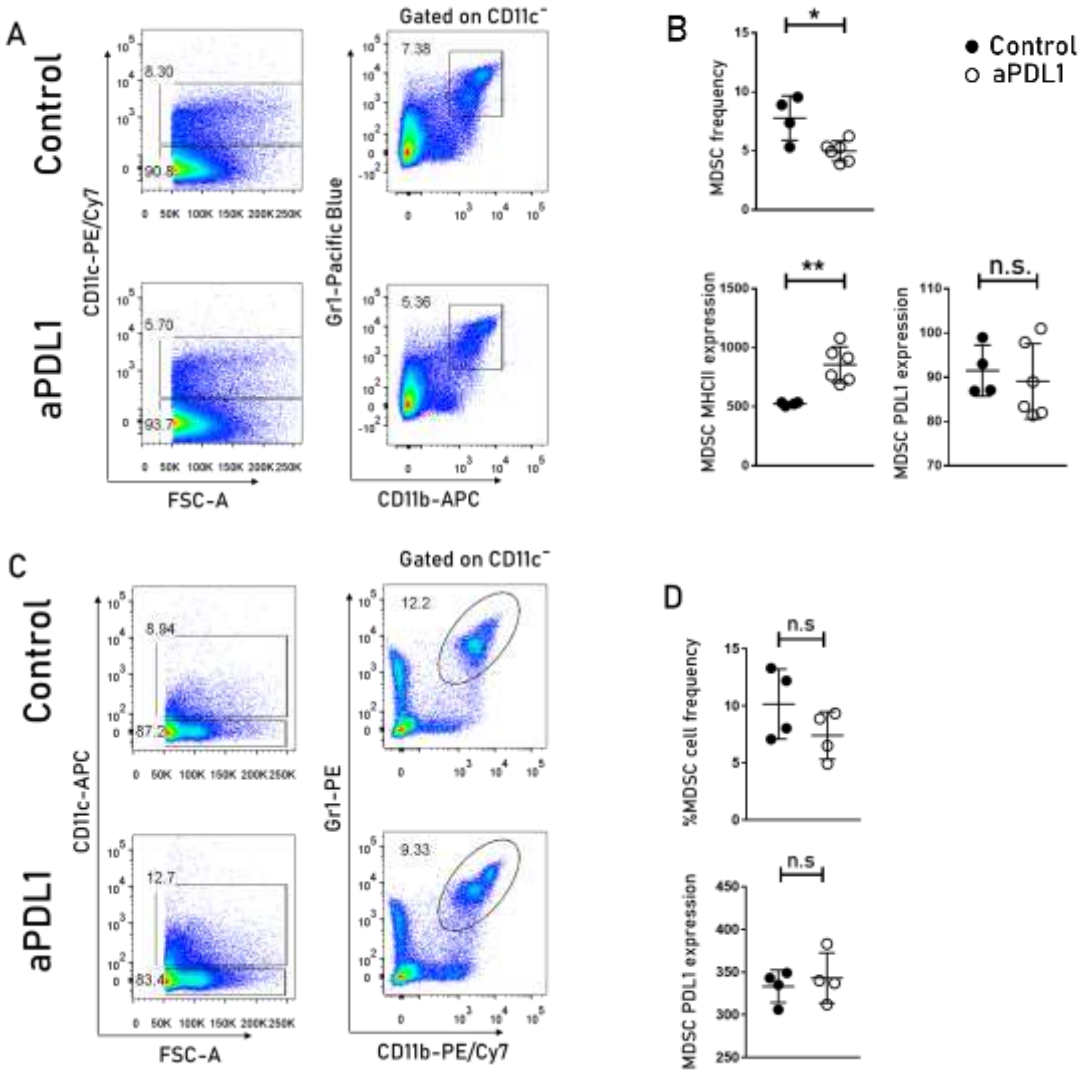


*Figure 9 Circulating and splenic MDSC of anti PD-L1 and control treated melanoma bearing mice. A) Representative gating strategy for the assessment of MDSC in spleen of tumor mice. B) Frequency and expression of MHCII and PD-L1 on splenic MDSC in tumor bearing mice, **P<0.01 *P<0.05 results are presented as means ± SD C) Representative gating strategy for the assessment of circulating MDSC of tumor mice. D) Frequency and PD-L1 expression of circulating MDSC in tumor bearing mice, results are presented as means ± SD. Representative results from 3 independent experiments for each tissue*

MDSC have been found to accumulate in peripheral tissues such as the blood, spleen and liver of cancer patients[152]. These cells are potent immunosuppressors leading to poor anti-tumor immune responses. MDSC are cells negative for the CD11c and positive for CD11b and Gr1 markers. Upon anti-PD-L1 immunotherapy we observed a decrease in the frequency of MDSC population in the spleen of melanoma bearing mice compared to controls (figure 9A, 9B) as well as an upregulation of the MHCII molecule, this molecule is responsible for presentation of antigens, mainly of extracellular origin to CD4+ helper T cell, possibly hinting to altered function of those cells. Also, in peripheral blood we reported a decrease in the circulating MDSC though to a lesser extent than that in the spleen (figure 9C, 9D). Interestingly and in contrast to the existing literature we did not observe any decrease in the mean fluorescent intensity (MFI) for the PD-L1 marker (an indicator that aPD-L1 therapy targets/binds to those cells). So, the decrease in the frequency of this cell type is probably mediated by another signal than the binding of PD-L1.

Anti-PD-L1 treatment leads to shifts in the frequencies of splenic MDSC subsets

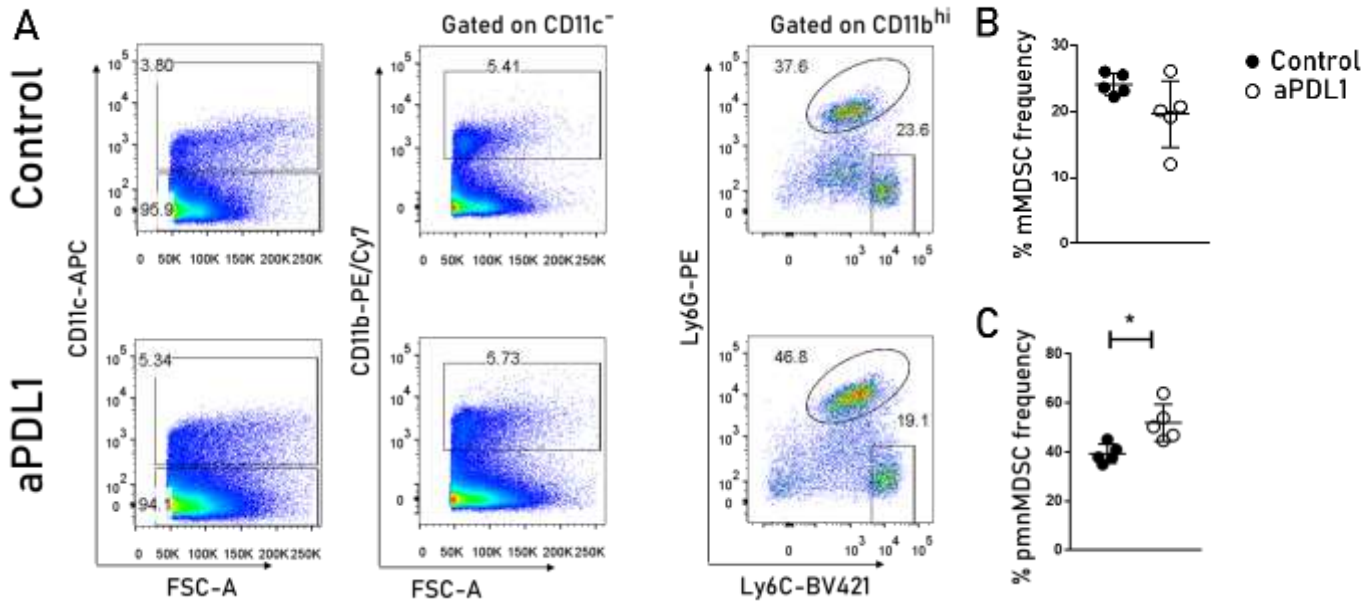


*Figure 10 Subsets of MDSC in spleen of anti PD-L1 and control treated melanoma bearing mice. A) Representative gating strategy for the assessment of MDSC subsets of in spleen of tumor mice. B) Frequency of splenic mMDSC in tumor bearing mice. C) Frequency of splenic pmnMDSC in tumor bearing mice, *P<0.05 results are presented as means ± SD. Representative results from 3 independent experiments*

The MDSC population is highly heterogeneous consisting of at least to distinct sub populations, the monocytic MDCS (mMDSC), characterized by their expression of the Ly6C marker and the lack of expression of the Ly6G marker, and the polymorphonuclear MDSC (pmnMDSC), characterized by high expression of the Ly6G marker and low expression of the Ly6C marker. Those two MDSC subsets can be distinguished by FACS analysis as shown in *figure 10A.* Upon anti-PD-L1 immunotherapy we observed that the composition of the MDSC population also changes. We reported an increase in the pmnMDSC compartment with a concomitant, though not statistically significant, decrease in the mMDSC compartment.

Anti-PD-L1 treatment reduces splenic DCs, but increases circulating DCs of tumor bearing mice

According to the literature another major cell population that expresses the PD-L1 marker are antigen presenting cells (e.g. DC, macrophages etc.)[153,154]. The function of DC as antigen presenting cells is greatly appreciated, in order to mount effective anti - tumor immune response. Herein we report that total dendritic cells frequency, assessed through the expression of the CD11c marker a common marker for all DC subsets, is decreased in the spleen of tumor bearing mice upon anti-PD-L1 treatment (*figure11A)*. We also show that expression of the PD-L1 marker, assessed as MFI, is lower for the group receiving anti-PD-L1 immunotherapy (*figure11B*). This effect could be explained by the binding of immunotherapy to the PD-L1 molecule expressed on DCs. Lastly, we report in change in the MHCII molecule expression in response to immunotherapy(*figure11B*). In peripheral blood of treated mice, we report an increase, non-significant statistically, in the frequency of total DC, we also found as in the spleen the same decrease in PD-L1 expression for the treated group (*figure11D, E*).

A subset of DC, the monocyte derived dendritic cells (moDC) (CD11c+ CD11b+ Gr1+ cells), has been shown to exacerbate autoimmunity, and induce anti-tumor immune response as they produce a vast array of immunostimulatory cytokines[155–157]. Because of their established role in anti-cancer immunity we sought to investigate their response to anti-PD-L1 immunotherapy. In that regard we report a decrease of the frequency of this cell type in the spleen of treated mice (*figure11A*). As is the case for the total DC population we report here also a decrease in the MFI for the PD-L1 marker, suggesting that immunotherapy binds to those cells, lastly, we report the increase of the MHCII expression in those cells (*figure11B*). In peripheral blood of treated mice moDC frequency seems to remain constant and the MFI for the PD-L1 marker is decreased (*figure11D, F*).

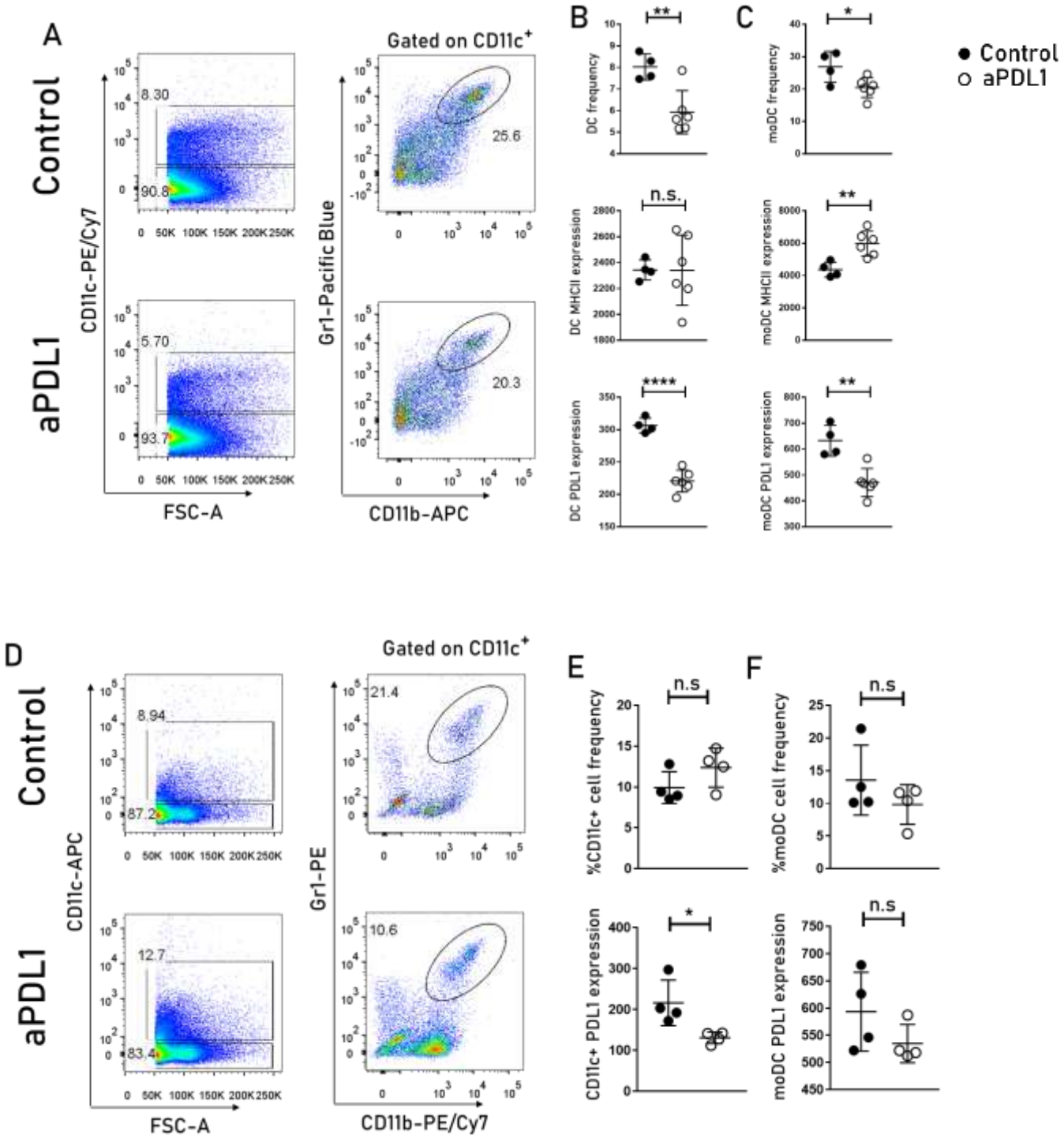


*Figure 11 Circulating and splenic DC and moDC of anti PD-L1 and control treated melanoma bearing mice. A) Representative gating strategy for the assessment of DC and subsets in spleen of tumor mice. B) Frequency and expression of MHCII and PD-L1 on splenic DC in tumor bearing mice, ****P<0.0001 **P<0.01 *P<0.05 results are presented as means ± SD. C) Frequency and expression of MHCII and PD-L1 on splenic moDC in tumor bearing mice, **P<0.01 *P<0.05 results are presented as means ± SD. D) Representative gating strategy for the assessment of circulating DC and subsets of tumor mice. E) Frequency and PD-L1 expression of circulating DC in tumor bearing mice, *P<0.05 results are presented as means ± SD. F) Frequency and PD-L1 expression of circulating moDC in tumor bearing mice, results are presented as means ± SD. Representative results from 3 independent experiments for each tissue*

PD-L1 immunotherapy leads to changes in lymphoid cell population composition in the periphery

Based on our results for the frequency as well as the activation status of the myeloid cells in the periphery upon anti-PD-L1 immunotherapy, and because some of those cell (e.g. DC, moDC) have been implicated on the activation of the adaptive immune compartment and especially T cell responses, we looked for the T cell responses to immunotherapy.

Treg cells are markedly reduced in the spleen of tumor bearing mice.

T cell in peripheral tissues have been used as predictive but also as read out for the success of immunotherapy. T cell receptor (TCR) expression is often accompanied by the expression of the CD3 molecule (accessory chain of TCR), so the expression of CD3 is capable of denoting cells expressing TCR. For further characterization of the T cell compartment into effector and cytotoxic cells the markers CD4 and CD8 are used respectively.

In spleen of treated mice, we report a decrease in the frequency of total CD3 expressing cells. We also found that inside the total CD3 population there was a marked decrease of the Cytotoxic T cells (CD8+ cells) while frequency of effector T cell (CD4+ cells) frequency remained unchanged (*figure 12A, B*).

The frequency of regulatory T cell population has often been associated with poor prognosis and is a marker for immunosuppression in the periphery for this reason we sought to determine the effect of anti-PD-L1 immunotherapy has to those cells. We observed decreased frequency of T regulatory cells in the spleen of treated mice (*figure 12A, B*).

The same effect was also reported for the tumor draining lymph nodes (Data not shown)

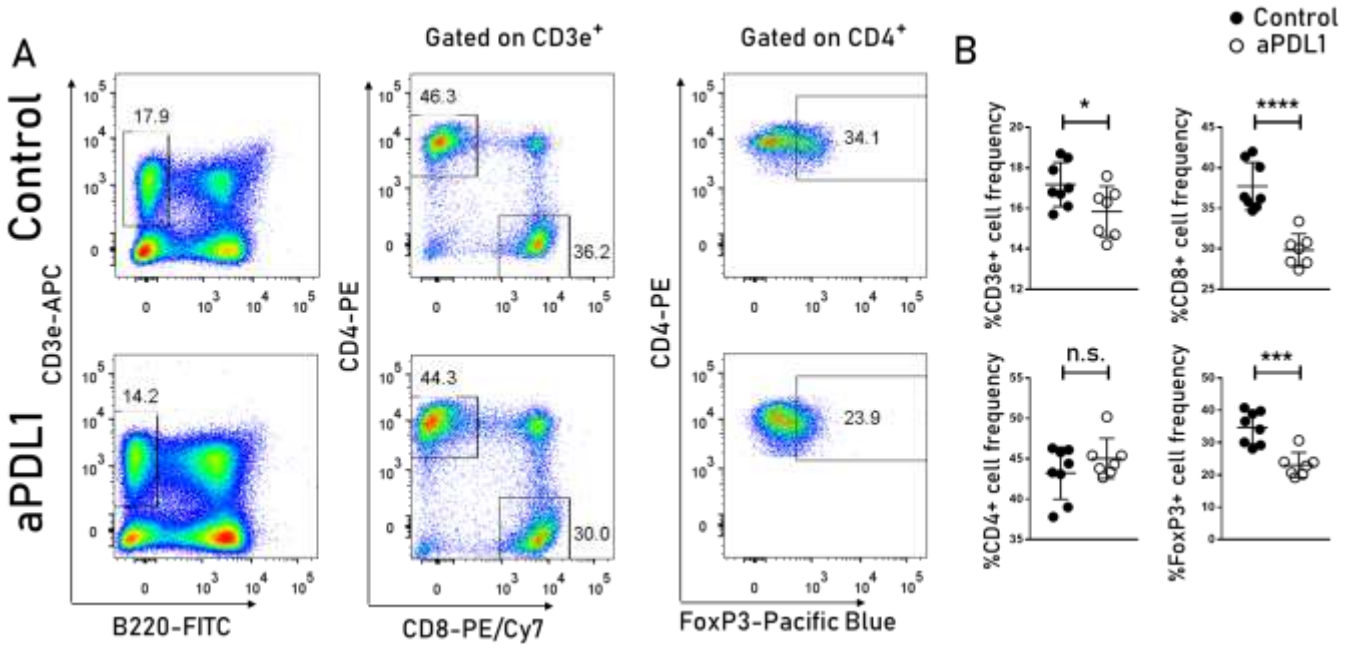


*Figure 12 Splenic Lymphoid cell analysis of anti PD-L1 and control treated melanoma bearing mice. A) Representative gating strategy for the assessment of T cells in spleen of tumor mice. B) Frequency of total T (CD3+), effector (CD3+CD4+), cytotoxic (CD3+CD8+) and T regulatory (CD3+CD4+FoxP3+) cells in tumor bearing mice, ****P<0.0001 ***P<0.001 *P<0.05 results are presented as means ± SD.*

PD-L1 immunotherapy induces changes in hematopoiesis

Stemming from the changes in the frequency of immune cells in the periphery we were inclined to investigate the generation of these cell types. The primary organ tasked with hematopoiesis, and as a result immune cell generation, is the bone marrow.

PD-L1 is expressed on LSK cells, and other Lin- cells in the bone marrow of tumor bearing mice.

In order to have a more comprehensive view of the hematopoietic prosses we first analyzed the bone marrow lineage negative compartment, cells which in the FACS setting express low to no levels of markers associated with mature cell types. The lineage negative cells in the bone marrow comprise a heterogeneous population and

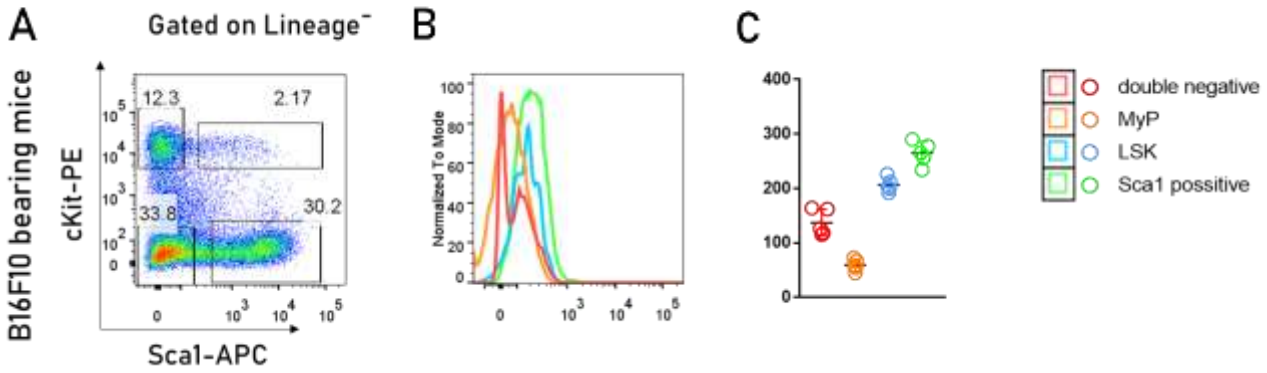


Figure 13 Bone marrow lineage negative cell express PD-L1. A) Representative gating strategy of lineage negative cells B) Histogram of the PD-L1 expression on each population, colors corresponding to the legend. C) MFI for the PD-L1 marker on the different lineage negative population, results are presented as means ± SD Representative results from 3 independent experiments

can be further divided based on expression of markers such as the SCF1R (cKit) and the stem cell antigen 1 (Sca1) molecule. Lineage negative cells positive for both the cKit and the Sca1 marker are termed the LSK and in the literature are considered to be an enriched population for the HSC[158]. Lineage negative cells that are positive for the cKit marker but negative for the Sca1 marker, termed from now on MyP, are heterogeneous population containing common myeloid progenitors as well as the granulocyte/ monocyte progenitors[159], while the lineage negative sca1 positive but cKit negative cells, Sca1 positive cells, are thought to contain some lymphoid potential[160].

To investigate the possibility that those cells might be targeted by anti-PD-L1 immunotherapy we determined the levels of PD-L1 expression during early melanoma formation and found that expression of PD-L1 was high on LSK and Sca1 positive cells, less so o double negative cells and almost absent in the MyP population(*Figure 12A, B, C*).

PD-L1 immunotherapy is associated with increased frequency of the LSK cell population

Based on the observation that LSK cells express high levels of PD-L1 and given the biologic importance of those cell, we interrogated the effect anti-PD-L1 immunotherapy might have on them. Upon anti-PD-L1 treatment, we report a massive increase of the LSK population and as a measure of the capacity of anti-PD-L1 immunotherapy to target those cells we report the decrease in the MFI for the PD-L1 marker.

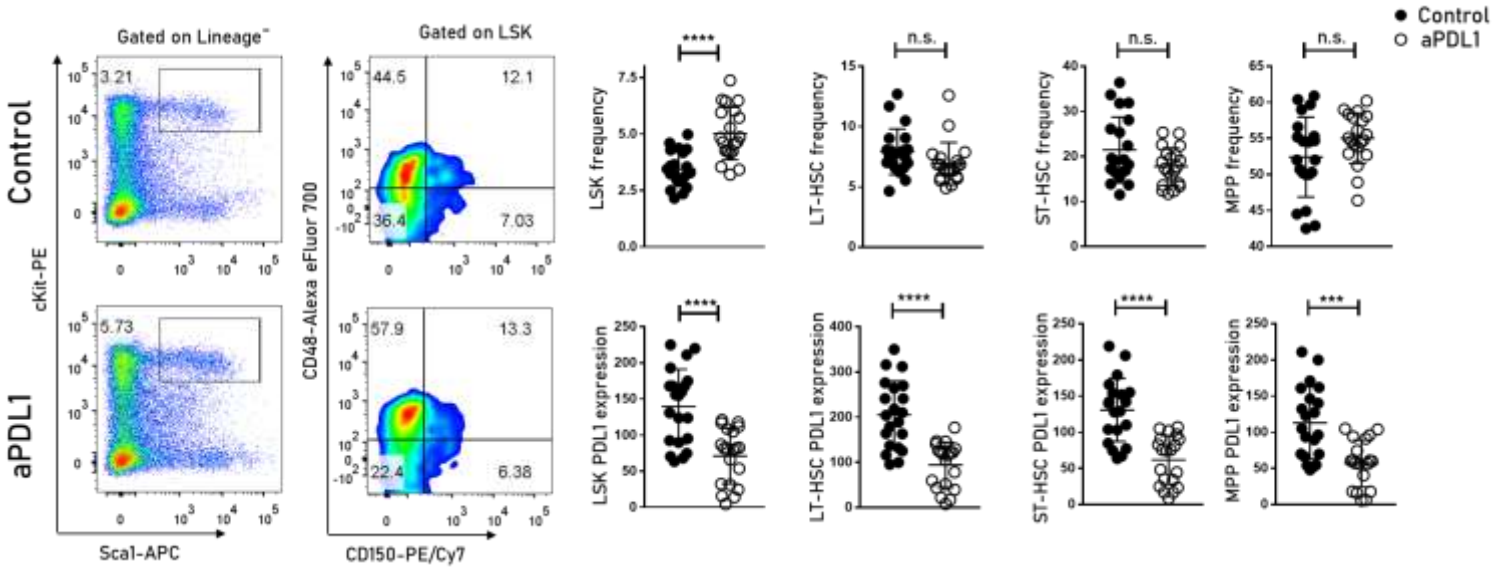


*Figure 14 LSK expand upon anti-PD-L1 treatment. A) representative gating strategy of LSK and their subset. B) Frequency and expression PD-L1 on bone marrow LSK and their subsets on tumor bearing mice, ****P<0.0001 ***P<0.001 results are presented as means ± SD. Combined results from 5 independent experiments*

The LKS compartment as previously stated is highly heterogeneous and can be further divided by the expression of markers such as CD150 and CD48 in order to identify bona fide HSC with long term- (CD150+ CD48- cell, LT-HSC), short term- (CD150-

CD48- cells, ST-HSC) repopulating capacity or progenitors with multilineage differentiation potential (CD150- CD48+ cells, MPP) Upon treatment we report only slight shifts in the frequency of each of those populations with the decrease of LT- and ST-HSC and an increase of the MPP population. The decrease of the MFI for PD-L1 is also evident in each of the LSK subpopulations(*Figure14*).

Increased LSK frequencies are accompanied by changes in their transcriptome profile

In order to delineate the intrinsic effect of anti-PD-L1 immunotherapy targeting of the LSK compartment, at the transcriptome level, we sorted highly purified LSK cells (sorting efficiency >90%) and performed bulk RNA-seq. Next, we performed gene expression analysis of sorted LSK cells from control and ant-PD-L1 treated mice. 279 DEGs (|FC|> 1.5, Pvalue<0.05) between control and treated mice, of which 228 were upregulated and 51 were downregulated (*Figure15*).

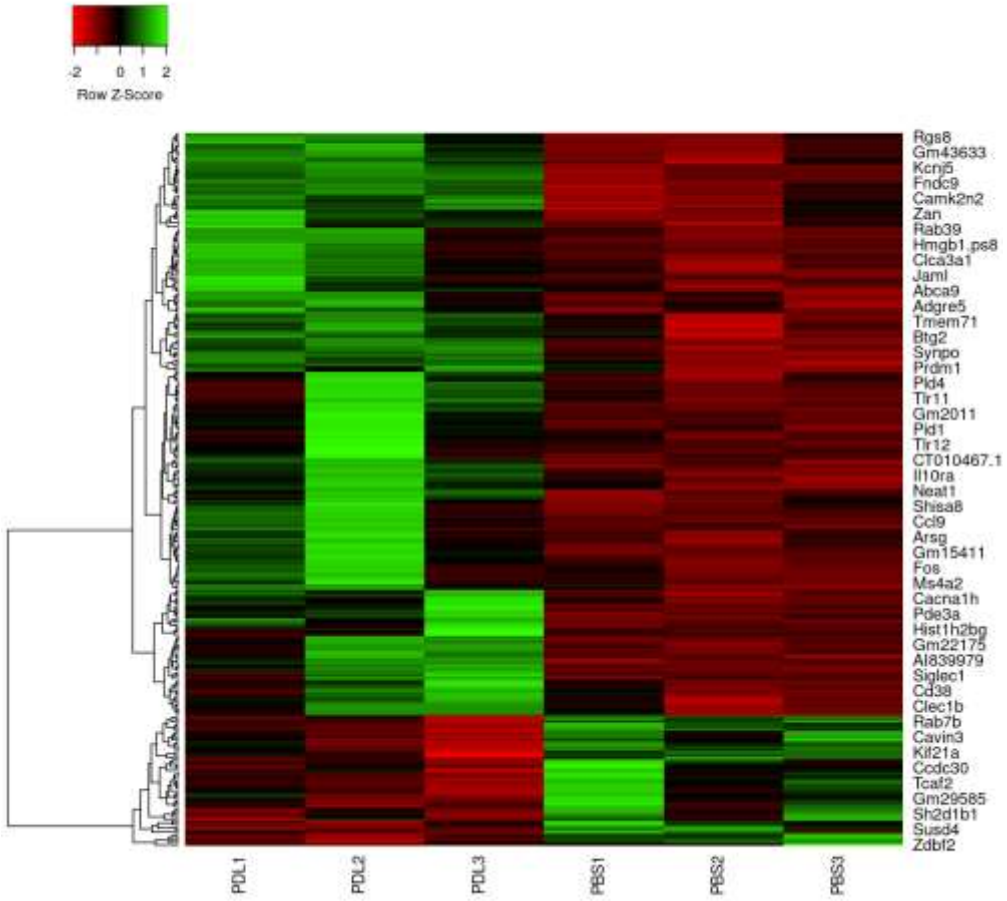
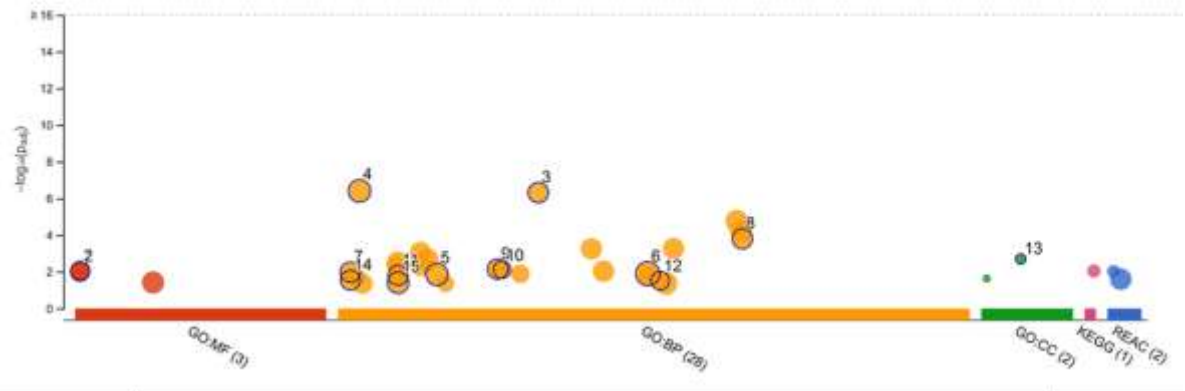


Figure 15 Heatmap of differentially expressed genes on LSK from treated versus control tumor bearing mice

Gene ontology and pathway analysis revealed that the DEGs were implicated in response to cytokine, hemopoiesis, leukocyte chemotaxis, cytokine production, leukocyte migration and inflammatory response(*Figure16*).



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0001216	DNA-binding transcription activator activity	
2	GO:MF	GO:0001228	DNA-binding transcription activator activity, RNA ...	
3	GO:BP	GO:0034097	response to cytokine	
4	GO:BP	GO:0002376	immune system process	
5	GO:BP	GO:0010628	positive regulation of gene expression	
6	GO:BP	GO:0048518	positive regulation of biological process	
7	GO:BP	GO:0001816	cytokine production	
8	GO:BP	GO:0071345	cellular response to cytokine stimulus	
9	GO:BP	GO:0030097	hemopoiesis	
10	GO:BP	GO:0030595	leukocyte chemotaxis	
11	GO:BP	GO:0006954	inflammatory response	
12	GO:BP	GO:0050900	leukocyte migration	
13	GO:CC	GO:0035976	transcription factor AP-1 complex	
14	GO:BP	GO:0001817	regulation of cytokine production	
15	GO:BP	GO:0006955	immune response	

Figure 16 Gene ontology and pathway enrichment analysis of differentially expressed genes. The analysis was done on the g:profiler online platform

Interestingly among the DEGs were genes associated with lymphoid cell differentiation. Upon treatment LSK cells were found to upregulate IKZF3 a transcription factor that leads to lymphoid differentiation[161,162], MycL an isoform of the c-myc transcription factor was found upregulated, this isoform in the literature has been found to be expressed in CLP at higher levels compared to LSK or MyP[163,164]. Lastly, we report the upregulation of Blimp1 a transcription factor that plays a central role on B cell maturation, possibly hinting to the activation of a lymphoid differentiation program[165,166].

Concurrent with the signatures described above we also report the upregulation of various genes related to myeloid differentiation upon treatment. To that effect we report the upregulation of both miR23a and miR27a, the upregulation of those two

miRNA have been shown to lead to increased myelopoiesis[167–169]. We also report the upregulation of the transcription factor Zfp366, this transcription factor has been found to promote DC differentiation[170].

Additionally, we found signature genes relating to quiescence to be deregulated in the anti-PD-L1 treated LSK, this signatures include the downregulation of TCF15 and Mpdz, two genes that are highly expressed on LT-HSC and have been found to be downregulated during exit from quiescence[171,172]. Upon treatment we also report the upregulation of the AP-1 transcription factor complex, this has been associated with increased proliferation[173].

PD-L1 immunotherapy lead in the composition of the bone marrow extracellular fluid

In order to fully comprehend the effect that anti-PD-L1 immunotherapy has on LSK we also sought to investigate possible changes in niche biochemistry that might be introduced upon treatment. To that end we flushed out the femur bones of control and treated mice, we then prepared bone marrow fluid as described on the Materials and Methods section. Upon analysis of those samples with mass spectrometry we found 272 proteins to be deferentially expressed (DEP) (|FC|> 1.5), of which 146 were up- and 126 were downregulated (*Figure17*). Unfortunately, the low number of samples pe group as well as the relative harshness of the algorithm used for the detection of the DEP did not reveal any statistically significant differences.

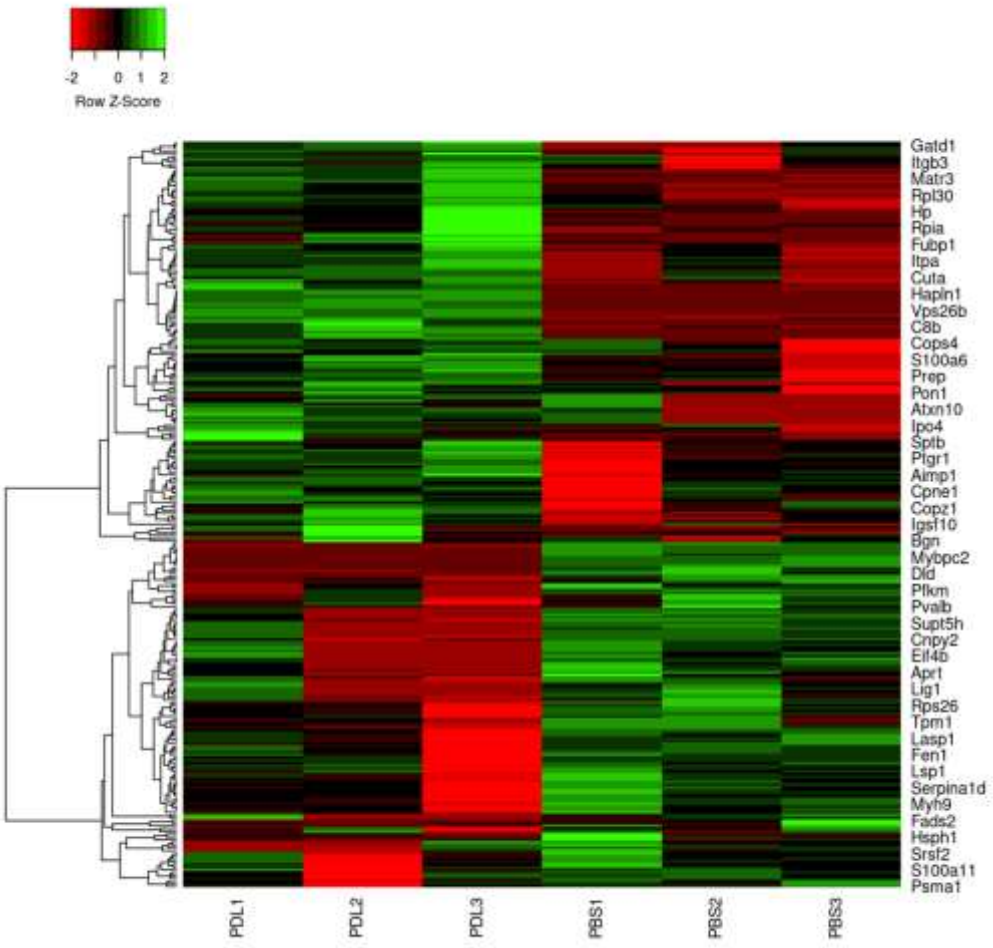
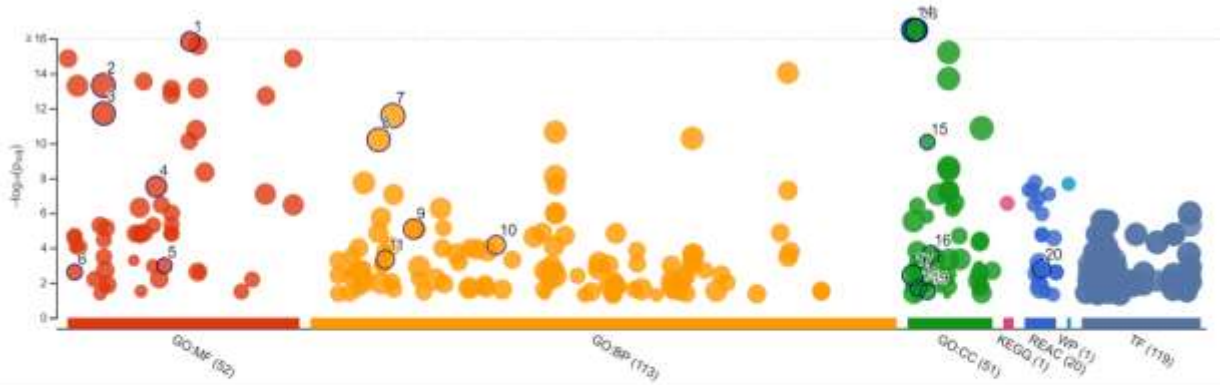


Figure 17 Heatmap of the differentially expressed proteins of the bone marrow extracellular fluid from control and anti-PD-L1 treated mice.

Even though no criteria for the statistical significance were used for the filtering of the differential expressed proteins we conducted Gene ontology and pathway enrichment analysis for the total of the DEP. As shown in *Figure 18* multiple molecular function, biological prosses and cellular component terms were found to be enriched in the list of DEP provided. Among those the most intriguing are the terms related to metabolic prosses, response to cytokine, extracellular matrix, focal adhesion and cell-substrate junction, as many of those have been implicated in HSC function and differentiation.



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:MF	GO:0036094	small molecule binding	1.453×10^{-16}
2	GO:MF	GO:0005488	binding	4.821×10^{-14}
3	GO:MF	GO:0005515	protein binding	1.957×10^{-12}
4	GO:MF	GO:0019899	enzyme binding	3.010×10^{-8}
5	GO:MF	GO:0031072	heat shock protein binding	1.039×10^{-2}
6	GO:MF	GO:0002020	protease binding	2.398×10^{-2}
7	GO:BP	GO:0009987	cellular process	2.628×10^{-12}
8	GO:BP	GO:0008152	metabolic process	6.296×10^{-11}
9	GO:BP	GO:0015031	protein transport	8.143×10^{-6}
10	GO:BP	GO:0034097	response to cytokine	6.749×10^{-5}
11	GO:BP	GO:0009306	protein secretion	4.614×10^{-4}
12	GO:CC	GO:0005737	cytoplasm	3.469×10^{-32}
13	GO:CC	GO:0005829	cytosol	3.674×10^{-28}
14	GO:CC	GO:0005622	intracellular	2.770×10^{-21}
15	GO:CC	GO:0022626	cytosolic ribosome	8.503×10^{-11}
16	GO:CC	GO:0031012	extracellular matrix	2.307×10^{-4}
17	GO:CC	GO:0005576	extracellular region	3.654×10^{-3}
18	GO:CC	GO:0005925	focal adhesion	1.059×10^{-2}
19	GO:CC	GO:0030055	cell-substrate junction	3.172×10^{-2}
20	REAC	REAC:R-MMU-6...	Neutrophil degranulation	1.588×10^{-3}

Figure 18 Gene ontology and pathway enrichment analysis of differentially expressed proteins. The analysis was done on the g:profiler online platform

PD-L1 immunotherapy is associated with shifts in myelopoiesis

In view of the transcriptomic changes in the LSK compartment that are associated with the induction of myelopoiesis we sought to study the later stages of myelopoiesis. To that goal we characterized with flow cytometry the myeloid progenitor compartment.

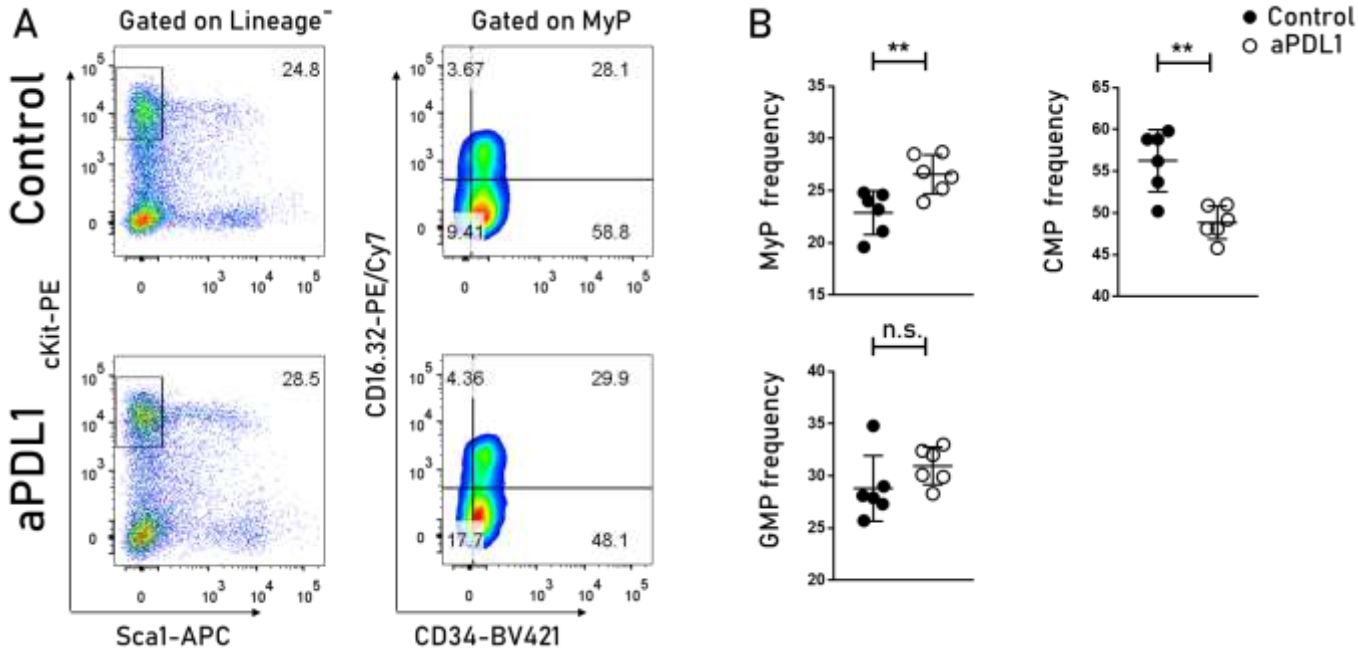


*Figure 19 Bone marrow myeloid progenitors (MyP) expand upon anti-PD-L1 immunotherapy. A) Representative gating strategy for MyP and their subsets in the bone marrow of tumor mice. B) Frequency of MyP, CMPs (lin-cKit+CD34+CD16.32- cells) and GMPs(lin-cKit+CD34+CD16.32+ cells) in tumor bearing mice, **P<0.01 results are presented as means ± SD. Representative of 3 experiments*

Upon therapy we denoted an increase in the frequency of MyP cell population(*Figure19B*). Because the MyP population is heterogeneous and contains functionally distinct populations we proceeded to investigate the common myeloid –(cKit+CD34+CD16.32-)as well as the granulocyte/monocyte progenitor (cKit+CD34+CD16.32+) population(*Figure19A*). We also report increased frequency of GMPs and decreased frequency of CMPs consistent with active myelopoiesis[159,174–176](*Figure19B*).

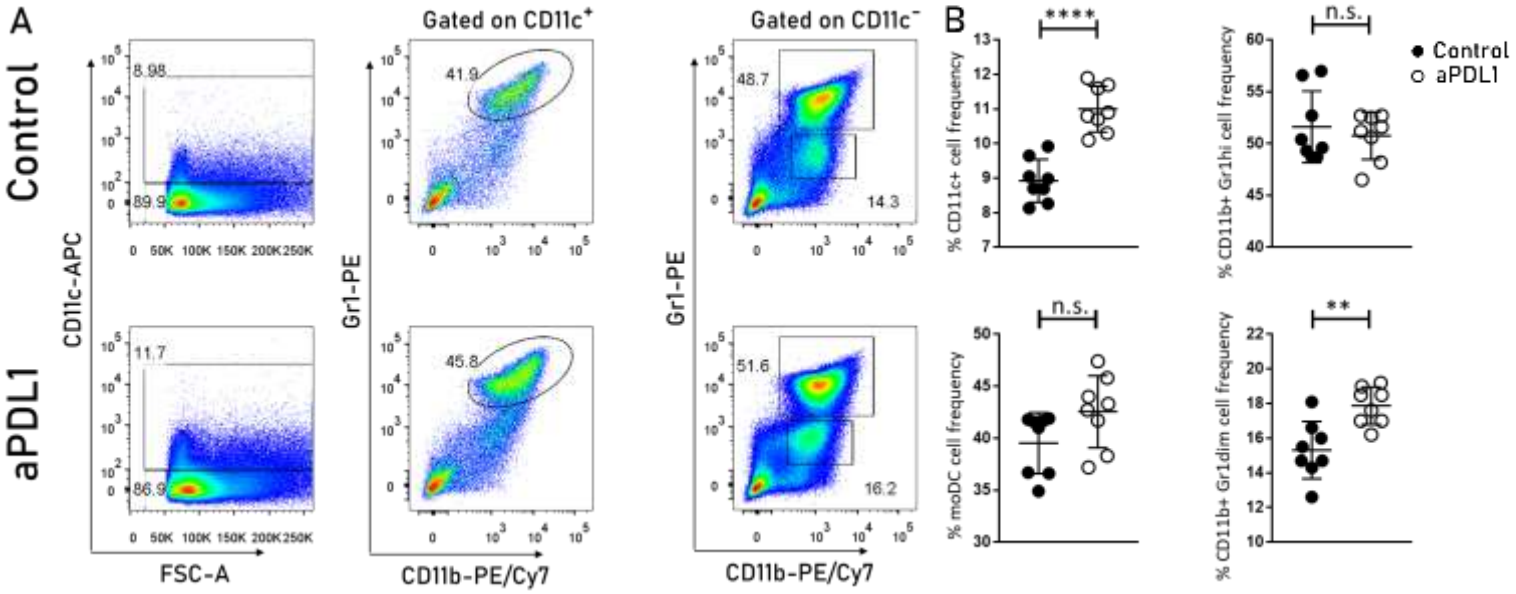


*Figure 20 Bone marrow myeloid compartment altered composition upon anti-PD-L1 immunotherapy. A) Representative gating strategy for myeloid cells and their subsets in the bone marrow of tumor mice. B) Frequency of DC (CD11c+), moDC (CD11c+CD11b+Gr1+) and CD11c-CD11b+Gr1hi or Gr1dim cells in tumor bearing mice, **P<0.01 results are presented as means ± SD. Combined results from two independent experiments*

Lastly, we interrogated the mature myeloid cells compartment in order to functionally characterized the output of myelopoiesis activation. We report the increased frequencies of total DCs (CD11c+ cells), with a slight increase in the frequency of moDCs (*Figure20B*). We also report the increase of a population reminiscent of immature myeloid cells (*Figure20B*). The CD11c- CD11b+ Gr1dim has been recently characterized in the murine bone marrow Yang and Luo et al showed that this cell type represents the immature monocyte population of the bone marrow and expresses the Ly6C marker[177]. No change in the frequency of the polymorphonuclear compartment (CD11c- CD11b+ Gr1high) was observed(*Figure20B*).

PD-L1 immunotherapy is associated with shifts in lymphopoiesis

To investigate the signatures related to the lymphoid lineage we sought to characterize the effect of anti-PD-L1 immunotherapy on downstream common lymphoid progenitors (CLPs). CLPs can be characterized by o expression of the cKit and Sca1 marker and also the expression of FLT3 and the IL7Ra[160,178](*Figure 22A*). Upon treatment we found that the parent gate of CLPs (cKitlo Sca1lo) had increased frequency, and that frequency of CLPs was also increased (*Figure 22B*).

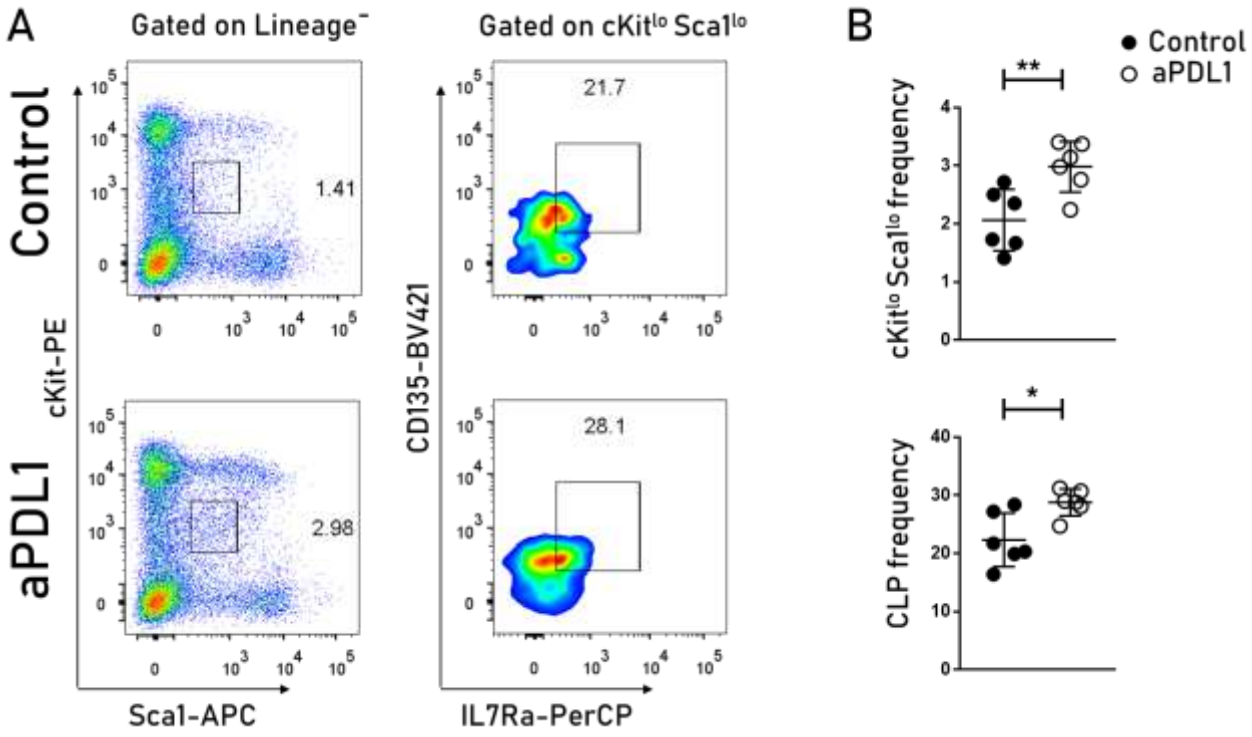


*Figure 21 Bone marrow lymphoid progenitors expand upon anti-PD-L1 immunotherapy. A) Representative gating strategy for CLP in the bone marrow of tumor mice. B) Frequency of the heterogeneous population cKitloSca1lo and the CLPs (cKitlo Sca1lo CD135+ IL7Ra+) in tumor bearing mice, **P<0.01 *P<0.05 results are presented as means ± SD. Representative of 3 experiments*

In order to have a more comprehensive understanding of the output of lymphopoiesis upon anti-PD-L1 immunotherapy we assessed the generation of mature lymphoid cell subsets in the bone marrow.

Total T cells were found to be unchanged in regard to their frequency(*Figure 21A, B*). The same effect was observed for CD4 and CD8 positive T cells(*Figure 21A, B*). Lastly

only a slight increase was reported in the frequency of the total B220+ cells(*Figure 21A, B*). This cell population encompasses the B cell population.

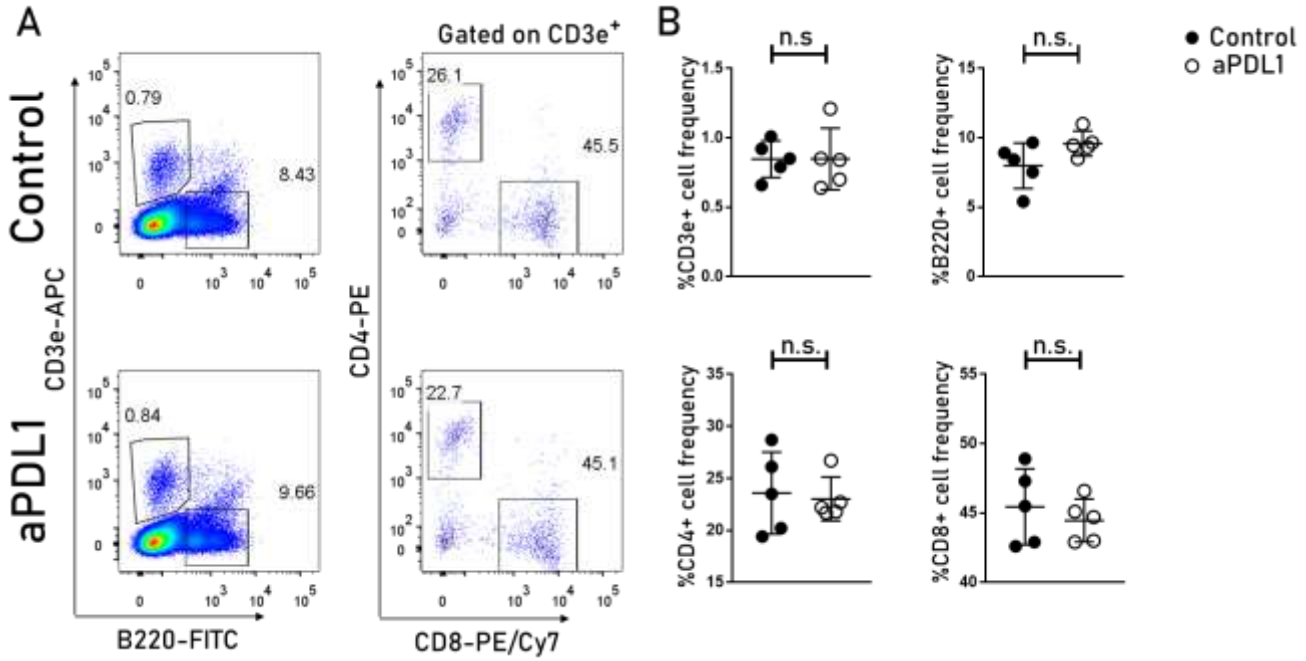


Figure 22 Bone marrow lymphoid compartment altered composition upon anti-PD-L1 immunotherapy. A) Representative gating strategy for lymphoid cells and their subsets in the bone marrow of tumor mice. B) Frequency of CD3+, B220+, CD4 T cells and CD8 T cells in tumor bearing mice, results are presented as means ± SD.

It is important to state that the results presented in Figure 21 have been repeated but the two independent experiment are not in agreement in respect to some of the population frequency presented. In order to have a clearer understanding on the generation of mature lymphoid cells the experiment must be repeated. Also, because of intricacies when attempting the fixation of bone marrow cells for intracellular cell staining, the analysis of FoxP3+ Treg cell was not possible. As Treg cells are an important part of cancer immune evasion and have been recently identified as niche components the investigation of these cells is necessary for a holistic understanding of the effect of anti-PD-L1 immunotherapy on lymphopoiesis in the bone marrow.

Increased frequency of LSK upon PD-L1 immunotherapy are mediated by the adaptive immune system.

Recently, it was shown that T cells are utilized by tumor cells that promote the secretion of TNFa from CD4 T cells, which in turn through the tumor vasculature reaches the bone marrow and leads to increased LSK frequencies and increased CMP and MDSC generation[110].

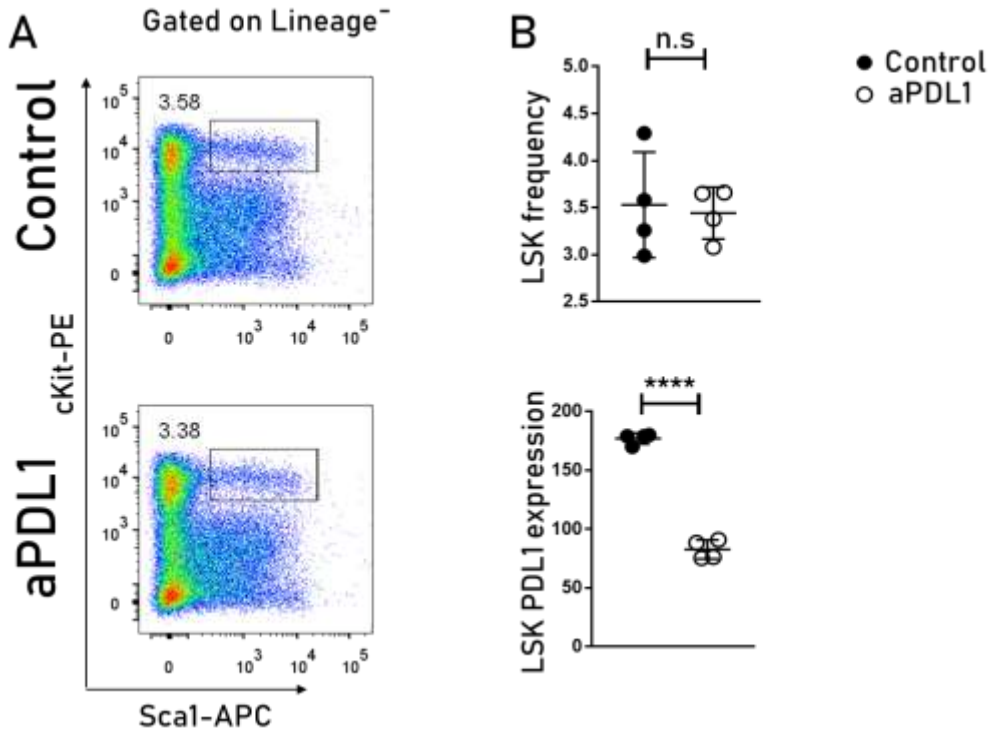


*Figure 23 LSK compartment remains unchanged upon anti-PD-L1 treatment on RAG1KO mice. A) representative gating strategy of LSK. B) Frequency and expression of PD-L1 on bone marrow LSK on tumor bearing Rag1KO mice, ****P<0.0001 results are presented as means ± SD.*

In order to address the contribution of the adaptive immune compartment in our setting we inoculated melanoma in Rag1 knock out (Rag1KO) mice, mice lacking the adaptive immune compartment, and subsequently analyzed their LSK compartment n the bone marrow(*Figure 23A*). We report that the effect seen in the C57BL/6 mice, the expansion of the LSK population, was abolished in the Rag1KO setting even though anti-PD-L1 immunotherapy did target those cells(*Figure 23B*). Together this results show that the adaptive immune compartment is probably responsible for the expansion of the LSK compartment in the bone marrow of tumor bearing mice. This effect could be mediated by T cell secreted TNFa as was described by Al Sayed et al.

Discussion

In this work we reasoned that that anti-PD-L1 therapy alters or reprograms the function of MDSCs from T cell suppressor to T-cell stimulators, and that this functional alteration might eventually lead to arrest of tumor development. We also theorized that the altered phenotype associated with this treatment scheme might stem from a central reprograming of the hematopoietic process. To address this, we investigated the generation of myeloid and lymphoid cells upon anti-PD-L1 immunotherapy.

Our data demonstrate that upon treatment PD-L1-expressing MDSC frequency is reduced in the spleen as well as the circulation of tumor bearing mice, a marked upregulation of the MHCII molecule is also observed on splenic MDSC, suggesting that upon treatment MDSC are activated to change their function form suppressive to immunogenic and possibly able to present antigens to the adaptive immune compartment. This was accompanied by the shift towards the pmn-MDSC phenotype which, according to the literature, is relatively less immunosuppressive[179,180], but can inhibit T cell function on an antigen dependent manner[181]. Among other myeloid cell population, anti-PD-L1 immunotherapy lead to the expansion of circulating DC and moDC, a subset of DC with established role in anti-tumor immune response [155–157], and in the spleen a reduction of their frequency associated whoever with increased levels of MHCII expression, suggesting once more the activation of these cell types[182–184].

However, upon treatment the adaptive immune compartment was found to be significantly affected, with a decrease in overall T cell population. Importantly the cytotoxic T cell (CTL) compartment was found to be greatly decrease in the spleen and tumor draining lymph nodes of treated tumor bearing mice. Although the frequency of the total CD4 population remained unchanged upon treatment, a decrease in the T regulatory population was observed.

When we interrogated the long-term response to anti-PD-L1 immunotherapy in regard to tumor volume kinetics, in brief tumor volume was measured daily from day 10 after tumor inoculation until day 15 using calipers. No significant change was observed between the control and treated groups (data not shown). This effect might

be explained due to the low frequency of immune cell capable of mounting an anti-tumor immune response observed at the earlier tumor stage .

Although this therapy scheme seems to activate the myeloid cells to contribute towards the anti-tumor immune response, the large absence of cells of the adaptive immunity capable of exerting this anti-tumor effect, like CLTs, might dampen the effect of anti-PD-L1 monotherapy. In order to be able to have a holistic understanding of the effect of anti-PD-L1 therapy in the periphery, it is crucial to interrogate the tumor immune contexture. This will enable us to investigate whether the significant decrease of adaptive immune cells is due to increased homing to the tumor site.

In order to have a better understanding of mechanisms responsible for the changes in the frequency as well as the activation status of cells in the periphery we then investigate their generation in the bone marrow. Interestingly, anti-PD-L1 therapy promoted the expansion of PD-L1-expressing Hematopoietic Stem and Progenitor cells (LSK) in the bone marrow.

The unexpected finding of the LSK expansion led us to theorize that the binding of anti-PD-L1 immunotherapy on the surface of HSPCs, determined as the decrease in MFI, might lead to transcriptomic changes that are responsible for the expansion effect we see with flowcytometry. We also hypothesized that this transcriptomic changes might influence the generation of committed progenitors from the HSPCs.

To that goal we sorted LSK from treated and control mice in order to perform bulk-RNA-seq. 279 DEG ($|FC| > 1.5$, $Pvalue < 0.05$) were found between control and treated mice, of which 228 were upregulated and 51 were downregulated. Gene ontology and pathway enrichment analysis was conducted. The DEG were enriched for signatures related to cytokine production, response to cytokine, hemopoiesis, leukocyte chemotaxis, leukocyte migration and inflammatory response.

Interestingly genes related to quiescence like TCF15 and Mpdz[171,172] were found to be downregulated in treated LSK, while the transcription factor complex AP1, that has been implicated in proliferation, was found upregulated[173]. Additionally genes related

to both myeloid (miR23a, miR27a, Zfp366) and lymphoid (Ikzf3, mycL, Blimp1) cell differentiation were found upregulated in treated LSK[162–165,167,168,170,185,186]. This signatures point towards a phenotype of accelerated hematopoiesis in which LSK cell differentiated towards both the myeloid and the lymphoid lineage at a high rate. This was further evident when myeloid and lymphoid progenitor cells were assessed with flowcytometry. We found decreased CMPs and increased GMPs frequency, characteristic of active myelopoiesis, also we found increased CLPs frequency consistent with the RNA-seq results.

In a recently published study Strauss et al. showed that upon melanoma inoculation CMP and GMP accumulate in the bone marrow, also that GMP upregulate the expression of the PD-1 checkpoint molecule. They lastly showed that genetic ablation of the PD-1 or pharmacological inhibition of PD-1, using anti-PD-1 antibody, was able to diminish GMP accumulation and lead to differentiation of myeloid cells with effector function[187]. This suggest checkpoint molecules are crucial for the induction of emergency myelopoiesis, and that checkpoint blockade therapy can reach the bone marrow affecting progenitor cell generation and differentation.

At this point we sought to address that although increased frequency of CLPs is observed in the bone marrow mature lymphoid cells have been found to be decreased. Whether an arrest in the differentiation of lymphoid cell process exist downstream of the CLP stages is a hypothesis that warrants further testing. Also, for mature T cells to exit eventually in the periphery, they must pass successfully through, positive as well as negative selection from the thymus, whether that is the point in T cell differentiation responsible for the decrease in mature T cell population in response to ant-PD-L1 treatment remains to be seen. At least one point of evidence hinting to that direction comes from Sage et al, who showed that PD-L1-PD-1 interaction leads to decreased follicular helper cell development and immunosuppressive function[188–190].

Lastly in order to delineate the involvement of the adaptive immune compartment in bone marrow response to immunotherapy we performed experiments in Rag1KO mice. We found that the when the adaptive immune compartment was absent, the

expansion of the LSK compartment was diminished. This evidence suggests, a cell extrinsic signal, stemming from the adaptive immune compartment, as the cause for the LSK expansion. This signal might act in tandem with anti-PD-L1 immunotherapy to produce the significant expansion of LSK we see upon treatment. Whether that is the case it remains to be seen. At least one point of evidence that supports the involvement of the adaptive immune compartment, and especially CD4 T cells, comes from Al Sayed et al who showed that, CD4 T cell produced, TNFa was able to expand LSK and myeloid progenitors and generate MDSC[110].

In conclusion, our results provide evidence that myeloid-specific PD-L1 targeting might mediate a possible myeloid cell-intrinsic effect, for the upregulation of molecules capable of initiating the anti-tumor immune response. This effect may represent a key mechanism by which PD-L1 blockade induces anti-tumor function not only through a periphery related effect, but also through the bone marrow compartment. Further investigation of the mechanisms involved and the additional search of way to stimulate or expand the adaptive immune compartment upon anti-PD-L1 immunotherapy might improve the outcome of cancer immunotherapy.

The literature on the response of patients to anti-PDL1 immunotherapy in regard to changes in their immune compartment is lacking, despite that fact a recently published paper has investigated the changes of immune cell population in peripheral blood of patient with NSCLC. Zhuo et al, showed that upon treatment the patient with partial response or stable disease had decreased frequency of Treg and MDSC populations compared to patients with progressive disease, they also reported that patients with progressive disease had decreased frequency of the total CD4 and CD8 T cell populations compared to the group of Stable disease or partial responders[191]. Evidence also suggests that atezolizumab treatment leads to increase in neoantigen-specific T cell in the peripheral blood of responding NSCLC patients[192]. Lastly in combination therapy of anti-PD-L1 immunotherapy with anti-VEGF therapy has been shown to lead to increase CD8 T cell homing to the tumor site on metastatic Renal cell carcinoma[193].

Future Directions

Assessment of the early tumor immune contexture

In order to have a holistic understanding of the effect of anti-PD-L1 immunotherapy on peripheral tissue resident cells, we will perform experiments according to *Figure8*. At day 8 of tumor inoculation we will isolate the tumor and through flowcytometry we will investigate the myeloid and lymphoid cell presence.

Single cell ATAC- and RNA-seq of HSC from control and anti-PD-L1 treated mice

The LSK population used for the bulk RNA-seq analysis has been revealed as highly heterogeneous and the low number of DEG might be attributed to that heterogeneity. In order to achieve results that will represent the transcriptomic and chromatin accessibility state on each of the subsets of the LSK compartment we will isolate LSK cells from anti-PD-L1 and control treated mice through FACS sorting and perform single cell ATAC- and RNA-seq analysis as described by Nestorowa et al[184–186]. This will allow an in-depth assessment of the changes in the epigenetic as well as the transcriptomic state of each HSC subset.

HSC response to other immunotherapy schemes

In order to investigate, weather our results are specific for anti-PD-L1 immunotherapy, we will inoculate B16F10 melanoma cells in mice that will receive PD-1, CTLA4 or control therapy according to the experimental setting shown in *Figure8*. On day8 upon tumor inoculation the frequency of the LSK compartment will be assessed through flowcytometry.

HSC response upon anti-PD-L1 treatment on Lewis Lung Carcinoma (LLC) inoculated mice

To inquire about the specificity of our results for melanoma, we will perform experiment, according to *Figure8*, with LLC inoculated mice. Upon Day 8 of tumor

inoculation we will perform flowcytometric analysis for the frequency as well as the expression of PD-L1 on the LSK compartment.

Investigation of anti-PD-L1 therapy response in human bone marrow samples

Unfortunately, bone marrow biopsy is not an approved procedure for melanoma patients. In order to translate our results in the human setting we will assess, through flowcytometric analysis, the frequency as well as PD-L1 expression on human bone marrow HSC form patient with lymphoma, as controls aged matched lymphoma patient that receive standard of care treatment.

Investigation of the differentiation potential of HSC form melanoma bearing PD-L1 knock out and WT mice.

In order to assess the functionality of PD-L1 interaction in HSC differentiation we will perform experiment, according to the experimental design shown on *Figure8*, on PD-L1 knock out (PD-L1ko) mice. In brief B16F10 melanoma cells will be inoculated in PD-L1ko mice (CD45.2 expressing) and WT controls (CD45.2 expressing). On Day 8 after melanoma inoculation bone marrow LSK cells, from both groups will be isolated and transplanted in a congenic mouse transplantation model (CD45.1 expressing host). In order to characterize HSC differentiation unperturbed from the regenerative environment introduced by myeloablation, we will perform transplantation experiments on NBSGW mice, mice that on steady state lack their immune compartment, that do not need to be irradiated before transplantation.

In vitro assessment of myeloid output of anti-PD-L1 treated melanoma bearing mice.

In order to directly link the changes in myeloid compartment, at peripheral tissues, to the effect we see in the bone marrow, we will isolate LSK cell from PD-L1 treated and control melanoma bearing mice. Subsequently LSK will be plated on MethoCult 3434 medium. In these setting LSK cells differentiate towards the myeloid lineage due

to cytokines present in the culture medium. This will help us to identify differences in the myeloid output of control vs PD-L1 treated LSK cells.

Bone marrow Treg response to anti-PD-L1 therapy

Bone marrow resident CD150+ T regulatory cells have been shown to contribute to HSC quiescence, engraftment potential on the transplantation setting and have been found in close proximity to HSC[68]. The fact that adaptive immunity seems to be indispensable for the expansion of LSK upon anti-PD-L1 treatment leads us to theorize that CD150+ Tregs in the bone marrow might be prime targets of PD-L1 immunotherapy. Due to intricacies in the process of nuclear staining in bone marrow cell suspension, Fox-P3-GFP mice will be used, following the experimental design shown on *Figure8*, to investigate the role of bone marrow Tregs on anti-PD-L1 immunotherapy. With flow cytometry we will investigate changes in the frequency as well as the activation status of bone marrow CD150+ Tregs. In order to gain insight to the localization of bone marrow CD150+ Treg cells upon anti-PD-L1 immunotherapy we will perform immunofluorescence experiment on bone marrow mount as described by Holzwarth et al [187,188]. The previously described experiment will bring into focus the response of bone marrow CD150+ Treg to immunotherapy and will serve as an indicator for the effect of anti-PD-L1 immunotherapy on HSC niche formation.

Acknowledgments

The present study was done at the Biomedical Research Foundation of the Academy of Athens under the supervision of Dr Verginis, were I was fortunate to interact with an array of incredibly supportive and kindhearted people. I express the warmest thanks to Dr Verginis for the assignment of this project, the supervision and advice during the course of my Master thesis. I am especially thankful to Dr Katerina Hatzioannou, Dr Themis Alissafi and Dr Maria Grigoriou for their valuable help, advice, training and technical support. I would also like to thank the members of our laboratory Athina Varveri, Miranta Papadopoulou, Iosif Papafragos, Elpida Neofotistou and Athina Boumpas for maintaining the most pleasant work environment I had ever participated in and their help and encouragement when trouble presented itself. Special thanks go to Athina Boumpas without the help of which, on the technical as well as the theoretical levels, these results would not be possible. Additionally, I would like to extend my thanks to the lab manager Nikos Malissovas for the continuous effort and care to keep the lab supplied with everything the project required.

Finally, I could not forget my family and friends. Without their help and their support, the completion of my study would not be feasible. I thank them for the support, help, immense understanding, patience and love.

References

1. Esfahani, K. *et al.* A review of cancer immunotherapy: from the past, to the present, to the future. *Curr. Oncol.* **27**, (2019).

2. Michot, J. M. *et al.* Immune-related adverse events with immune checkpoint blockade: a comprehensive review. *European Journal of Cancer* **54**, 139–148 (2016).

3. Michot, J.-M. *et al.* Immune-related bone marrow failure following anti-PD1 therapy. *European Journal of Cancer* **80**, 1–4 (2017).

4. Shiuan, E. *et al.* Thrombocytopenia in patients with melanoma receiving immune checkpoint inhibitor therapy. *j. immunotherapy cancer* **5**, 8 (2017).

5. Swann, J. B. & Smyth, M. J. Immune surveillance of tumors. *J. Clin. Invest.* **117**, 1137–1146 (2007).

6. Lou, Y. *et al.* Epithelial–Mesenchymal Transition Is Associated with a Distinct Tumor Microenvironment Including Elevation of Inflammatory Signals and Multiple Immune Checkpoints in Lung Adenocarcinoma. *Clin Cancer Res* **22**, 3630–3642 (2016).

7. Mak, M. P. *et al.* A Patient-Derived, Pan-Cancer EMT Signature Identifies Global Molecular Alterations and Immune Target Enrichment Following Epithelial-to-Mesenchymal Transition. *Clinical Cancer Research* **22**, 609–620 (2016).

8. Ghiringhelli, F. *et al.* Tumor cells convert immature myeloid dendritic cells into TGF-β–secreting cells inducing CD4+CD25+ regulatory T cell proliferation. *Journal of Experimental Medicine* **202**, 919–929 (2005).

9. Ugel, S., De Sanctis, F., Mandruzzato, S. & Bronte, V. Tumor-induced myeloid deviation: when myeloid-derived suppressor cells meet tumor-associated macrophages. *Journal of Clinical Investigation* **125**, 3365–3376 (2015).

10. Birbrair, A. & Frenette, P. S. Niche heterogeneity in the bone marrow: Cellular complexity of the HSC niche in the BM. *Ann. N.Y. Acad. Sci.* **1370**, 82–96 (2016).

11. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation. *Cell* **144**, 646–674 (2011).

12. Giles, A. J. *et al.* Activation of Hematopoietic Stem/Progenitor Cells Promotes Immunosuppression Within the Pre–metastatic Niche. *Cancer Res* **76**, 1335–1347 (2016).

13. Eble, J. A. & Niland, S. The extracellular matrix in tumor progression and metastasis. *Clin Exp Metastasis* **36**, 171–198 (2019).

14. Gong, L. *et al.* Promoting effect of neutrophils on lung tumorigenesis is mediated by CXCR2 and neutrophil elastase. *Mol Cancer* **12**, 154 (2013).

15. Houghton, A. M. *et al.* Neutrophil elastase–mediated degradation of IRS-1 accelerates lung tumor growth. *Nat Med* **16**, 219–223 (2010).

16. Campbell, D. J. Control of Regulatory T Cell Migration, Function, and Homeostasis. *J.I.* **195**, 2507–2513 (2015).

17. Awad, R. M., De Vlaeminck, Y., Maebe, J., Goyvaerts, C. & Breckpot, K. Turn Back the TIMe: Targeting Tumor Infiltrating Myeloid Cells to Revert Cancer Progression. *Front. Immunol.* **9**, 1977 (2018).

18. Levental, K. R. *et al.* Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell* **139**, 891–906 (2009).

19. Noy, R. & Pollard, J. W. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* **41**, 49–61 (2014).

20. Giraldo, N. A. *et al.* The clinical role of the TME in solid cancer. *Br J Cancer* **120**, 45–53 (2019).

21. Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nat Med* **19**, 1423–1437 (2013).

22. Cen, Y. *et al.* The characteristic of tumor immune microenvironment in pulmonary carcinosarcoma. *Immunotherapy* **12**, 323–331 (2020).

23. Chew, V., Toh, H. C. & Abastado, J.-P. Immune Microenvironment in Tumor Progression: Characteristics and Challenges for Therapy. *Journal of Oncology* **2012**, 1–10 (2012).

24. Spranger, S., Dai, D., Horton, B. & Gajewski, T. F. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell* **31**, 711-723.e4 (2017).

25. Marcucci, F., Rumio, C. & Corti, A. Tumor cell-associated immune checkpoint molecules – Drivers of malignancy and stemness. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer* **1868**, 571–583 (2017).

26. Rittmeyer, A. *et al.* Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *The Lancet* **389**, 255–265 (2017).

27. Gun, S. Y., Lee, S. W. L., Sieow, J. L. & Wong, S. C. Targeting immune cells for cancer therapy. *Redox Biology* **25**, 101174 (2019).

28. Palucka, A. K. & Coussens, L. M. The Basis of Oncoimmunology. *Cell* **164**, 1233–1247 (2016).

29. Schlecker, E. *et al.* Tumor-Infiltrating Monocytic Myeloid-Derived Suppressor Cells Mediate CCR5-Dependent Recruitment of Regulatory T Cells Favoring Tumor Growth. *J.I.* **189**, 5602–5611 (2012).

30. Zamarron, B. F. & Chen, W. Dual Roles of Immune Cells and Their Factors in Cancer Development and Progression. *Int. J. Biol. Sci.* **7**, 651–658 (2011).

31. Bubeník, J. Tumour MHC class I downregulation and immunotherapy (Review). *Oncol Rep* (2003) doi:10.3892/or.10.6.2005.

32. DeNardo, D. G. *et al.* Leukocyte Complexity Predicts Breast Cancer Survival and Functionally Regulates Response to Chemotherapy. *Cancer Discovery* **1**, 54–67 (2011).

33. Vilgelm, A. E. & Richmond, A. Chemokines Modulate Immune Surveillance in Tumorigenesis, Metastasis, and Response to Immunotherapy. *Front. Immunol.* **10**, 333 (2019).

34. Payne, A. S. & Cornelius, L. A. The Role of Chemokines in Melanoma Tumor Growth and Metastasis. *Journal of Investigative Dermatology* **118**, 915–922 (2002).

35. Garner, H. & de Visser, K. E. Immune crosstalk in cancer progression and metastatic spread: a complex conversation. *Nat Rev Immunol* (2020) doi:10.1038/s41577-019-0271-z.

36. Raman, D., Baugher, P. J., Thu, Y. M. & Richmond, A. Role of chemokines in tumor growth. *Cancer Letters* **256**, 137–165 (2007).

37. Vesely, M. D., Kershaw, M. H., Schreiber, R. D. & Smyth, M. J. Natural Innate and Adaptive Immunity to Cancer. *Annu. Rev. Immunol.* **29**, 235–271 (2011).

38. Rigo, A. *et al.* Macrophages may promote cancer growth via a GM-CSF/HB-EGF paracrine loop that is enhanced by CXCL12. *Mol Cancer* **9**, 273 (2010).

39. Biswas, S. K. & Mantovani, A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol* **11**, 889–896 (2010).

40. Mishra, P., Banerjee, D. & Ben-Baruch, A. Chemokines at the crossroads of tumor-fibroblast interactions that promote malignancy. *Journal of Leukocyte Biology* **89**, 31–39 (2011).

41. Lau, T.-S. *et al.* Cancer cell-derived lymphotoxin mediates reciprocal tumour-stromal interactions in human ovarian cancer by inducing CXCL11 in fibroblasts: Lymphotoxin and tumour-stromal interactions. *J. Pathol.* **232**, 43–56 (2014).

42. Bhatia, M., Bonnet, D., Murdoch, B., Gan, O. I. & Dick, J. E. A newly discovered class of human hematopoietic cells with SCID-repopulating activity. *Nat Med* **4**, 1038–1045 (1998).

43. Chou, S. & Lodish, H. F. Fetal liver hepatic progenitors are supportive stromal cells for hematopoietic stem cells. *Proc Natl Acad Sci USA* **107**, 7799–7804 (2010).

44. Roy, V. & Verfaillie, C. M. Expression and function of cell adhesion molecules on fetal liver, cord blood and bone marrow hematopoietic progenitors:

Implications for anatomical localization and developmental stage specific regulation of hematopoiesis. *Experimental Hematology* 11 (1999).

45. Mazo, I. B., Massberg, S. & von Andrian, U. H. Hematopoietic stem and progenitor cell trafficking. *Trends in Immunology* **32**, 493–503 (2011).

46. Tober, J. *et al.* Maturation of hematopoietic stem cells from prehematopoietic stem cells is accompanied by up-regulation of PD-L1. *J. Exp. Med.* **215**, 645–659 (2018).

47. Pietras, E. M., Warr, M. R. & Passegué, E. Cell cycle regulation in hematopoietic stem cells. *The Journal of Cell Biology* **195**, 709–720 (2011).

48. King, K. Y. & Goodell, M. A. Inflammatory modulation of HSCs: viewing the HSC as a foundation for the immune response. *Nat Rev Immunol* **11**, 685–692 (2011).

49. Spangrude, G. J., Heimfeld, S. & Weissman, I. L. Purffication and Characterization of Mouse Hematopoietic Stem Cells. 5.

50. Seita, J. & Weissman, I. L. Hematopoietic stem cell: self-renewal versus differentiation: Hematopoietic stem cell. *WIREs Syst Biol Med* **2**, 640–653 (2010).

51. Kiel, M. J. *et al.* SLAM Family Receptors Distinguish Hematopoietic Stem and Progenitor Cells and Reveal Endothelial Niches for Stem Cells. *Cell* **121**, 1109–1121 (2005).

52. Upadhaya, S. *et al.* Kinetics of adult hematopoietic stem cell differentiation in vivo. *Journal of Experimental Medicine* **215**, 2815–2832 (2018).

53. Nerlov, C. & Graf, T. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes & Development* **12**, 2403–2412 (1998).

54. DeKoter, R. P. & Singh, H. Regulation of B Lymphocyte and Macrophage Development by Graded Expression of PU.l. **288**, 3 (2000).

55. DeKoter, R. P., Lee, H.-J. & Singh, H. PU.1 Regulates Expression of the Interleukin-7 Receptor in Lymphoid Progenitors. *Immunity* **16**, 297–309 (2002).

56. Wilson, A. & Trumpp, A. Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* **6**, 93–106 (2006).

57. Smith, J. N. P. & Calvi, L. M. Concise Review: Current Concepts in Bone Marrow Microenvironmental Regulation of Hematopoietic Stem and Progenitor Cells. *STEM CELLS* **31**, 1044–1050 (2013).

58. Nilsson, S. K., Johnston, H. M. & Coverdale, J. A. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* **97**, 2293–2299 (2001).

59. Arai, F. *et al.* Tie2/Angiopoietin-1 Signaling Regulates Hematopoietic Stem Cell Quiescence in the Bone Marrow Niche. *Cell* **118**, 149–161 (2004).

60. Visnjic, D. *et al.* Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* **103**, 3258–3264 (2004).

61. Stier, S. *et al.* Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. *Journal of Experimental Medicine* **201**, 1781–1791 (2005).

62. Petit, I. *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* **3**, 687–694 (2002).

63. Takubo, K. & Suda, T. Roles of the hypoxia response system in hematopoietic and leukemic stem cells. *Int J Hematol* **95**, 478–483 (2012).

64. Shima, H. *et al.* Reconstitution activity of hypoxic cultured human cord blood CD34-positive cells in NOG mice. *Biochemical and Biophysical Research Communications* **378**, 467–472 (2009).

65. Kopp, H.-G., Avecilla, S. T., Hooper, A. T. & Rafii, S. The Bone Marrow Vascular Niche: Home of HSC Differentiation and Mobilization. *Physiology* **20**, 349–356 (2005).

66. Yin, T. The stem cell niches in bone. *Journal of Clinical Investigation* **116**, 1195–1201 (2006).

67. Hirata, Y. *et al.* CD150high Bone Marrow Tregs Maintain Hematopoietic Stem Cell Quiescence and Immune Privilege via Adenosine. *Cell Stem Cell* **22**, 445-453.e5 (2018).

68. Nilsson, S. K. *et al.* Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. *Blood* **106**, 1232–1239 (2005).

69. Cordeiro Gomes, A. *et al.* Hematopoietic Stem Cell Niches Produce Lineage-Instructive Signals to Control Multipotent Progenitor Differentiation. *Immunity* **45**, 1219–1231 (2016).

70. Ding, L., Saunders, T. L., Enikolopov, G. & Morrison, S. J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457–462 (2012).

71. Ding, L. & Morrison, S. J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231–235 (2013).

72. Greenbaum, A. *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**, 227–230 (2013).

73. Zhou, B. O. *et al.* Bone marrow adipocytes promote the regeneration of stem cells and haematopoiesis by secreting SCF. *Nat Cell Biol* **19**, 891–903 (2017).

74. Poulos, M. G. *et al.* Endothelial Jagged-1 Is Necessary for Homeostatic and Regenerative Hematopoiesis. *Cell Reports* **4**, 1022–1034 (2013).

75. de Graaf, C. A. & Metcalf, D. Thrombopoietin and hematopoietic stem cells. *Cell Cycle* **10**, 1582–1589 (2011).

76. Hinge, A. & Filippi, M.-D. Deconstructing the Complexity of TGFβ Signaling in Hematopoietic Stem Cells: Quiescence and Beyond. *Curr Stem Cell Rep* **2**, 388–397 (2016).

77. Bruns, I. *et al.* Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat Med* **20**, 1315–1320 (2014).

78. CD82/KAI1 Maintains the Dormancy of Long-Term Hematopoietic Stem Cells through Interaction with DARC-Expressing Macrophages | Elsevier Enhanced Reader. https://reader.elsevier.com/reader/sd/pii/S193459091600014X?token=C8D35A35

8D4C177AB316853C5F7DE3B1A415EB526B2691415682F3F10FCA9EC757DCE68E6
06BAF3D3B10599AA66B7283 doi:10.1016/j.stem.2016.01.013.

79. Kitamura, T., Qian, B.-Z. & Pollard, J. W. Immune cell promotion of metastasis. *Nat Rev Immunol* **15**, 73–86 (2015).

80. Wolf, A. M. *et al.* Increase of Regulatory T Cells in the Peripheral Blood of Cancer Patients. *Clin Cancer Res* **9**, 606–612 (2003).

81. Liu, Z., Kim, J. H., Falo, L. D. & You, Z. Tumor Regulatory T Cells Potently Abrogate Antitumor Immunity. *J Immunol* **182**, 6160–6167 (2009).

82. Prevalence of Regulatory T Cells Is Increased in Peripheral Blood and Tumor Microenvironment of Patients with Pancreas or Breast Adenocarcinoma | The Journal of Immunology. https://www.jimmunol.org/content/169/5/2756.long.

83. Youn, J.-I., Nagaraj, S., Collazo, M. & Gabrilovich, D. I. Subsets of Myeloid-Derived Suppressor Cells in Tumor Bearing Mice. *J Immunol* **181**, 5791–5802 (2008).

84. Tumor-induced Tolerance and Immune Suppression Depend on the C/EBPbeta Transcription Factor - PubMed. https://pubmed.ncbi.nlm.nih.gov/20605485/.

85. de Vrij, J. *et al.* Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells: Glioblastoma Vesicles Affect Monocytic Cells. *Int. J. Cancer* **137**, 1630–1642 (2015).

86. Wu, W.-C. *et al.* Circulating hematopoietic stem and progenitor cells are myeloid-biased in cancer patients. *Proceedings of the National Academy of Sciences* **111**, 4221–4226 (2014).

87. Takizawa, H., Boettcher, S. & Manz, M. G. Demand-adapted regulation of early hematopoiesis in infection and inflammation. *Blood* **119**, 2991–3002 (2012).

88. Mitroulis, I., Kalafati, L., Hajishengallis, G. & Chavakis, T. Myelopoiesis in the Context of Innate Immunity. *J Innate Immun* **10**, 365–372 (2018).

89. Takizawa, H. & Manz, M. G. Impact of inflammation on early hematopoiesis and the microenvironment. *Int J Hematol* **106**, 27–33 (2017).

90. Taghipour Fard Ardekani, M. *et al.* Evaluation of Pre-Treatment Serum Levels of IL-7 and GM-CSF in Colorectal Cancer Patients. *Int J Mol Cell Med* **3**, 27–34 (2014).

91. Revoltella, R. P., Menicagli, M. & Campani, D. Granulocyte–macrophage colony-stimulating factor as an autocrine survival-growth factor in human gliomas. *Cytokine* **57**, 347–359 (2012).

92. Kast, R. E. *et al.* Glioblastoma-synthesized G-CSF and GM-CSF contribute to growth and immunosuppression: Potential therapeutic benefit from dapsone, fenofibrate, and ribavirin. *Tumour Biol.* **39**, 101042831769979 (2017).

93. Greenbaum, A. M. & Link, D. C. Mechanisms of G-CSF-mediated hematopoietic stem and progenitor mobilization. *Leukemia* **25**, 211–217 (2011).

94. Christopher, M. J., Rao, M., Liu, F., Woloszynek, J. R. & Link, D. C. Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *J. Exp. Med.* **208**, 251–260 (2011).

95. Wilson, A. *et al.* Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell* **135**, 1118–1129 (2008).

96. Berardi, R. *et al.* Prognostic models to predict survival in patients with advanced non-small cell lung cancer treated with first-line chemo- or targeted therapy. *Oncotarget* **7**, 26916–26924 (2016).

97. el Aziz, L. M. A. Blood neutrophil-lymphocyte ratio predicts survival in locally advanced cancer stomach treated with neoadjuvant chemotherapy FOLFOX 4. *Med. Oncol.* **31**, 311 (2014).

98. Yildirim, M., Demir Cendek, B. & Filiz Avsar, A. Differentiation between benign and malignant ovarian masses in the preoperative period using neutrophil-to-lymphocyte and platelet-to-lymphocyte ratios. *Mol Clin Oncol* **3**, 317–321 (2015).

99. Szkandera, J. *et al.* External validation of the derived neutrophil to lymphocyte ratio as a prognostic marker on a large cohort of pancreatic cancer patients. *PLoS ONE* **8**, e78225 (2013).

100. Yao, A. *et al.* High neutrophil-to-lymphocyte ratio predicts poor clinical outcome in patients with castration-resistant prostate cancer treated with docetaxel chemotherapy. *Int. J. Urol.* **22**, 827–833 (2015).

101. Galizia, G. *et al.* Neutrophil to lymphocyte ratio is a strong predictor of tumor recurrence in early colon cancers: A propensity score-matched analysis. *Surgery* **158**, 112–120 (2015).

102. Pistelli, M. *et al.* Pre-treatment neutrophil to lymphocyte ratio may be a useful tool in predicting survival in early triple negative breast cancer patients. *BMC Cancer* **15**, 195 (2015).

103. Gao, F. *et al.* Pretreatment neutrophil-lymphocyte ratio: an independent predictor of survival in patients with hepatocellular carcinoma. *Medicine (Baltimore)* **94**, e639 (2015).

104. Menetrier-Caux, C. *et al.* Inhibition of the Differentiation of Dendritic Cells From CD34+ Progenitors by Tumor Cells: Role of Interleukin-6 and Macrophage Colony-Stimulating Factor. *Blood* **92**, 4778–4791 (1998).

105. Ratta, M. *et al.* Dendritic cells are functionally defective in multiple myeloma: the role of interleukin-6. *Blood* **100**, 230–237 (2002).

106. Park, S.-J. *et al.* IL-6 Regulates In Vivo Dendritic Cell Differentiation through STAT3 Activation. *The Journal of Immunology* **173**, 3844–3854 (2004).

107. Hayashi, T. *et al.* Ex vivo induction of multiple myeloma–specific cytotoxic T lymphocytes. *Blood* **102**, 1435–1442 (2003).

108. Menetrier-Caux, C., Thomachot, M. C., Alberti, L., Montmain, G. & Blay, J. Y. IL-4 Prevents the Blockade of Dendritic Cell Differentiation Induced by Tumor Cells. *Cancer Res* **61**, 3096–3104 (2001).

109. Berberoglu, U., Yildirim, E. & Celen, O. Serum levels of tumor necrosis factor alpha correlate with response to neoadjuvant chemotherapy in locally advanced breast cancer. *Int. J. Biol. Markers* **19**, 130–134 (2004).

110. Al Sayed, M. F. *et al.* T-cell–Secreted TNFα Induces Emergency Myelopoiesis and Myeloid-Derived Suppressor Cell Differentiation in Cancer. *Cancer Res* **79**, 346–359 (2019).

111. Kamran, N. *et al.* Melanoma induced immunosuppression is mediated by hematopoietic dysregulation. *Oncoimmunology* **7**, (2017).

112. Mroczko, B., Szmitkowski, M. & Czygier, M. [Interleukin 3 (IL-3) in diagnosis and monitoring of non-small-cell lung cancer]. *Prz. Lek.* **56**, 763–766 (1999).

113. Ng, L. *et al.* Post-Operative Plasma Osteopontin Predicts Distant Metastasis in Human Colorectal Cancer. *PLoS One* **10**, (2015).

114. Thoms, J. W. *et al.* Plasma osteopontin as a biomarker of prostate cancer aggression: relationship to risk category and treatment response. *Br J Cancer* **107**, 840–846 (2012).

115. Fremder, E. *et al.* Tumor-derived microparticles induce bone marrow-derived cell mobilization and tumor homing: A process regulated by osteopontin. *International Journal of Cancer* **135**, 270–281 (2014).

116. McAllister, S. S. *et al.* Systemic Endocrine Instigation of Indolent Tumor Growth Requires Osteopontin. *Cell* **133**, 994–1005 (2008).

117. Lund, S. A. *et al.* Osteopontin mediates macrophage chemotaxis via α_4 and α_9 integrins and survival via the α_4 integrin. *J. Cell. Biochem.* **114**, 1194–1202 (2013).

118. Weber, G. F. *et al.* Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. *Journal of Leukocyte Biology* **72**, 752–761 (2002).

119. O'Donnell, R. K. *et al.* VEGF-A/VEGFR Inhibition Restores Hematopoietic Homeostasis in the Bone Marrow and Attenuates Tumor Growth. *Cancer Research* **76**, 517–524 (2016).

120. Kaplan, R. N. *et al.* VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* **438**, 820–827 (2005).

121. Qu, P., Yan, C. & Du, H. Matrix metalloproteinase 12 overexpression in myeloid lineage cells plays a key role in modulating myelopoiesis, immune suppression, and lung tumorigenesis. *Blood* **117**, 4476–4489 (2011).

122. Ning, Y. *et al.* Interleukin-8 is associated with proliferation, migration, angiogenesis and chemosensitivity in vitro and in vivo in colon cancer cell line models. *Int. J. Cancer* **128**, 2038–2049 (2011).

123. Guichard, C. *et al.* Interleukin-8-induced Priming of Neutrophil Oxidative Burst Requires Sequential Recruitment of NADPH Oxidase Components into Lipid Rafts. *J. Biol. Chem.* **280**, 37021–37032 (2005).

124. Chakrabarti, S. & Patel, K. D. Regulation of matrix metalloproteinase-9 release from IL-8-stimulated human neutrophils. *Journal of Leukocyte Biology* **78**, 279–288 (2005).

125. Li, X. *et al.* Lung tumor exosomes induce a pro-inflammatory phenotype in mesenchymal stem cells via NFκB-TLR signaling pathway. *J Hematol Oncol* **9**, 42 (2016).

126. Kaplan, R. N., Psaila, B. & Lyden, D. Bone marrow cells in the 'pre-metastatic niche': within bone and beyond. *Cancer Metastasis Rev* **25**, 521–529 (2007).

127. Kaplan, R. N., Psaila, B. & Lyden, D. Niche-to-niche migration of bone-marrow-derived cells. *Trends in Molecular Medicine* **13**, 72–81 (2007).

128. Peinado, H. *et al.* Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* **18**, 883–891 (2012).

129. Topalian, S. L., Weiner, G. J. & Pardoll, D. M. Cancer Immunotherapy Comes of Age. *JCO* **29**, 4828–4836 (2011).

130. Chen, D. S., Irving, B. A. & Hodi, F. S. Molecular Pathways: Next-Generation Immunotherapy—Inhibiting Programmed Death-Ligand 1 and Programmed Death-1. *Clin Cancer Res* **18**, 6580–6587 (2012).

131. Kitsou, M., Ayiomamitis, G. D. & Zaravinos, A. High expression of immune checkpoints is associated with the TIL load, mutation rate and patient survival in colorectal cancer. *International Journal of Oncology* **57**, 237–248 (2020).

132. Hargadon, K. M., Johnson, C. E. & Williams, C. J. Immune checkpoint blockade therapy for cancer: An overview of FDA-approved immune checkpoint inhibitors. *International Immunopharmacology* **62**, 29–39 (2018).

133. Francisco, L. M., Sage, P. T. & Sharpe, A. H. The PD-1 Pathway in Tolerance and Autoimmunity. *Immunol Rev* **236**, 219–242 (2010).

134. Butte, M. J., Keir, M. E., Phamduy, T. B., Freeman, G. J. & Sharpe, A. H. PD-L1 interacts specifically with B7-1 to inhibit T cell proliferation. *Immunity* **27**, 111–122 (2007).

135. Paterson, A. M. *et al.* The PD-L1:B7-1 pathway restrains diabetogenic effector T cells in vivo. *J Immunol* **187**, 1097–1105 (2011).

136. Zhao, Y. *et al.* PD-L1:CD80 Cis-Heterodimer Triggers the Co-stimulatory Receptor CD28 While Repressing the Inhibitory PD-1 and CTLA-4 Pathways. *Immunity* **51**, 1059-1073.e9 (2019).

137. Atefi, M. *et al.* Effects of MAPK and PI3K Pathways on PD-L1 Expression in Melanoma. *Clin Cancer Res* **20**, 3446–3457 (2014).

138. Dermani, F. K., Samadi, P., Rahmani, G., Kohlan, A. K. & Najafi, R. PD-1/PD-L1 immune checkpoint: Potential target for cancer therapy. *Journal of Cellular Physiology* **234**, 1313–1325 (2019).

139. Rizvi, N. A. *et al.* Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. *The Lancet Oncology* **16**, 257–265 (2015).

140. Robert, C. *et al.* Pembrolizumab versus Ipilimumab in Advanced Melanoma. *New England Journal of Medicine* **372**, 2521–2532 (2015).

141. Weber, J. *et al.* Phase I/II study of metastatic melanoma patients treated with nivolumab who had progressed after ipilimumab. *Cancer Immunol Res* **4**, 345–353 (2016).

142. Weber, R. *et al.* Myeloid-Derived Suppressor Cells Hinder the Anti-Cancer Activity of Immune Checkpoint Inhibitors. *Front. Immunol.* **9**, 1310 (2018).

143. Weber, J. S. *et al.* Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a

randomised, controlled, open-label, phase 3 trial. *The Lancet Oncology* **16**, 375–384 (2015).

144. Oliver, A. J. *et al.* Tissue-Dependent Tumor Microenvironments and Their Impact on Immunotherapy Responses. *Front Immunol* **9**, (2018).

145. Martins, F. *et al.* Adverse effects of immune-checkpoint inhibitors: epidemiology, management and surveillance. *Nature Reviews Clinical Oncology* **16**, 563–580 (2019).

146. Sullivan, R. J. *et al.* Atezolizumab plus cobimetinib and vemurafenib in BRAF - mutated melanoma patients. *Nature Medicine* **25**, 929–935 (2019).

147. Hamid, O. *et al.* Clinical activity, safety, and biomarkers of MPDL3280A, an engineered PD-L1 antibody in patients with locally advanced or metastatic melanoma (mM). *JCO* **31**, 9010–9010 (2013).

148. Bronte, V. *et al.* Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat Commun* **7**, 12150 (2016).

149. Umansky, V., Blattner, C., Gebhardt, C. & Utikal, J. The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression. *Vaccines* **4**, 36 (2016).

150. Groth, C. *et al.* Immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) during tumour progression. *Br J Cancer* **120**, 16–25 (2019).

151. Klarquist, J. S. & Janssen, E. M. Melanoma-infiltrating dendritic cells. *Oncoimmunology* **1**, 1584–1593 (2012).

152. Tannenbaum, C. S. *et al.* Mediators of Inflammation-Driven Expansion, Trafficking, and Function of Tumor-Infiltrating MDSCs. *Cancer Immunol Res* **7**, 1687–1699 (2019).

153. Lin, H. *et al.* Host expression of PD-L1 determines efficacy of PD-L1 pathway blockade–mediated tumor regression. *Journal of Clinical Investigation* **128**, 805–815 (2018).

154. Xiong, H. *et al.* Anti–PD-L1 Treatment Results in Functional Remodeling of the Macrophage Compartment. *Cancer Res* **79**, 1493–1506 (2019).

155. Kuhn, S., Yang, J. & Ronchese, F. Monocyte-Derived Dendritic Cells Are Essential for CD8+ T Cell Activation and Antitumor Responses After Local Immunotherapy. *Front. Immunol.* **6**, (2015).

156. Segura, E. *et al.* Human Inflammatory Dendritic Cells Induce Th17 Cell Differentiation. *Immunity* **38**, 336–348 (2013).

157. Dranoff, G. *et al.* Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 3539–3543 (1993).

158. Fan, Z. *et al.* Bone Marrow Derived Hematopoietic Stem and Progenitor Cells Infiltrate Allogeneic and Syngeneic Transplants. *Am J Transplant* **14**, 2869–2873 (2014).

159. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I. L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193–197 (2000).

160. Kumar, R., Fossati, V., Israel, M. & Snoeck, H.-W. Lin-Sca1+Kit- bone marrow cells contain early lymphoid-committed precursors that are distinct from common lymphoid progenitors. *J Immunol* **181**, 7507–7513 (2008).

161. Park, S.-M. *et al.* IKZF2 Drives Leukemia Stem Cell Self-Renewal and Inhibits Myeloid Differentiation. *Cell Stem Cell* **24**, 153-165.e7 (2019).

162. Morganl, B., Sun, L. & Avitahl, N. Aiolos, a lymphoid restrictedtranscriptionfactor that interactswith lkarosto regulatelymphocyte differentiation. 10.

163. Laurenti, E. *et al.* Hematopoietic Stem Cell Function and Survival Depend on c-Myc and N-Myc Activity. *Cell Stem Cell* **3**, 611–624 (2008).

164. Anderson, D. A., Murphy, T. L., Eisenman, R. N. & Murphy, K. M. The MYCL and MXD1 transcription factors regulate the fitness of murine dendritic cells. *Proc Natl Acad Sci USA* **117**, 4885–4893 (2020).

165. Chan, Y.-H. *et al.* Absence of the Transcriptional Repressor Blimp-1 in Hematopoietic Lineages Reveals Its Role in Dendritic Cell Homeostatic Development and Function. *J Immunol* **183**, 7039–7046 (2009).

166. Fu, S.-H., Yeh, L.-T., Chu, C.-C., Yen, B. L.-J. & Sytwu, H.-K. New insights into Blimp-1 in T lymphocytes: a divergent regulator of cell destiny and effector function. *J Biomed Sci* **24**, 49 (2017).

167. Kurkewich, J. L. *et al.* The miR-23a~27a~24-2 microRNA cluster buffers transcription and signaling pathways during hematopoiesis. *PLoS Genet* **13**, e1006887 (2017).

168. Mullenix, J. *et al.* The MiR-23a MicroRNA Cluster Inhibits B Cell Development. *Blood* **114**, 1465–1465 (2009).

169. Ben-Ami, O., Pencovich, N., Lotem, J., Levanon, D. & Groner, Y. A regulatory interplay between miR-27a and Runx1 during megakaryopoiesis. *Proceedings of the National Academy of Sciences* **106**, 238–243 (2009).

170. Chopin, M. *et al.* Transcription Factor PU.1 Promotes Conventional Dendritic Cell Identity and Function via Induction of Transcriptional Regulator DC-SCRIPT. *Immunity* **50**, 77-90.e5 (2019).

171. Ali, M. A. E. *et al.* Functional dissection of hematopoietic stem cell populations with a stemness-monitoring system based on NS-GFP transgene expression. *Sci Rep* **7**, 11442 (2017).

172. Rodriguez-Fraticelli, A. E. *et al.* Single-cell lineage tracing unveils a role for TCF15 in haematopoiesis. *Nature* **583**, 585–589 (2020).

173. Lee, S.-Y. *et al.* The Role of Heterodimeric AP-1 Protein Comprised of JunD and c-Fos Proteins in Hematopoiesis. *J. Biol. Chem.* **287**, 31342–31348 (2012).

174. Mitroulis, I. *et al.* Modulation of Myelopoiesis Progenitors Is an Integral Component of Trained Immunity. *Cell* **172**, 147-161.e12 (2018).

175. Hérault, A. *et al.* Myeloid progenitor cluster formation drives emergency and leukaemic myelopoiesis. *Nature* **544**, 53–58 (2017).

176. Griseri, T., McKenzie, B. S., Schiering, C. & Powrie, F. Dysregulated Hematopoietic Stem and Progenitor Cell Activity Promotes Interleukin-23-Driven Chronic Intestinal Inflammation. *Immunity* **37**, 1116–1129 (2012).

177. Yang, Y.-W. & Luo, W.-H. Recruitment of bone marrow CD11b+Gr-1+ cells by polymeric nanoparticles for antigen cross-presentation. *Sci Rep* **7**, 44691 (2017).

178. Kondo, M., Weissman, I. L. & Akashi, K. Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell* **91**, 661–672 (1997).

179. Cuenca, A. G. *et al.* A Paradoxical Role for Myeloid-Derived Suppressor Cells in Sepsis and Trauma. *Mol Med* **17**, 281–292 (2011).

180. Dolcetti, L. *et al.* Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. *European Journal of Immunology* **40**, 22–35 (2010).

181. Nagaraj, S. *et al.* Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. *Nat Med* **13**, 828–835 (2007).

182. Cho, K.-J., Ishido, S., Eisenlohr, L. C. & Roche, P. A. Activation of Dendritic Cells Alters the Mechanism of MHC Class II Antigen Presentation to CD4 T Cells. *The Journal of Immunology* (2020) doi:10.4049/jimmunol.1901234.

183. Walseng, E. *et al.* Dendritic Cell Activation Prevents MHC Class II Ubiquitination and Promotes MHC Class II Survival Regardless of the Activation Stimulus. *J Biol Chem* **285**, 41749–41754 (2010).

184. Villadangos, J. A. *et al.* MHC Class II Expression Is Regulated in Dendritic Cells Independently of Invariant Chain Degradation. *Immunity* **14**, 739–749 (2001).

185. Wilson, A. c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. *Genes & Development* **18**, 2747–2763 (2004).

186. Mitchell, J. L., Seng, A. & Yankee, T. M. Expression patterns of Ikaros family members during positive selection and lineage commitment of human thymocytes. *Immunology* **149**, 400–412 (2016).

187. Strauss, L. *et al.* Targeted deletion of PD-1 in myeloid cells induces antitumor immunity. *Sci. Immunol.* **5**, eaay1863 (2020).

188. Sage, P. T. *et al.* Dendritic Cell PD-L1 Limits Autoimmunity and Follicular T Cell Differentiation and Function. *The Journal of Immunology* **200**, 2592–2602 (2018).

189. Sage, P. T., Francisco, L. M., Carman, C. V. & Sharpe, A. H. The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nat Immunol* **14**, 152–161 (2013).

190. Zeng, W. *et al.* PDL1 blockage increases fetal resorption and Tfr cells but does not affect Tfh/Tfr ratio and B-cell maturation during allogeneic pregnancy. *Cell Death Dis* **11**, 119 (2020).

191. Zhuo, M. *et al.* The potential predictive value of circulating immune cell ratio and tumor marker in atezolizumab treated advanced non-small cell lung cancer patients. *CBM* **22**, 467–476 (2018).

192. Fehlings, M. *et al.* Late-differentiated effector neoantigen-specific CD8+ T cells are enriched in peripheral blood of non-small cell lung carcinoma patients responding to atezolizumab treatment. *j. immunotherapy cancer* **7**, 249 (2019).

193. Wallin, J. J. *et al.* Atezolizumab in combination with bevacizumab enhances antigen-specific T-cell migration in metastatic renal cell carcinoma. *Nat Commun* **7**, 12624 (2016).