

**On the diversity of neutrophils in blood and tumor of
cancer patients**

*CD45 expression discriminates low-density neutrophils with
different suppressive capacities*

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*A mes grands-parents, qui m'ont accompagné dans ces longues études
mais partis trop tôt que pour assister à cette ultime consécration*

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Abstract

Low-density neutrophils (LDN) have pro-tumor properties and were often described as myeloid-derived suppressors cells (MDSC). However, protocols and approaches used to study LDN in cancer are diverse and thus, the field still lacks consistency. Moreover, direct comparisons between LDN isolated from different cancer types or different environments (e.g., blood and tumor) are currently rare.

We analyzed LDN in the blood and tumors of ovarian cancer patients and lung cancer patients. We observed a higher frequency of circulating LDN in lung cancer patients compared to ovarian cancer patients. We also showed that circulating LDN transcriptomically differ from both high-density neutrophils (HDN) and tumor LDN.

We observed that circulating LDN are composed of two subsets that express different levels of CD45. CD45^{high} LDN have a mature morphology and suppress T-cell proliferation *in vitro*, unlike immature CD45^{dim} LDN. Compared to CD45^{high} LDN, CD45^{dim} LDN surprisingly express higher levels of *ARG-1* and *NOX-2*, which are often described as important suppressive mechanisms of MDSC. Finally, we unexpectedly observed that HDN from blood of lung cancer patients suppressed T-cell proliferation *in vitro* similarly to CD45^{high} LDN, which was not case of HDN isolated from non-cancerous donors.

Our data highlights the heterogeneity of LDN and suggests redefining the concept of MDSC as immature cells. Also, analysis of circulating HDN and tumor LDN should not be neglect to study the role of neutrophils in cancer.

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Introduction

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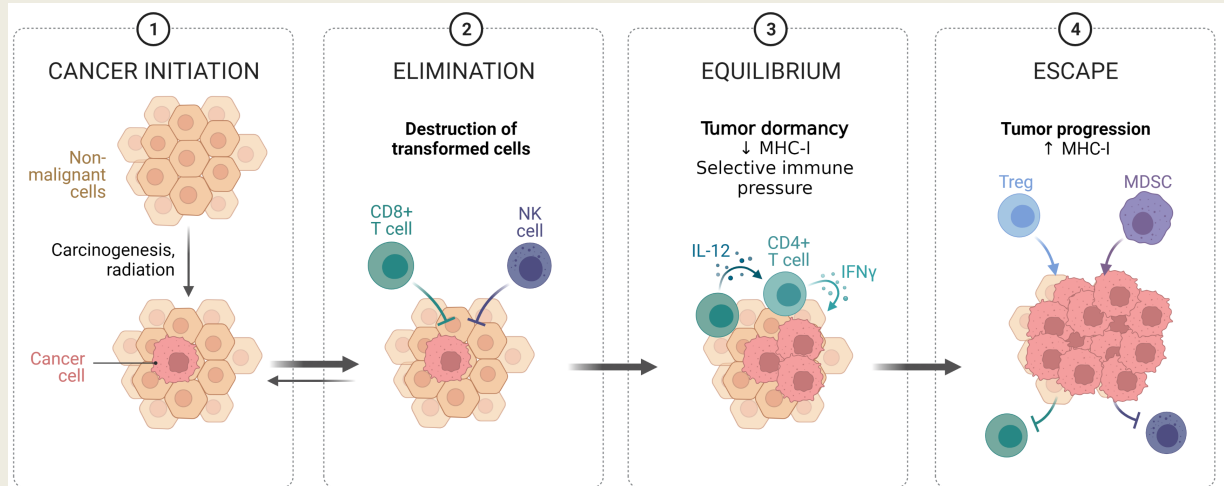
Preamble

Our immune system is a complex and finely regulated network of biological processes that ensure protection against pathogens (bacteria, viruses, ...). Some immune cells, like NK cells and T-cells, recognize and eliminate (pre-) malignant cells [1], [2]. However, some tumor cells develop mechanisms to evade immunosurveillance, leading to tumor progression. Immunotherapies, like immune checkpoint inhibition (ICI), show promising results to prevent cancer progression. But some patients fail to respond to ICI. Immunotherapy shows limited efficacy particularly in melanoma, lung cancer or ovarian cancer [3]–[6].

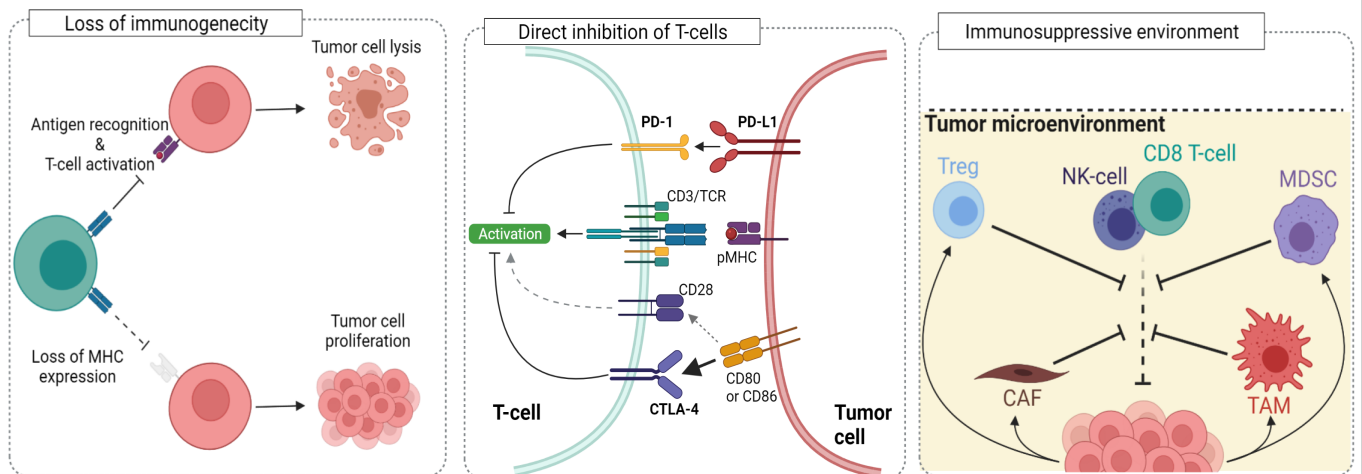
To evade immune control, tumors reshape their environment, which in turn plays a major role in cancer progression. Tumor cells metastasize to sites where the local microenvironment is favorable, according to the *seed and soil* theory by Paget [7]. In the past decades, immune-regulatory cells, termed “MDSC” for myeloid-derived suppressor cells, were discovered in the blood and tumor microenvironment (TME) of cancer patients.

In this introduction, I will first briefly summarize the known mechanisms of tumor evasion. Then, I will focus on MDSC. I will detail how they are identified and isolated from both humans and mice. I will then describe the different mechanisms of suppression in both species and how they are tested *in vitro* and *in vivo*. Finally, I will discuss the presence of MDSC in lung and ovarian cancer and describe current clinical trials targeting MDSC.

Box 1. Cancer immunoediting



Cancer immunoediting is a dynamic interplay between immune cells and fast-growing tumor cells[1], [2], [8]–[10]. This selection of immune-resistant tumor cells proceeds through distinct phases: **initiation** of carcinogenesis, **elimination** of tumor cell by CD8⁺ T-cells and NK cells, an **equilibrium** state where resistant tumor cells remain dormant while the immune system prevents outgrowth of non-resistant cells and an **evasion** phase characterized by the uncontrolled expansion of the tumor. Several mechanisms are involved in the failure of the immune system to control tumor expansion, such as the loss of immunogenicity in tumor cells, direct inhibition of effector cells and generation of an immunosuppressive environment.



Tumor cells may stop presenting antigen to T-cell by downregulating class I MHC, having a deficiency in the antigen processing pathway and/or presenting poorly immunogenic peptide to T-cell.

Upon activation, T-cells express checkpoint inhibitor receptors that restrain their functions. PD-1 binds to PD-L1 expressed on the tumor cell surface and inhibit T-cell activation. CTLA-4 is a receptor that compete with CD28 for the co-stimulatory molecules CD80 and CD86.

Tumor cells reshape their environment by recruiting immunosuppressive cells such as tumor-associated macrophage (TAM), cancer-associated fibroblast (CAF), regulatory T-cell (Treg) and myeloid-derived suppressor cells (MDSC). These regulatory actors inhibit NK and T-cell function in return

Myeloid cells as regulators of tumoral immunity¹

In 1957, Thomas and Burnet formulated the hypothesis of immunosurveillance, a process by which the immune system actively detects and kills (pre-)malignant cells before they progress to cancer [1], [2]. In addition to showing the reality of this surveillance, Thierry Boon and his group also observed occurrences of tumor immune escape through loss of antigen presentation [8], [9]. It is now clear that besides eliminating tumor cells, the immune system also promotes tumor progression by exerting selective pressure on malignant cells that favor the survival of immune-resistant cells in a process called cancer immunoediting (Box 1) [10]. One of the mechanisms involved in tumor immune evasion is the establishment of an immunosuppressive tumor microenvironment.

Myeloid-derived suppressor cells: definition and origins

Within the TME, myeloid-derived suppressor cells (MDSCs) are a rare group of myeloid cells with immunosuppressive capacities. MDSC accumulate in individuals with conditions including cancer, sepsis or chronic infections [11]. MDSC are thought to originate from bone marrow (BM) and recruited to the tumor site by chemokines like ligands of C-C chemokine receptor (CCR) 2, CCR5 or C-X-C Motif Chemokine Receptor (CXCR) 1/2 receptors (Figure 1) [12]–[14]. The mechanisms driving the expansion of MDSC are still not well understood. It has been suggested that these cells originate from alterations in myelopoiesis in pathological contexts (Figure 1)

¹ This introduction is adapted from my first-author review for an MDSC-focused issue of J. Clin. Med. (2021), hence the terminology “MDSC” used in this section [285].

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[15], [16]. Cytokines regulating normal myelopoiesis, like granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and macrophage colony-stimulating factor (M-CSF), are believed to be key actors in the generation of MDSC [17], [18]. Inflammatory cytokines (e.g. interleukin-6 (IL-6)), may stimulate expansion and activation of MDSC through the signal transducer and activator of transcription (STAT) 3 signaling pathway [19]. STAT3 and STAT5 downregulate IFN- γ Regulatory Factor (IRF) 8 in the early stage of granulopoiesis, which leads to accumulation of PMN-MDSC in mice [20]–[22]. Suppressive capacities of MDSC were also associated with expression of CCAAT/enhancer-binding protein (C/EBP)- β , whose is a positive regulator of MDSC immunosuppressive functions and associated with emergency granulopoiesis [22]–[25]. Its expression is induced by IL-6, G-CSF, and GM-CSF through the JAK-STAT3 pathway. Lechner *et al.* cultivated human PBMC *ex vivo* in the presence of IL-6, IL-1 β or tumor necrosis factor alpha (TNF- α) combined with GM-CSF to generate myeloid cells with immunosuppressive capacities similar to MDSC [26].

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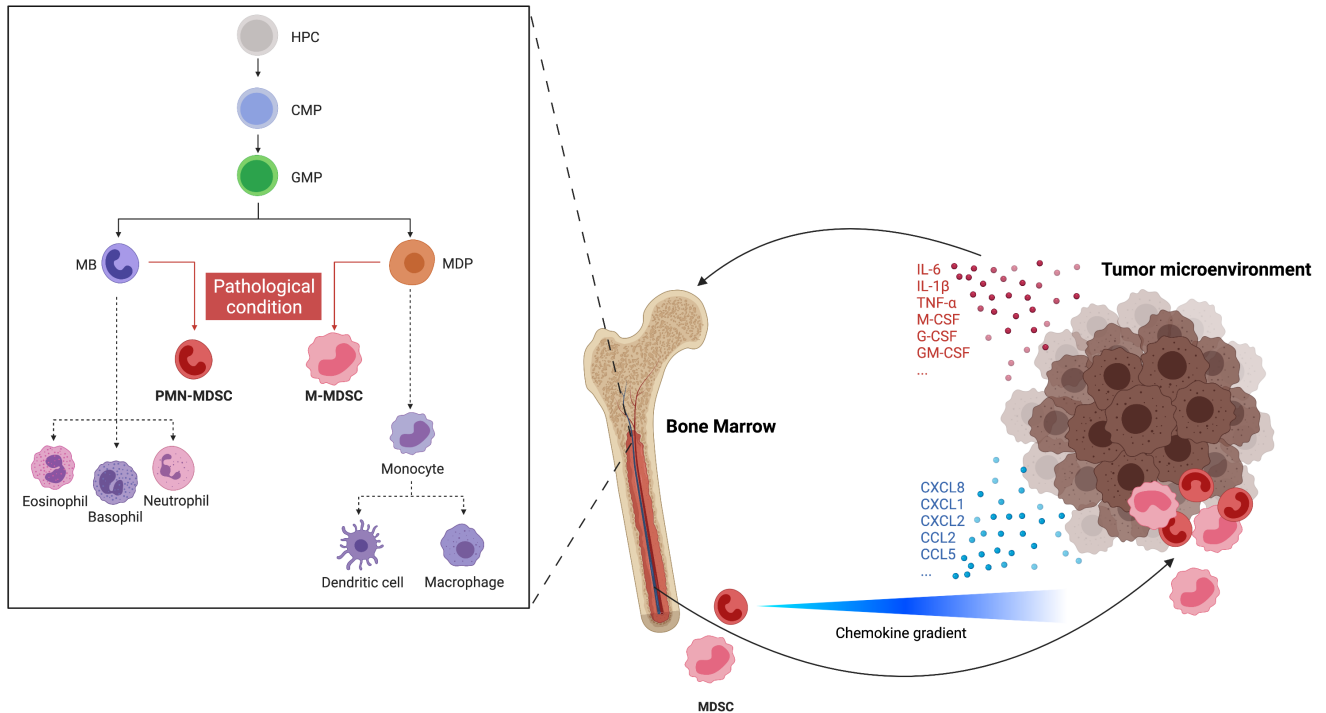


Figure 1. The tumor microenvironment might trigger abnormal myelopoiesis, resulting in the expansion and recruitment of MDSC subsets at the tumor site. Under healthy conditions, myeloid cells (including monocytes, dendritic cells and granulocytes) are produced in the bone marrow. In the context of cancer, tumor cells produced GM-CSF, G-CSF and M-CSF that will stimulate the production of myeloid cells. This emergency myelopoiesis in the presence of inflammatory cytokines (IL-6, IL-1 β or TNF- α) causes the release of MDSC into the circulation. The MDSC are recruited to the tumor site by chemokines (CXCL8, CXCL1, CXCL2, CCL2 or CCL5 e.g.).

Classically, MDSC are divided into monocytic MDSC (M-MDSC) and polymorphonuclear or granulocytic MDSC (PMN- or G-MDSC), according to their lineage and phenotype. In humans, early-stage MDSC (E-MDSC) represent a third progenitor subset that lacks monocytic and/or granulocytic markers [27]–[29]. While MDSC were first described as immature or progenitor cells, recent studies extended the definition to include mature subpopulations. In fact, some research groups think that MDSC represent a state of activation rather than a distinct population (Box 2) [30]–[32].

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In humans, increased MDSC levels are correlated to poor prognoses for cancer outcome [29], [33]–[35]. Ablating MDSC in mice results in improved tumor control [36]. Thus, MDSC are potentially good predictors for disease outcome or targets for future immunotherapeutic strategies. However, the current depth of knowledge and the technical sophistication with which MDSC are approached differ between the species.

Box 2. MDSC: a distinct population or a state of activation that mediates suppression?

Because MDSC share many phenotypic and functional elements with neutrophils and monocytic cells, uncertainty remains about whether MDSC are distinct populations. For instance, tumor-associated macrophages and tumor-associated neutrophils display immunosuppressive mechanisms and favor cancer progression [37], [38]. It raises the possibility that PMN-MDSC, for example, are merely pathologically activated neutrophils [37], [39]–[41].

The definition of MDSC as immature cells was recently challenged: some research groups show that PMN-MDSC with a mature phenotype and morphology had higher immunosuppressive activity than immature cells [29], [42]. In human and mice, mature neutrophils are found in the light-density fraction of a density-gradient separation after activation, priming or exposure to TGF- β [39], [43], [44]. These low-density neutrophils (LDN) display a mature phenotype (expression of CD16, e.g.). Therefore, some consider PMN-MDSC as a pathological state of activation of neutrophils rather than a distinct population [37]. We share this point of view and believe that PMN-MDSC do not represent a specific subpopulation that arise to fulfill one specific function.

However, we kept the term “MDSC” in the introduction because this section was based on a review we wrote about MDSC and their suppressive mechanisms. In the results and discussion sections, we use the terms “LDN” and HLA-DR^{low} monocytes to refer to the phenotype of the cells we isolated.

Identification and isolation

MDSC are rare in healthy individuals but expand in pathological conditions [37]. Thus, high sample volumes are required to obtain sufficient MDSC for experimentation. Today, no MDSC-specific surface marker is known. Discerning between human MDSC and other myeloid cells relies on physical properties like cell density or differences in the intensity of surface marker expression [27]. Identifying MDSC correctly is further complicated by their definition as immunosuppressive cells, which requires confirmation of their suppressive capacities through *in vitro* assays [27].

MDSC in mice

Murine MDSC are mainly isolated from the spleen, the bone marrow or the lung [38]–[41]. These tissues offer the greatest yields of up to 10^7 to 10^8 cells [42]. It is also possible to isolate MDSC from lymph nodes (by mechanical disruption), liver (using Percoll), tumor (by mechanical and enzymatic disruption) or peripheral blood (by cardiac puncture) [43]–[45]. However, it might be necessary to combine the blood of several mice to achieve MDSC yields with which experiments can be performed. While all tissues require different pre-treatments (e.g. the spleen requires mechanical disruption and erythrocyte depletion, while the lung requires enzyme-based digestion [38], [39]), all MDSC are typically isolated using either commercial magnetic bead kits or flow cytometry [46]–[48].

Originally, all murine MDSC were described as CD11b⁺ and Gr-1⁺. Gr-1 is composed of subunit from Ly6 family and cells are further classified into M- (Ly6C⁺) or PMN- (Ly6G⁺) MDSC (Figure 2) [27], [46]. To date, no E-MDSC have been discovered in mice.

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CD84, CD244, fatty acid transporter protein 2 (FATP2) and CD36 have all been suggested as markers to identify M-MDSC and PMN-MDSC more effectively (Figure 2) [11], [49]–[52]. For instance, CD11b⁺Ly6C^{low}Ly6G^{high}CD244⁺ PMN-MDSC isolated from mouse models for thymoma, melanoma or colorectal cancer suppressed CD8⁺ T-cells more effectively than CD11b⁺Ly6C^{low}Ly6G^{high}CD244⁻ populations [49]. In mice models of lymphoma, lung, colorectal and pancreatic cancer, Veglia *et al.* observed an upregulation of FATP2 in splenic PMN-MDSC compared to PMN from healthy controls [51]. Also, knocking-down FATP2 in neutrophils abrogated the suppressive activity of PMN-MDSC and reduced tumor growth in comparison to wild-type mice. However, the authors did not analyze expression of FATP2 in PMN from tumor-bearing mice and thus it is uncertain whether FATP2 is a specific marker of PMN-MDSC or simply upregulated in neutrophils within a tumor context. Therefore, a specific marker for either M-MDSC or PMN-MDSC is still elusive as all markers mentioned can be expressed by macrophages, neutrophils or dendritic cells (DC).

Biochemical features that underlie MDSC-mediated immunosuppression - e.g., expression of Arginase-1 (ARG-1), inducible nitric oxide synthase (iNOS) or NADPH oxidase 2 (NOX2) - can be used in the phenotypic analyses of MDSC. However, these enzymes are expressed intracellularly, which means that labelling them is incompatible with isolating live cells. Furthermore, the listed enzymes are also expressed by immunosuppressive macrophages or activated neutrophils [53], [54].

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—◇ Minimal recommended panel
—● Additional markers

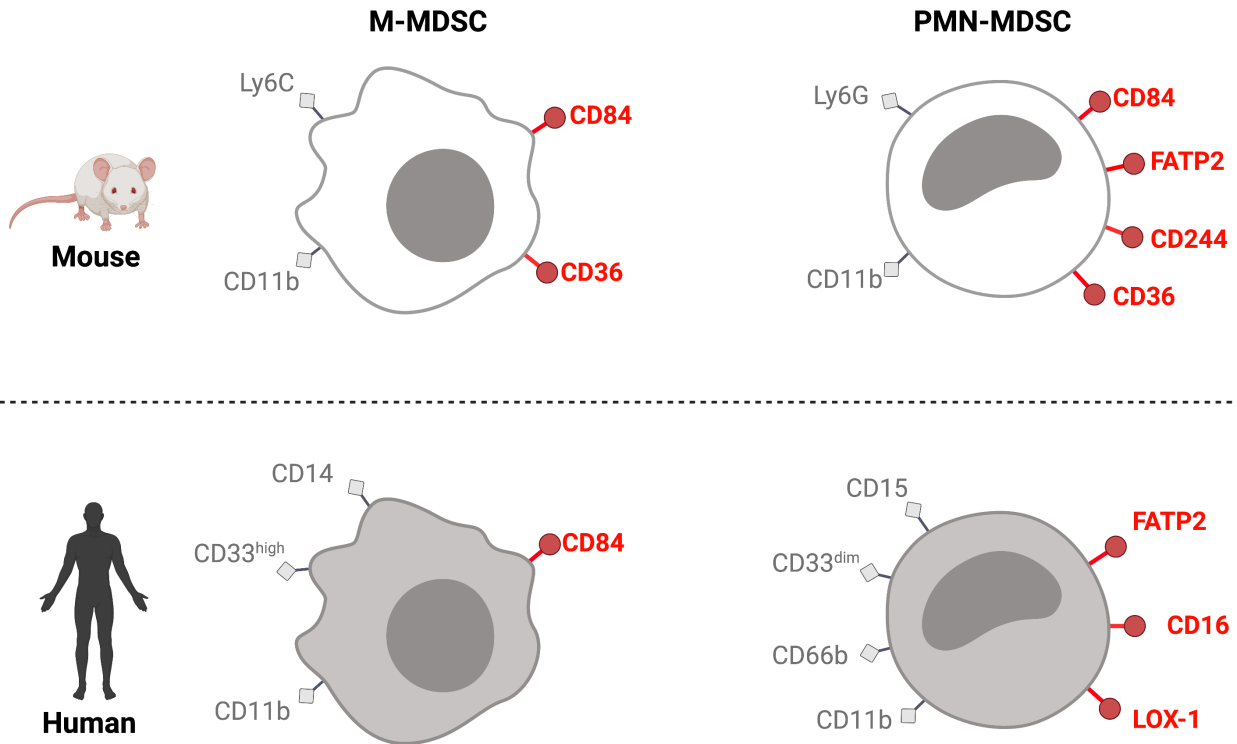


Figure 2. Phenotype M-MDSC and PMN-MDSC in mice and humans. Grey markers represent the minimal recommended panel described by Bronte et al. for isolating M- and PMN-MDSC from human and mice [27]. Red proteins represent additional surface markers whose expression is increased in MDSC compared to non-suppressive myeloid cells. FATP2: Fatty-acid transporter protein 2 ; LOX-1: Oxidized low-density lipoprotein receptor 1 (OLR1).

MDSC in humans

Human MDSC are most commonly isolated from the peripheral blood of patients [28], [50], [55]. Sometimes, MDSC are isolated from tumors following mechanical tissue disruption and enzyme-based digestion [56], [57]. Additionally, the presence and intra-tumoral distribution of MDSC (but not their immunosuppressive functions) are analyzed by immunohistochemistry [58]. In rare cases, MDSC are isolated from spleen [59], BM aspirates [60] or cancer ascites [61].

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As access to human clinical samples can be challenging, protocols have been established to differentiate myeloid cells into MDSC *in vitro*. For example, culturing peripheral blood monocytes with GM-CSF, IL-4 and prostaglandin-E₂ (PGE₂) or PGE₂-expressing tumor cells generates cells with immunosuppressive functions and phenotypes similar to M-MDSC [24], [62]. Similar results were obtained by culturing BM cells with G-CSF, GM-CSF and IL-6 [63], [64]. While generating MDSC *in vitro* is enticing, myeloid cells are highly heterogeneous and plastic cells. The suitability of *in vitro*-generated MDSC as models for MDSC with clinical relevance still needs to be confirmed in genetic and transcriptomic comparisons to patient-isolated MDSC.

The 2016 seminal review by Bronte *et al.* clearly defined standard minimal protocols to identify and isolate human MDSC: M-MDSC are CD11b⁺CD14⁺CD15⁻HLA-DR^{low/-} (Figure 2) [27]. Instead of CD11b, the myeloid marker CD33 can be used. It is expressed in high levels on monocytic cells [65]. The low expression of HLA-DR distinguishes M-MDSC from monocytes [27]. PMN-MDSC are CD11b⁺CD14⁻CD15⁺HLA-DR⁻ [27]. CD33 is dimly expressed on granulocytic cells [65]. Either CD15 or CD66b can be used as a granulocyte marker. Phenotypically, PMN-MDSC are currently indistinguishable from mature neutrophils. Instead, PMN-MDSC and neutrophils are separated by density [27]. Higher expression levels of CD11b and CD16 on PMN-MDSC correlated with stronger suppression of T-cell functions in head and neck (HN) cancer patients [29].

As in mice, although no MDSC-specific marker is known to date, several candidate surface markers that are upregulated on MDSC have been identified (Figure 2). CD84 is upregulated on murine M- and PMN-MDSC, and on human M-MDSC that were generated *in vitro* from PBMC [50]. FATP2 was also upregulated on PMN-MDSC from HN, lung and breast cancer compared to PMN from healthy

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donors. However, the authors did not analyze its expression on PMN from cancer patients [51]. Thus, it is impossible to know whether FATP-2 is expressed in PMN-MDSC only or in whole neutrophil population during cancer. Lectin-type oxidized LDL receptor 1 (LOX-1) is overexpressed by PMN-MDSC from non-small cell lung carcinoma (NSCLC) patients and HN cancer patients in comparison to PMN-MDSC and neutrophils from the blood of healthy donors [55]. Higher levels of LOX-1 are expressed by PMN-MDSC of hepatocellular carcinoma and pancreatic cancer patients compared to healthy donors [66], [67]. Additionally, LOX-1-expressing tumor-associated neutrophils (TAN) from HN cancer patients also expressed ARG-1 [58]. Higher frequencies of LOX-1⁺ TAN at the tumor site correlated to decreased overall survival [58]. However, all of these surface markers emerged recently and require further characterization to be confirmed as MDSC-specific.

Considering the complex phenotype of human MDSC and their rarity, it is recommended to isolate MDSC by flow cytometry [27], [68]. Magnetic selection of CD33⁺ or CD11b⁺ cells could be used as an enrichment step, but preferably not as the principal method of isolation because both markers are present on all myeloid cells. Today, no commercial isolation kits exist for human MDSC.

Common challenges in handling murine and human MDSC

Because of their rarity, only few MDSC are accessible for *in vitro* study. In patients with pathologies like breast or colorectal cancers, PMN-MDSC and M-MDSC usually represent between 0,1% to 5% of PBMC. But in some individuals, e.g. ovarian carcinoma patients, the frequency of MDSC is as low as 0.01% of PBMC ([28] and own observation). Realistic MDSC yields are further limited by the constricted

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volumes of blood that are practically and ethically obtainable from tumor-bearing mice or cancer patients (around 2.5 and 50 ml respectively).

MDSC samples need to be processed carefully because rough handling or cold temperatures easily activate granulocytes. If and how this could affect the suppressive functions of PMN-MDSC remains unclear [68]. Careful handling of MDSC samples is especially important when the cells are isolated from tissues other than blood, as it is unknown if and how mechanical and/or enzymatical digestion affects MDSC phenotypes or functions [69].

Following isolation, MDSC need to be tested in experiments immediately because PMN-MDSC are cryosensitive and have short *ex vivo* life-spans [70], [71]. For context, neutrophils typically survive 24 hours *in vivo* [72].

Immunosuppressive mechanisms of MDSC

The principal characteristic of MDSC is their ability to suppress immune responses [27]. Consequently and in the absence of specific markers for immunosuppressive MDSC, a part of each MDSC sample should be tested in *in vitro* immunosuppression assays [68], [73]. T-cells are the most documented target of MDSC-mediated immunosuppression. But MDSC can also affect the functions of B-cells and natural killer (NK)-cells [74]–[78]. In this chapter, we will explore the known mechanisms by which MDSC suppress T-cell and NK cell functions in each species (Table 1).

Amino acid deprivation

Upon encountering their antigen, T-cells shift from a quiescent state based on oxidative respiration to an active state that relies on glycolysis. This allows for rapid

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growth and effector cell functions (i.e. cytokine secretion or cell lysis) [79], [80]. Proliferation and effector functions require protein synthesis and thus activated T-cells increase their uptake of amino acids, like glutamine [79], [81], [82]. T-cells detect the abundance of amino acids through mammalian target of rapamycin (mTOR) signaling pathways, and deprivation of amino acids through general control nonderepressible-2 kinase (GCN2) [83], [84]. GCN2 is activated when it binds to uncharged transfer-RNA (tRNA). To inhibit further protein synthesis GCN2 phosphorylates the α -subunit of eukaryotic translation initiation factor 2 (eIF2 α) and to promote catabolism and cell autophagy GCN2 inhibits the mTOR pathway [83], [84] (Figure 3).

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Table 1. Summary of immunosuppressive mechanisms related to MDSC. IDO: indoleamine 2,3-dioxygenase, ROS: reactive oxygen species, NO: nitric oxide, iNOS: inducible nitric oxide synthase, NOX2: NADPH oxidase 2, TCR: T-cell receptor, pMHC: peptide-MHC complex, ADCC: antigen-dependent cell cytotoxicity, FasL: Fas ligand, PD-L1: programmed-death ligand 1, TME: tumor microenvironment, Treg: regulatory T-cells.

Mechanisms of suppression	Mouse	Ref.	Human	Ref.
Amino-acid depletion	L-Arginine	PMN-MDSC express Arginase-1 ^a . Through Arginase-1, PMN-MDSC inhibit proliferation, cytokine secretion and decrease CD3 ζ expression in T-cells during <i>in vitro</i> co-culture. <i>Tumors regress in vivo after inhibition of Arginase-1.</i>	[85]–[88] <i>PMN-MDSC and activated PMN express Arginase-1. T-cells activated in the presence of Arginase-1 or L-arginine-depleted medium arrest the cell-cycle.</i>	[89]–[93]
	L-Cysteine	M- and PMN-MDSC sequester cysteine. Cysteine concentrations are lower in the serum of tumor-bearing mice.	[95] -	[68], [69], [72]
	L-Tryptophan	<i>Bone marrow (in vitro)-derived M-MDSC express IDO. Inhibiting IDO restores T-cell proliferation.</i>	[60] <i>E-MDSC isolated from blood and metastasis of cancer patients express IDO. IDO expression is correlated with Foxp3⁺ T-cell expansion.</i>	[96]
Oxidative stress	PMN-MDSC produce ROS (peroxynitrite) by NOX2. M-MDSC produce NO by iNOS. Inhibiting ROS or ROS-producing enzyme blocks suppressive capacities of M- and PMN-MDSC.	[97]–[100]	<i>PMN-MDSC isolated from cancer patients produce ROS and increased levels of nitrotyrosine are present in CD8⁺ T-cell.</i>	[76], [78]
	M- and PMN-MDSC mediate nitration of the TCR <i>in vivo</i> and <i>in vitro</i> , which disrupts pMHC binding to CD8 ⁺ T-cells.	[100]	<i>T-cells co-cultured with iNOS-expressing PMN-MDSC isolated from cancer patients express diminished levels of CD3ζ.</i>	[94]
	M- and PMN-MDSC produce ROS, which inhibits proliferation, cytokine secretion, ADCC and granzyme-B production by NK cells.	[101]	M- and PMN-MDSC from cancer patient inhibit cytokine secretion and ADCC of NK cell through NO production by iNOS.	
Treg	M-MDSC produce IL-10 and TGF- β . Foxp3 ⁺ Treg expand <i>in vivo</i> and <i>in vitro</i> in IL-10-, TGF- β -, arginase- and CD40L-dependant manners.	[102]–[104]	M-MDSC induce Treg in an IL-10-dependant manner.	[105]
	M-MDSC produce CCR5-ligand <i>in vivo</i> and <i>in vitro</i> , which recruits of Treg.	[106]	M- and PMN-MDSC that express CD39 and CD73 generate adenosine and inhibit T-cell and NK cell proliferation and cytokine secretion. <i>Accumulation of M-MDSC correlate with increased frequency of circulating Foxp3⁺ T-cells in renal transplant patients.</i>	[85], [86] [109]

Table 1. Cont.

Mechanisms of suppression		Mouse	Ref.	Human	Ref.
Direct MDSC/ T-cell interaction	Immune checkpoint blockade	<i>PD-L1⁺ M- and PMN-MDSC present at higher frequency in tumor microenvironment compared to peripheral blood.</i>	[110], [111]	<i>PD-L1⁺ M- and PMN-MDSC accumulate in blood of cancer patients. PD-L1⁺ PMN-MDSC accumulate in TME.</i>	[112]
		<i>Myeloid-specific ablation of PD-1 decreases tumor growth.</i>	[113]	<i>PMN-MDSC from non-responding cancer patients (ipilimumab) express more PD-L1⁺.</i>	[114]
	Cell death ligand	FasL-expressing PMN-MDSC induce CD8 ⁺ T-cell apoptosis.	[115]	-	

Direct causality of MDSC shown – *Indirect observation or correlation.*

^a Rodriguez *et al* (2008) initially defined MDSC as mature Gr-1⁺CD11b⁺ myeloid suppressor cells before using MDSC terminology in later reviews. Miret *et al* (2019) used only anti-CD11b to isolate MDSC

Arginine metabolism

Arginine is a non-essential amino acid. Some pathological conditions increase the overall arginine demand; and high concentrations of arginine correlate with better survival and anti-tumor activity in cancer [116]. Low arginine levels in the TME can lead to the loss of T-cell functions, including proliferation [117], [118].

Arginine is metabolized by the arginase family enzymes, which convert arginine to ornithine and urea, and nitric oxide synthases (NOS), which degrade arginine to produce nitric oxide (NO) and citrulline [116]. Both mechanisms are found in MDSC, with the NOS pathway being particularly prominent in M-MDSC [48]. Downstream metabolites of arginine, like spermine or spermidine, also modulate T-cell differentiation [119].

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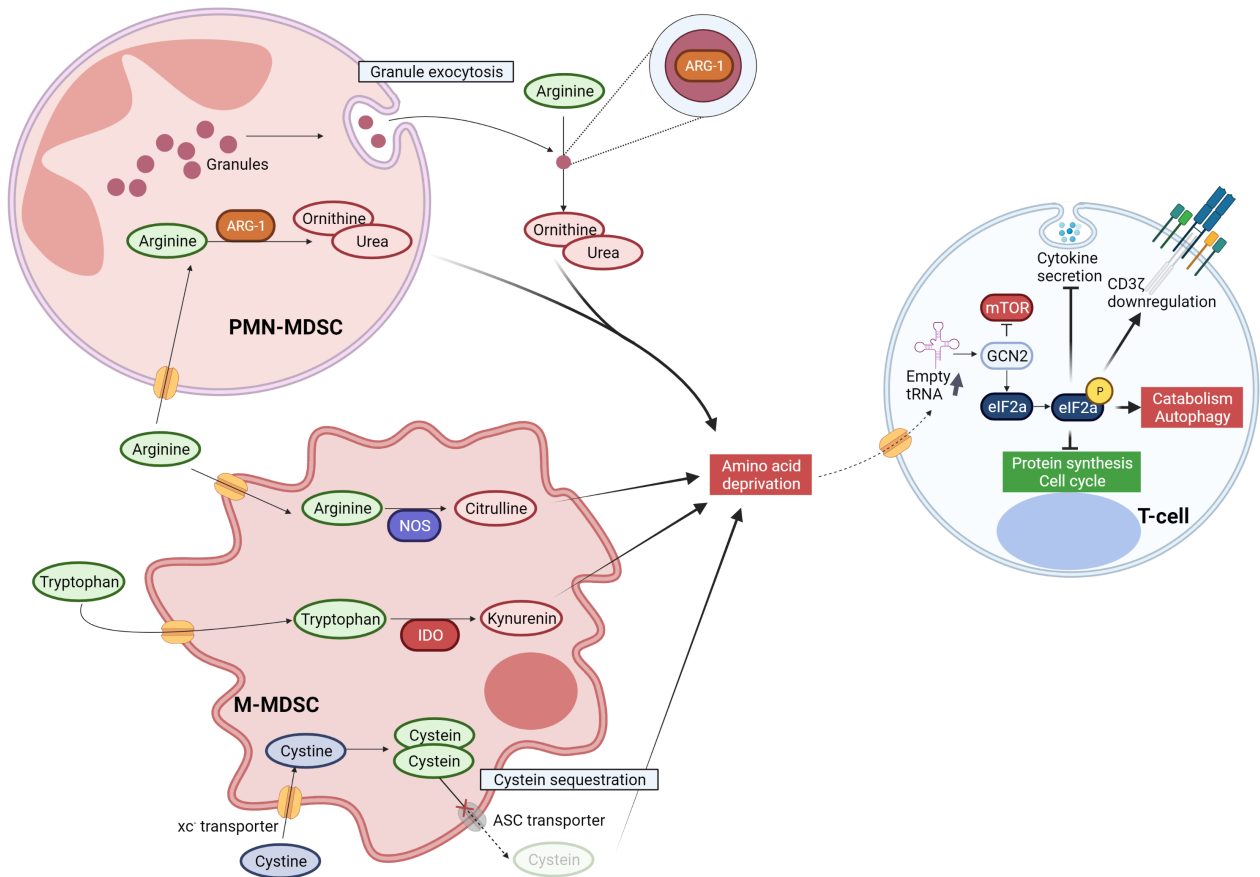


Figure 3. Amino-acid depletion by M- and PMN-MDSC cause T-cell dysfunction through CD3 downregulation, inhibition of cytokine secretion and cell cycle arrest. Arginine can be depleted through expression of Arginase-1 (ARG-1) by PMN-MDSC and/or expression of iNOS in M-MDSC. Human PMN-MDSC produce granules containing ARG-1 while murine MDSC express the enzyme in their cytosol. Arginine is imported in cytosol through transporter like SLC7A2. M-MDSC deplete tryptophan by expressing IDO and sequester cysteine due to their lack of ASC transporter expression. T-cells sense amino acid deprivation via empty rRNA activating GCN2, which in turn activate eIF2α pathway but inhibit mTOR.

In mice, arginine deprivation is described as one of the main mechanisms by which MDSC suppress T-cells. However, direct evidence of arginine depletion by MDSC is sparse. In 3LL murine lung carcinoma, Gr-1⁺CD11b⁺ myeloid suppressor cells expressed increased levels of intra-cytosolic ARG-1 and the cationic amino acid transporter 2B (SLC7A2), which imports extracellular L-arginine [85]. T-cells cultured in the presence of myeloid suppressor cells proliferated slower, secreted fewer

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cytokines and downregulated the expression of the CD3 ζ . Adding the ARG-1 inhibitor N-hydroxy-nor-L-arginine restored the impaired T-cell functions. COX2 induction was responsible for upregulating ARG-1 in Gr-1⁻CD11b⁺ myeloid suppressor cells. Blocking COX2 *in vivo* decreased the tumor volume in a mouse model of lung carcinoma [86]. However, despite being referred to as MDSC in later reviews [87], [120], the described cells expressed low levels of Gr-1, and thus, it is uncertain whether these cells should be considered as MDSC [17]. Inhibiting Arginase-1 *in vivo* in a NSCLC mouse model caused tumor regression and improvement of T-cell functions [88]. In mice bearing 4T1 tumors, inhibiting iNOS and/or Arginase-1 rescued antibody-dependent cell-mediated cytotoxicity of NK cells [78]. Nevertheless, it is important to remember that arginine depletion by ARG-1 or iNOS is a mechanism shared by other myeloid cells, which are thus not specific to MDSC [53], [121]. Furthermore, Bian *et al.* showed that ARG-1 was not expressed by M- and PMN-MDSC, even though they show potent suppressive capacities in mouse models of lymphoma, melanoma or colorectal cancer [122]. Also, induction of ARG-1 expression in those MDSC did not lead to an increase of suppressive potential. Therefore, depletion of arginine by MDSC in mice requires further analysis, e.g. by specifically inhibiting ARG-1 in MDSC if specific markers are discovered.

In humans, no direct evidence that MDSC suppress immune responses by depleting arginine exists. PMN-MDSC and PMN express Arginase-1 in granules [89]–[91], [123], and T-cells arrest their cell cycle in the absence of L-arginine or in the presence of Arginase-1 [92], [93]. Inhibiting ARG-1 in myeloma patients enhanced the functions of T-cells co-cultured with PMN [118]. In renal carcinoma patients, the presence of ARG-1-expressing PMN-MDSC was correlated with decreased CD3 ζ expression and impaired cytokine secretion by PBMC, which was restored by

depleting PMN-MDSC [90], [91]. In NSCLC patients, PMN-MDSC that express ARG-1 caused downregulation of CD3 ζ in CD8⁺ T-cells and Jurkat cells during *in vitro* co-culture [94].

Cysteine metabolism

Cysteine is a non-essential amino acid. Cysteine deprivation inhibits the formation of glutathione, a major cellular process that prevents damage caused by reactive oxygen species (ROS) [124]. Cysteine is generated in two ways: (1) by converting intracellular methionine to cysteine through cystathionase; (2) by importing cystine, the oxidized form of cysteine, through the xc⁻ transporter and reducing it to cysteine with cystathionase [95], [124]. However, naïve T-cells express neither the xc⁻ transporter nor cystathionase. Instead, naïve T-cells directly import cysteine through the alanine-serine-cysteine (ASC) family of transporters, which includes SLC1A4 and SLC1A5 [125]. Since naïve T-cells are unable to generate their own cysteine, they rely on antigen presenting cells to convert cystine to cysteine, which is then exported through ASC transporters.

In mice, Srivastava *et al.* (2010) showed that pan-MDSC deplete cysteine from the serum [95]. MDSC expressed high levels of the xc⁻ transporter but low levels of ASC transporters, meaning that MDSC import extracellular cystine without releasing cysteine [95]. Cysteine sequestration by MDSC lead to an arrest of T-cell proliferation. Supplementing a reducing agent, e.g. β -mercaptoethanol, or stable cysteine abolished the effect.

In humans, there is no evidence that either M- or PMN-MDSC sequester cysteine. Although neither ASC nor xc⁻ transporters are expressed on naïve CD4⁺ T-cells, their expression is upregulated upon activation [126]. This suggests that activated human T-cells directly import both cystine and cysteine. Thus, the impact

of MDSC in the metabolism of cysteine and cystine in T-cells is likely negligible in human.

Tryptophan metabolism

Tryptophan is an essential amino acid. Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) degrade tryptophan to N-formylkynurenine [127]. T-cells and NK cells sense tryptophan deprivation or the presence of tryptophan metabolites by GCN2 (see *Amino acid deprivation introduction*), arresting T-cell proliferation, causing anergy and triggering the differentiation of CD4⁺ T-cells into regulatory T-cells (Treg) [127]–[130]. Tryptophan metabolites, like kynurenine, 3-hydroxykynurenine or picolinic acid, dampen T-cell and NK cell functions and proliferation [130], [131], and induce Treg generation [128].

IDO-KO mice lack functional MDSC compared to WT specimen in a model of graft-versus-host disease [132]. However, direct evidence of IDO expression in M- or PMN-MDSC is still lacking. Only one study shows that murine M-MDSC express IDO, which contributes to the suppressive effects of MDSC on T-cells [60]. However, the MDSC were generated *in vitro* from bone marrow and were never compared to *ex vivo* MDSC phenotypically or transcriptomically.

In humans, direct evidence that MDSC suppress T-cells by depleting tryptophan is still missing. Yu *et al.* (2013) observed that IDO is expressed by CD33⁺CD14⁺CD15⁺ cells, corresponding to E-MDSC, isolated from the blood or metastasis of breast cancer patients [96]. E-MDSC inhibited CD3⁺ T-cell proliferation in *in vitro* co-cultures and proliferation was restored in the presence of 1-methyl-L-tryptophan, an IDO inhibitor.

Generation of oxidative stress

Reactive oxygen species (ROS) are short-lived oxygen-containing molecules that are chemically reactive due to unpaired electrons [133]. The main enzymes involved in ROS production are NADPH oxidase (NOX) and nitric oxide (NO) synthase (NOS) (Figure 4). NOX converts NADPH and oxygen into NADP^+ and superoxide radicals. Superoxide dismutase (SOD) catalyzes superoxide radicals into the less reactive H_2O_2 . NOS generates NO when it degrades L-arginine to L-citrulline. NO reacts with O_2^- to form peroxynitrite ONOO^- . ROS generate oxidative stress that damages nucleic acids, proteins and lipids, thus impairing T-cell functions and viability [133]. For example, oxidative stress downregulates or induces post-translational modifications in TCR-signaling proteins like lymphocyte-specific protein tyrosine kinase (LCK) or CD3 ζ , leading to dysfunction and/or proteasomal degradation [134]–[136]. Impaired TCR-signaling results in hyporesponsive T-cells [135], [136]. Effector CD4^+ T-cells are more sensitive to death induced by ROS than Treg [137], [138].

In mice, strong evidence shows that MDSC rely on both NOX2 and iNOS enzymes to produce ROS and inhibit T-cell or NK cell functions. Murine PMN-MDSC express NOX2 but not iNOS, and release peroxynitrite [97]. For example, PMN-MDSC in two different viral infection models inhibited NK cell functions by releasing ROS [101], [139]. Murine M-MDSC express iNOS but not NOX2, and produce more NO [97]. NO-based reactive molecules can cause nitration of the TCR *in vivo*, which leads to CD8^+ T-cells ineffectively binding their peptide-MHC (pMHC) complex, and chemokine nitration, which impairs T-cell recruitment to tumor site [100], [140]. Blocking NOX2 (in $\text{gp91}^{-/-}$ mice), iNOS (with L-NMMA or in $\text{iNOS}^{-/-}$ mice) or peroxynitrite (with AT38 inhibitor or uric acid) abolishes the immunosuppressive effects of MDSC on T-cells [97]–[99]. NK cell functions like MHC-I-dependent cell

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cytotoxicity, antigen-dependent cell cytotoxicity (ADCC) and IFN- γ secretion were inhibited by pan-MDSC in a mouse model of breast cancer [78]. ADCC by NK cells was restored with L-NIL, an iNOS inhibitor, and in *Nos2*^{-/-} mice. L-NIL administration in EMT6-HER2, a mouse model of breast cancer, reduced tumor growth.

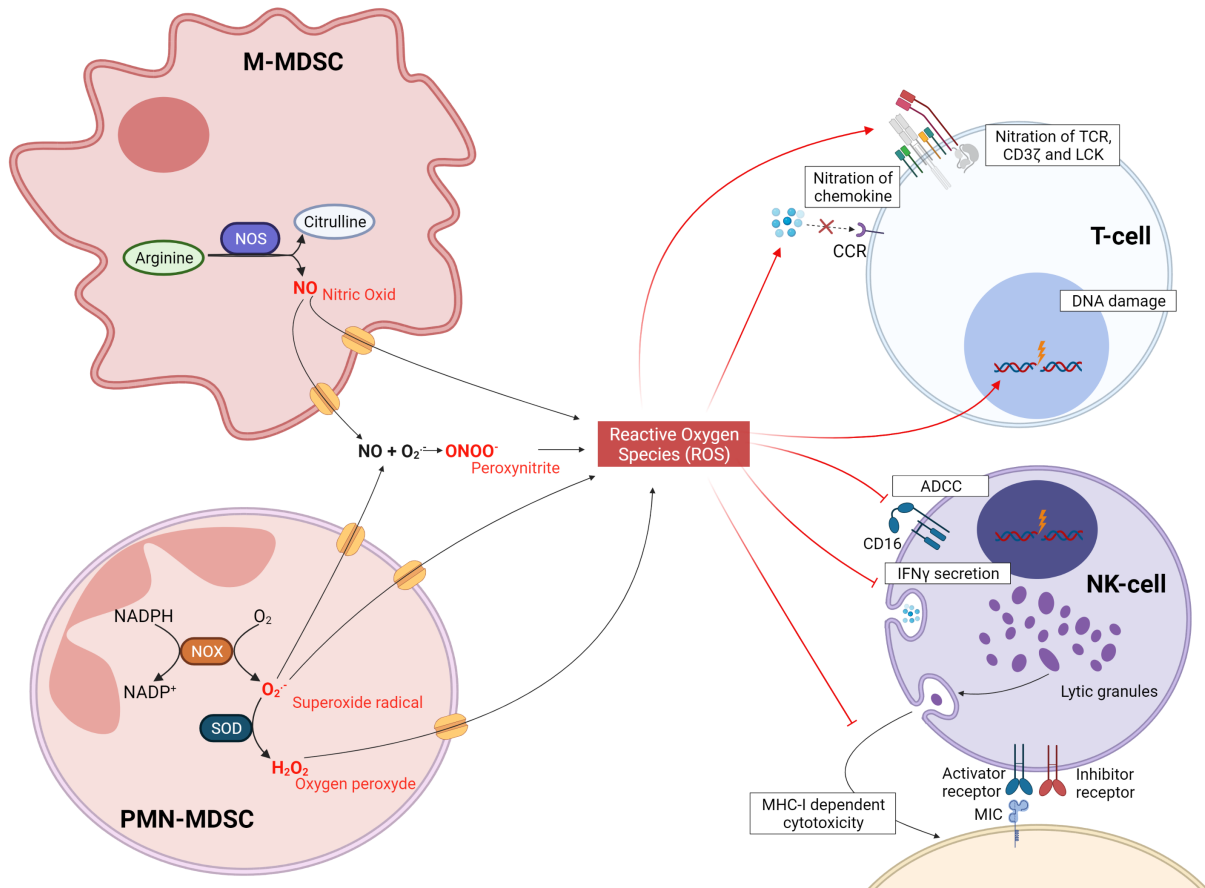


Figure 4. M- and PMN-MDSC generate reactive-oxygen species that impair T-cell and NK cell functions in tumor-bearing mice and potentially cancer patients. Nitric Oxide (NO) is produced as a product of the conversion of arginine to citrulline through nitric oxide synthase (NOS) in M-MDSC. PMN-MDSC express NADPH oxidase (NOX) and super oxide dismutase (SOD) that produce superoxide radicals and oxygen peroxide, respectively. NO and superoxide radicals react with each other to form peroxynitrite, another ROS. Within T-cells, ROS cause DNA damage, nitration of proteins involved in TCR signaling (such as TCR, CD3 or LCK) and chemokines like CCL2. NK cells show impaired cytokine secretion, and both antibody-dependent cellular cytotoxicity (ADCC) and MHC-1-dependent cytotoxicity in the presence of ROS.

In humans, our knowledge of ROS-dependent immunosuppressive mechanisms is fragmentary. Upon PMA activation, CD11b⁺CD14⁻ cells from cancer

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patients produce higher levels of ROS compared to CD11b⁺CD14⁻ cells isolated from healthy donors [98]. Blood PMN-MDSC in NSCLC patients express similar levels of iNOS than monocytes [94]. Co-culturing MDSC from NSCLC patients with CD8⁺ T-cells decreased the expression of CD3 ζ [94]. However, it is important to remember that ROS production and immunosuppression are not exclusive to cells traditionally called MDSC. For instance, activated neutrophils from healthy donors or cancer patients suppress CD4⁺ and CD8⁺ T-cells through NOX2 and ROS [141]. Neutrophils treated with ROS inhibitors or neutrophils from patients with chronic granulomatous disease - a genetic disease that impacts NOX2 function – failed to suppress T-cells. Pan-MDSC impaired ADCC and IFN- γ secretion of NK cells in *in vitro* co-cultures and this inhibition was reversed with the iNOS inhibitor L-NIL [78].

MDSC and accumulation of regulatory T-cells

Besides MDSC, other tolerogenic immune cells, like regulatory T-cells (Treg), tightly regulate immune responses [142] (Figure 5). Treg regulate the activation and proliferation of NK cells, CD4⁺ T cells and CD8⁺ T-cells [143]. The differentiation and functions of Treg are linked to the immunosuppressive cytokines IL-10 and TGF- β [144]. In mice, the expression of forkhead box P3 (Foxp3) is associated with regulatory functions and thus defines the Treg subset. In humans, however, Foxp3⁺ expression alone is insufficient to define Treg. Foxp3 is transiently upregulated in all T-cells upon activation [145], [146]. Only the stable expression of Foxp3, which is associated with DNA demethylation of a specific region in the Foxp3 locus, defines Treg [147].

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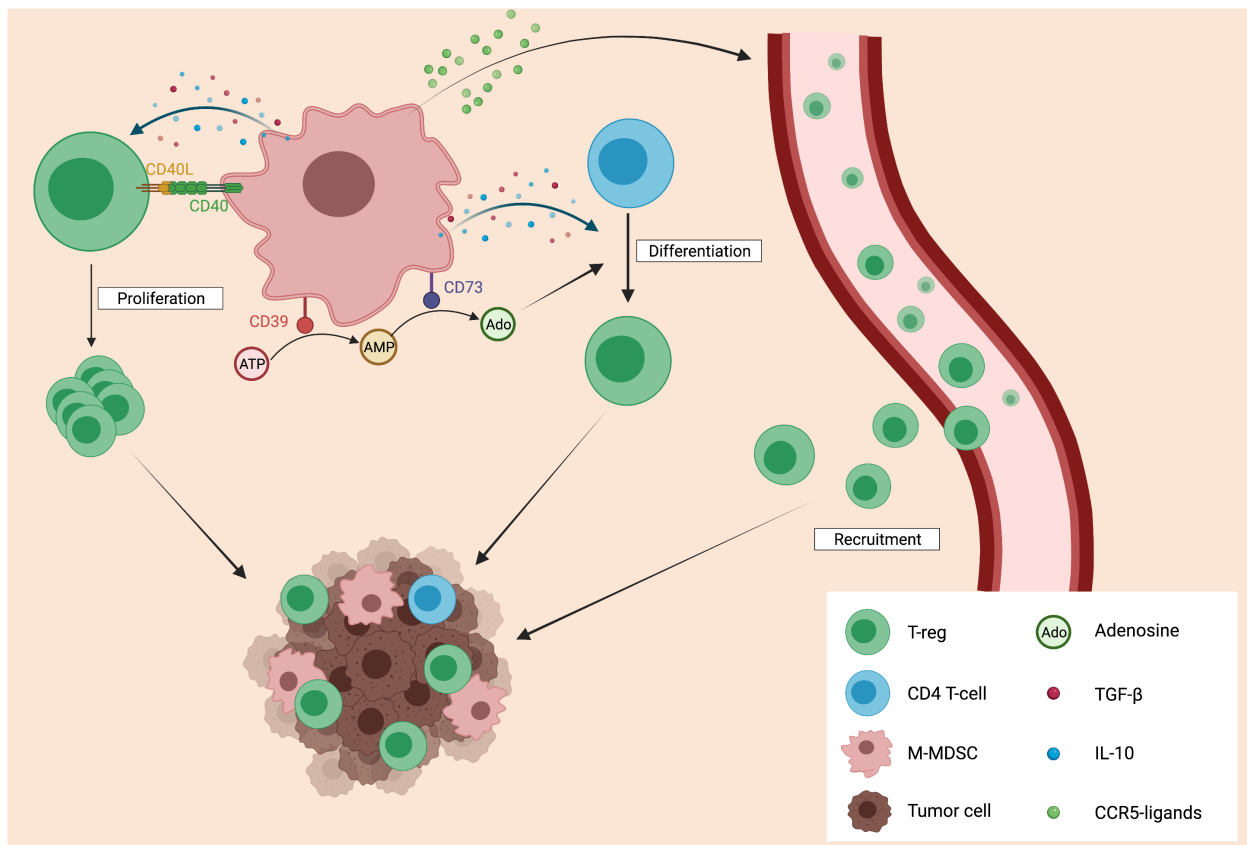


Figure 5. M-MDSC are involved in Treg expansion in tumors and participate in the generation of an immunosuppressive microenvironment. Expansion of Treg at tumor sites are either caused by (i) recruitment through CCR5-ligand chemokine produced by M-MDSC, (ii) differentiation from CD4⁺ T-cells in the presence of TGF- β and IL-10 secreted by M-MDSC or (iii) proliferation of Treg mediated through CD40/CD40L axis. In parallel, M-MDSC can also convert pro-inflammatory ATP into suppressive adenosine by expressing ectonucleoside triphosphate diphosphohydrolase 1 (CD39) and ecto-5'-nucleotidase (CD73).

In mice, there is a direct link between murine MDSC presence and Treg differentiation, expansion and/or recruitment (Figure 5). For instance, CD115⁺Gr-1⁺ M-MDSC secrete IL-10 and TGF- β in a colon-carcinoma model and induce the differentiation of Foxp3⁺ Treg *in vitro* and *in vivo* in an IL-10- and IFN- γ -dependent manner [102]. In a lymphoma model, CD11b⁺ cells promoted the antigen- and ARG-1-dependent expansion of Foxp3⁺ Treg cells when co-cultured with CD4⁺ T-cells

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[103]. MDSC recruit Treg *in vitro* and *in vivo* by secreting the chemokine CCR5-ligand [106]. Pan *et al.* showed that MDSC promote Treg proliferation in a contact-dependent manner through CD40 and CD40L. Knocking-down CD40 or blocking CD40 with antibodies reduced the MDSC-mediated *in vitro* expansion of Treg by two-fold in comparison to wild-type mice [104].

In humans, as most studies use only Foxp3 to define Treg, direct evidence of Treg induction or expansion mediated by MDSC is still lacking. M-MDSC isolated from hepatocellular carcinoma patients secrete IL-10 and differentiate naïve CD4⁺ T-cells into Foxp3⁺CD25⁺ T-cells in *in vitro* cultures - an effect that was contact-dependent and blocked with anti-IL-10 antibodies [105]. The Foxp3⁺CD25⁺ T-cells differentiated in the presence of MDSC suppressed autologous naïve CD4⁺ T-cells. In patients with kidney transplantations, accumulating MDSC were correlated to increased frequencies of Foxp3-expressing T-cells [109]. When co-cultured with activated CD4⁺ T-cells, M-MDSC mediated higher expansion of Foxp3⁺ T-cells compared to the control where CD4⁺ T-cells were activated alone [109]. In NSCLC and ovarian cancer patients, blocking CD39 and CD73 expressed on MDSC partially decreased their immunosuppressive effect on CD8⁺ and NK cells [107], [108]. CD39 and CD73 convert pro-inflammatory extracellular adenosine tri-phosphate (ATP) into adenosine (ADO), which favors Treg proliferation and CD8⁺ T-cell inhibition [148].

Expression of immune checkpoint receptors

Immune checkpoint receptors are transmembrane proteins that act as regulators of effector T-cells in particular. Tumor cells express immune checkpoint ligands such as the Programmed Cell Death-1 (PD-1) ligand (PD-L1), which binds to PD-1 on T-cells

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and inhibits anti-tumor responses [149]. Anti-PD-1 or -PD-L1 recombinant antibodies, in combination with antibodies targeting immune checkpoint receptor CTLA-4, today represent the most effective cancer immunotherapy strategy. However, only up to 56% of patients with melanoma respond to anti-checkpoint receptor immunotherapy and it is difficult to predict who these patients will be [150], [151].

In mice, evidence of immunosuppression mediated through direct contact between T-cells and MDSC remains scarce. Murine M- and PMN-MDSC express PD-L1 and it is often used to characterize MDSC phenotypically but not functionally [48], [110], [113]. In a colon-carcinoma model, M- and PMN-MDSC expressing PD-L1 were more frequent in the tumor microenvironment compared to peripheral blood or spleen [110]. Recently, it was discovered that myeloid cells, including M- and PMN-MDSC, also express PD-1 [113]. While its exact role and impact on M- and PMN-MDSC function remain unclear, myeloid-specific PD-1 ablation in tumor-bearing mice more effectively decreased tumor growth compared to tumor-bearing mice with T-cell-specific PD-1 ablation [113]. The myeloid-specific PD-1 ablation (PD-1^{f/fLysMcre}) prevented the accumulation of MDSC and myeloid progenitors while increasing the presence of effector T-cells at the tumor site. However, combining checkpoint inhibitor blockade with inhibition of PMN-MDSC improved treatment efficacy in HN cancer, suggesting that MDSC impair T-cell functions with mechanisms independent of PD-L1 expression [152]. In a melanoma mouse model that was resistant to combination of anti-PD-L1 and anti-CTLA-4 treatment, PMN-MDSC induced T-cell apoptosis by expressing FasL [115]. The effect was reversed by blocking Fas expression on CD8⁺ T-cells or by using anti-Fas antibodies.

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In humans, MDSC express PD-L1. But it is unclear to what extent the expression of immune checkpoint receptors or death ligand contributes to MDSC-mediated immunosuppression. The frequency of PD-L1⁺ MDSC in the blood of NSCLC patients was higher compared to that of healthy donors [112]. PD-L1⁺ PMN-MDSC, but not M-MDSC, were up to twice as frequent at the tumor site compared to peripheral blood in patients with adenocarcinoma or squamous cell carcinoma [112]. In melanoma patients that failed to respond to ipilimumab treatment against CTLA-4, PMN-MDSC expressed up to 3-fold higher levels of PD-L1 compared to responding patients at the beginning of treatment [35], [114].

*Assessing immunosuppression *in vitro* and *in vivo**

As no specific MDSC marker has been identified, phenotype alone is insufficient to define MDSC. Therefore, functional validation of MDSC is required [27].

Immunosuppression assays in mice

In vitro, assessing proliferation, cytokine secretion or Treg induction is often used to evaluate the impact of MDSC on T-cells [85], [102]. MDSC suppression can be enhanced or inhibited by adding drugs or antibodies to the co-cultures. Arginase-1 expression is analyzed using qRT-PCR or Western Blot, and its activity is directly measured by the production of urea [88]. The presence of nitrite is measured directly through Griess reaction and peroxynitrite by measuring nitrotyrosine residue by ELISA [97], [153]. Nitrosylation of proteins is assessed with specific anti-nitrotyrosine antibodies [99]. Alternatively, the suppressive activity of MDSC isolated from mice deficient for the enzymes related to ROS production is compared to that of MDSC isolated from wild-type mice [48]. Sex, genetic background or *in*

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vitro differentiation protocols for MDSC have all been shown to influence the immunosuppressive capacities of MDSC [154] [155] [156].

In vivo studies modulate the functions of MDSC in ongoing disease by using genetically altered mice, small molecule inhibitors or blocking antibodies. Alternatively, MDSC have been adoptively transferred into mice that do not harbor functional MDSC [100], [157]. The lack of MDSC-specific markers or transcription factors limits the options available to modulate MDSC specifically *in vivo*. The antibodies used in depletion assays target the Gr-1 subunits Ly6C and Ly6G. However, Ly6C targets both M-MDSC and monocytes, and Ly6G both PMN-MDSC and neutrophils. Technical limitations to depletion assays need to be considered, such as the rapid reconstitution of the depleted myeloid compartment [158], [159]. Similarly, the immunosuppressive mechanisms discussed in the previous section are not exclusively employed by MDSC. Other cells of the myeloid lineage - e.g. tumor-associated macrophages (TAM) or neutrophils (TAN) - deplete amino acids, produce ROS, secrete IL-10/TGF- β or express PD-1/PD-L1. Therefore, targeting one mechanisms within the myeloid lineage will target multiple subpopulations besides MDSC [113].

An elegant approach to assess the effect of MDSC *in vivo* is adoptively transferring purified MDSC and T-cells into mice. For example, Nagaraj *et al.* (2007) intravenously injected OT-1-specific T-cells into naïve animals, then transferred MDSC and finally immunized the mice against the relevant peptide (SIINFEKL) [100]. T-cell functions, e.g. proliferation or cytokine secretion, were impaired after a few days in the presence of functional MDSC. In contrast, Sierra *et al.* (2014) loaded the SIINFEKL peptide directly onto MDSC before transferring them into mice to analyze whether MDSC required antigen-presentation for their suppressive activities [157].

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This approach showed that T-cells exposed to peptide-loaded MDSC were dysfunctional compared to T-cell exposed to peptide-loaded dendritic cells (DC).

Immunosuppression assays in humans

T-cell proliferation and cytokine secretion assays are most commonly used to test the immunosuppressive activities of human MDSC [73]. However, it is difficult to compare the results among different research groups because the protocols vary widely [28], [73]. In all protocols T-cells are co-cultured with MDSC in 96-well tissue culture plates. T-cells are stimulated with anti-CD3- and anti-CD28-antibodies that are plate-bound and/or soluble. Stimulation beads are also used, but researchers should consider that myeloid cells phagocytose the beads, which could introduce bias to the assay. T-cell proliferation is either measured by tracking dye dilution or tritiated thymidine incorporation. Cytokine secretion, mostly IFN- γ , IL-2, IL-10 or TGF- β , is assessed by ELISA. Effector cells include autologous and allogeneic cells, PBMC, CD3⁺, CD4⁺, CD8⁺ and NK cells. Autologous T-cells show no allogeneic reaction to the MDSC and might reflect physiological reactions better. But allogeneic T-cells represent consistent responders across experiments and can be interchanged with other laboratories [68]. Other mechanisms of suppression studied *in vitro* include Foxp3⁺ CD4⁺ Treg induction, increased PD-1 expression, CD3 ζ downregulation or the presence of nitrotyrosine in CD8⁺ T-cells [60], [94], [100].

Most studies rely on *ex vivo* samples from blood or biopsies to analyze the expression of surface markers and the presence of possible suppressive mechanisms (e.g. ROS production or Treg induction) [160], [161] [73]. *In vivo* studies in humans observe the effects of chemo- or immunotherapy on MDSC levels or myeloid cell maturation [162]. However, as the therapies usually impact all myeloid cells, it is

difficult to draw MDSC-specific conclusions. Humanized mouse models could represent an interesting surrogate to human *in vivo* studies. For example, NOD scid γ (NSG) mice are immunodeficient and allow human immune cells to develop after human CD34⁺ hematopoietic stem cells engraftment [163]. Moreover, NSG mice can be crossed with mice deficient for the proto-oncogene c-kit, a receptor tyrosine kinase [164]. The resulting NBSGW mice can then be engrafted with human hematopoietic stem cells without requiring prior irradiation. However, these mice do not support the expansion of human myeloid cells because they lack the necessary human cytokines. Human tumor cells can be injected in these mice before transferring tumor-specific T-cell that had been cultured with pan-MDSC or control cells [165].

Neutrophils in cancer

Although this introduction is adapted from a review focusing on MDSC, the presence and role of high-density neutrophils in cancer cannot be overlooked, especially considering their close proximity to PMN-MDSC (Box 2). Up to 70% of the total circulating leucocytes are high-density neutrophils. The neutrophil population expands during cancer. Indeed, parameters like the neutrophil-to-lymphocyte ratio (NLR) and the presence of tumor-associated neutrophils are used as prognosis factors in cancer [53].

Neutrophils originate from the bone marrow during granulopoiesis. They differentiate from granulocyte-monocyte progenitors in the presence of mediators like G-CSF and GM-CSF. Neutrophilic precursors have non-segmented nuclei, with a round (neutrophil early precursor or NeP) or curved (band cells) form. In contrast, mature neutrophils have hyper-segmented nuclei [166]. Immature neutrophils are

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retained in the bone marrow through the expression of CXCR4, which is the receptor of the chemokine CXCL12 that is released by stromal cells. During their maturation, neutrophils lose the expression of CXCR4 and begin expressing CXCR2, allowing them to leave the bone marrow and join the peripheral blood.

It is important to remember that the main role of neutrophils is to clear pathogens during acute infections. However, neutrophils are highly plastic and short-lived cells that acquire different functions depending on their biological context (e.g. inflammation). In cancer patients or tumor-bearing mice, neutrophils show both pro- and anti-tumor activities for neutrophils. Fridlender *et al.* mirrored the M1/M2 macrophage nomenclature and described neutrophil polarization towards N1 (anti-tumor) or N2 (pro-tumor) phenotypes in tumor bearing mice [167]. N1 neutrophils, for instance, can kill tumor cells both *in vitro* and *in vivo*. They have higher cytotoxic activity towards highly metastatic cells, which can prevent tumor dissemination leading to metastasis [168]–[170]. However, in the presence of mediators like TGF- β , neutrophils can switch to an immunosuppressive N2 phenotype and the ability to suppress other immune cells [168].

Circulating neutrophils in tumor-bearing mice and cancer patients

Increases in the NLR are correlated with poor outcomes for cancer patients and is used as prognosis factor for survival in, among others, melanoma, breast, CRC and lung cancer [168], [171]–[175]. A meta-analysis including 40,559 patients from one hundred studies confirmed an association between high NLR (defined as > 4) and a hazard ratio of 1.81 for overall survival [176]. However, the value of the NLR as a prognostic factor is affected by type of cancer [168], [176]. For instance,

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mesothelioma showed higher hazard ratio (2.35) than hepatocellular carcinoma (1.45) for overall survival.

Whether circulating neutrophils in cancer patients differ from circulating neutrophils in healthy donors remains unclear. For instance, neutrophils isolated from head and neck cancer patients produced more ROS and underwent less apoptosis than neutrophils from healthy donors [177]. Non-activated circulating neutrophils from healthy donor do not suppress T-cell functions, unless activated with compounds like N-formylmethionyl-leucyl-phenylalanine (fMLF) [141], [178]. In contrast, neutrophils from the blood of melanoma patients inhibit T-cell proliferation *in vitro* [179]. Conversely, no suppressive activity was observed in circulating neutrophils from metastatic HN, lung or mammary cancer patients [55], [141]. However, studies comparing the suppression capacity of neutrophils to that of PMN-MDSC in cancer patient remain rare and the functions of circulating neutrophils in cancer require further investigation [141].

Tumor-associated neutrophils

In many cancer types, neutrophils infiltrate the tumor-microenvironment. Like circulating neutrophils, TAN are defined as CD11b⁺ and CD66b⁺ cells. However, TAN are heterogeneous cells that are shaped by their biological context (stimulus or tumor histology), leading to various phenotypes and opposite (pro- and anti-tumor) functions [53], [166], [168]. Therefore, no unique signature defines TAN compared to circulating neutrophils. However, the expression of several genes like myeloperoxidase (MPO), PD-L1 or ARG-1 is highly expressed in TAN in lung cancer patients [53], [180]. As TAN are usually analyzed by immunohistochemistry or

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directly sorted after tumor digestion, little is known about their buoyancy. Thus it is unclear whether TAN include both LDN and HDN.

TAN have both pro- and anti-tumoral functions. Their effects on tumor progression were comprehensively described by Fridlender's group in their recent review [168]. In brief, the anti-tumor effects of TAN include killing primary or metastatic tumor cells (either through the release of ROS or NETosis). Additionally, degranulation of TAN impairs angiogenesis and tumor-cell mobility, reducing the development of metastasis. In contrast, TAN have pro-tumoral effects because degranulation and the release of proteases and ROS impairs T-cell functions or viability, leading to the remodeling of TME, triggering angiogenesis and thus promoting the development of premetastatic niche. *"The net impact of neutrophils in cancer is the sum of a complex balance between contradicting effects occurring at the same time"* [168].

High infiltration of TAN is also associated with poor outcomes. For instance, a meta-analysis of the expression signatures from ~18,000 human tumors revealed that the TAN-to-plasma cell ratio in tumors was a strong predictor for overall survival [53], [181]. Also, correlation between neutrophil infiltration and poor prognosis was also shown in melanoma, HN cancer and hepatocellular cancer [182]–[184]. However, the value of neutrophil infiltration in tumor as prognosis factor is contrasted in CRC and lung cancer. For instance, increased frequency of TAN was correlated with both poor prognosis [185] and favorable outcomes [53], [186], [187]. In lung cancer, Rakaee *et al.* also showed that the impact of neutrophil infiltration in lung cancer is different according to the subtype of the tumor: in SCLC high densities of intratumoral TAN was a positive prognostic factor, in adenocarcinoma it was a negative one [188]. Conversely, another study highlighted the opposite effect. TAN

infiltration was correlated with poor outcomes in SCLC patients [189]. These studies highlight once again that the impact of neutrophils on cancer progression still requires further investigation.

MDSC in different human cancer types

In ovarian cancer

Due to its asymptomatic nature and its propensity to metastasize, ovarian cancer represents one of the most common causes of female cancer death in developed countries [190]. In 2015, classification guidelines for ovarian cancer were revised according to the tissue and mutations involved [191]. Type I or low-grade tumors include those that arise from transitional or germ cells, conditions like endometriosis or tissues such as fallopian tubes. Type II groups high-grade tumors like high-grade serous carcinoma, undifferentiated carcinoma and carcinosarcoma. While type I cancers tend to stay local and metastasize late. Type II cancers are more aggressive and patients present to clinicians at late stages [192].

Current treatments of ovarian carcinomas include surgery and platinum-based chemotherapy, to which most patients respond initially. However, around 70% of the patients will relapse within the first 2 years [190], [193], [194]. Immunotherapy (including ICI or chimeric antigen receptor (CAR) T-cell) show limited efficacy, with less than 15% of patients responding to anti-PD-L1 treatment and 35% responding to a combination of anti-PD-1 and anti-CTLA-4 [195]–[197]. The tumoral microenvironment in ovarian cancer is involved in resistance to chemotherapy, lack of T-cell infiltration and intratumoral immunosuppression [198], [199]. For instance, cells like cancer-associated fibroblast (CAFs) promote

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angiogenesis and tissue remodeling. Immature DC and TAM inhibit CD8⁺ and CD4⁺ T-cell functions. Furthermore, cellular composition, genomic and mutation analysis of TME revealed impaired T-cell infiltration in tumor of ovarian cancer patient [200].

Elevated numbers of MDSC in ovarian cancer patients are correlated to poor prognosis for responses to treatment and overall survival [64], [108], [165], [201]. On the flip side, low numbers of MDSC is associated with favorable outcomes: type II diabetic patients with ovarian cancer with low MDSC counts and higher CD8⁺ T-cell counts in blood had better overall survival with metformin treatment in [108]; and ovarian cancer patients with BRCA1 and BRCA2 mutations showed reduced frequencies (by about 40%) of circulating MDSC and 2-times higher CD8⁺ T-cell count and had a better response to chemotherapy than BRCA wild-type ovarian cancer patients [202]. However, whether MDSC represent a useful indicator to predict outcomes in ovarian cancer patients requires further investigation because of inconsistencies in the studies available to date regarding MDSC phenotyping, sample size or lack of detailed clinical information [194]. Currently, only one clinical trial (NCT02868255, available on ClinicalTrials.gov) examines MDSC frequencies in ovarian cancer patients.

In lung cancer

In 2020, lung cancer was the most diagnosed and most deadly cancer, accounting for 11.4% of all cancer cases and 18% of all cancer-related death [203]. Lung cancer is broadly classified in two main groups: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) which account approximatively for 15% and 85% of all lung cancers, respectively [204], [205]. SCLC is a poorly differentiated neuroendocrine tumor associated with heavy smoking history [206], [207]. SCLC is characterized by

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a high proliferation rate, a fast-doubling time, and a propensity for early metastasis [205]. NSCLC are divided into three histological subtypes : adenocarcinoma (ADC), squamous cell carcinoma (SqCC) and large-cell carcinoma (LCC) [208]. Since 2004, the histologically-based NSCLC classification have been combined with the analysis of molecular alterations in oncogenes and tumor-suppressor genes displayed by tumor cells [209]. NSCLC harboring an actionable molecular alteration such as epithelial growth factor (EGFR), anaplastic lymphoma kinase (ALK), RET, BRAF, ROS1, NTRK, MET and KRAS proto-oncogene (KRAS) is treated with specific inhibitor as a standard of care (SOC) for molecularly defined populations [210].

Currently, treatment choice is driven by cancer type, disease staging, molecular mutations and levels of PD-L1 expression on tumor cells [211]. All SCLC are treated with platinum-based chemotherapy. Stage I and II, as well as locally advanced stage III SCLC are treated with platinum-etoposide chemotherapy associated with radiotherapy. Metastatic SCLC which accounts for 86% of the cases are treated with platinum etoposide chemotherapy in combination with anti-PD-1/anti-PD-L1 immune checkpoint inhibitor such as durvalumab, atezolizumab or pembrolizumab [212], [213]. The median OS of metastatic SCLC treated with platinum-etoposide and atezolizumab is 12.3 months (95% CI 10.8-15.9 months) versus 10.3 months (95% CI 9.3-11.3 months) with platinum-etoposide chemotherapy [214].

The classical treatment for stage I, II and some stage IIIA NSCLC is surgical resection. Postoperative radiotherapy is not recommended anymore as a routine because it provided neither harmful nor beneficial effect to patients [215]. Surgery is also associated with chemotherapy in large tumor or with metastatic lymph node [211]. Unresectable stage III or metastatic (stage IV) NSCLC treatment first aims to

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target specific molecular alteration with, for instance, osimertinib or alectinib, which are inhibitors of EGFR and ALK respectively [216]. PD-L1 expression on tumor also guide the ICI treatments in NSCLC without targetable oncogenic driver. ICI treatment with pembrolizumab, atezolizumab or cemiplimab monotherapy is the SOC of stage IV NSCLC with high PD-L1 expression ($\geq 50\%$). In patient with tumor PD-L1 expression $<50\%$, pembrolizumab combined with platinum based-chemotherapy is the SOC of stage IV NSCLC [216], [217].

However, despite these significant improvements in treatments, patient overall survival remains poor and median progression-free survival rarely exceeds 2 years with the current treatments [211], [218]. Due to its physiological functions, the lung microenvironment is unique and dynamic, and contains a large array of immune cells and commensal microorganisms [219]. T-reg, TAM and MDSC, which are potent immunosuppressor of anti-tumoral immune response, can be found in TME of lung cancer patient. Therefore, the impact on TME on tumor progression raised interest, especially to improve current strategies of treatment [218].

The presence of MDSC is linked to prognosis, development and treatment response in lung cancer [112], [218], [220], [221]. For instance, PMN-MDSC that express ARG-1 inhibited CD3- ζ expression in CD8⁺ T-cell and were increased in patients who did not respond to treatment [94]. Also, M-MDSC that express S100A9 protein were associated with resistance to chemotherapy, metastasis and shortened progression-free survival [33], [221]. MDSC are also potential indicators of response to immunotherapy approaches as LOX-1⁺ PMN-MDSC were higher in non-responder compared to responder patients that received nivolumab e.g. [222]. Limagne *et al* have also shown that galectin-9 (Gal-9) M-MDSC, associated with a high level of Tim-3 expression on lymphoid cells, were involved in resistance to anti-PD-1 treatment

in metastatic NSCLC patient [223]. However, the effect of MDSC on lung cancer development and treatment still requires further investigation, especially due to the current inconsistency regarding MDSC phenotyping, isolation and *in vitro* testing [218].

Current therapeutic approaches target MDSC either by (i) eliminating them or promoting their differentiation into non-suppressive cells, (ii) impairing their recruitment to the tumor site or (iii) inhibiting their suppressive capacities [218], [224]. Currently, several drugs are on ongoing clinical trial in NSCLC tumor. For instance, vinorelbine have potential cytotoxic effects on MDSC (NCT03801304). Oleclumab is an antibody targeting CD73 ectonucleotidase (NCT04262388). The AB928 compounds targets adenosine receptors A2aR and A2bR (NCT03846310). Finally, the agonist RGX-104 have the potential to directly kill MDSC (NCT02922764) [224], [225].

In other cancer types

MDSC are also present in other cancer types [224]. Their presence is associated with tumor and metastasis development, and poor prognosis [220]. MDSC mediate resistance to ICI therapies and therefore, represent potential therapeutic targets [226].

Breast cancer is, with lung cancer, the most diagnosed cancer the and main cause of cancer-related death in women [203]. Breast cancer types are defined by the expression of the estrogenic receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER2) [227]. TAM, Treg and MDSC in breast tumors lead to poor immunogenicity and reduce the efficacy of immunotherapy [228]. For instance, the percentage and absolute numbers of MDSC

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were proportionally increased with disease stage, and the frequency of MDSC correlated to poor overall survival [62], [220]. A combination of CXCR2 antagonism and CSFR-1 inhibitor was used to decrease PMN-MDSCs populations and improve ICI treatment efficacy in breast cancer [229], [230]. Additionally, inhibitor of histone deacetylase (Entinostat) combined with ICI reprogrammed PMN-MDSC and significantly improved tumor-free survival in both the HER2/neu transgenic breast cancer [231]. CXCR2 antagonist and histone deacetylase are targeted in current clinical trials (NCT02370238 and NCT02453620 respectively).

Colorectal carcinoma (CRC) is the second most common cause of cancer deaths [203]. CRC is favored by chronic inflammation, like colitis, which are also associated with expansion of MDSC [232], [233]. ARG-1-expressing PMN-MDSC (but not M-MDSC) are expanded in both blood and tumor tissue of CRC patients compared to healthy donors and normal tissue [234]. Several clinical trials that target MDSC in CRC are ongoing, including anti-TRAIL-R2 agonist antibody DS-8273a (NCT2076451), which selectively depletes MDSC [235].

Melanomas are skin tumors that are treated by resection if spotted at early stages. However, melanoma is also the most aggressive form of skin cancer and metastasizes fast, reducing the median survival of patient below one year [236], [237]. Once metastatic, melanoma is usually treated with chemotherapeutic and/or anti-PD1 and anti-CTLA-4 mono- or combined ICI. However, almost half of patients fail to respond to this approach because of primary or acquired resistances [151], [238]. Once again, an immunosuppressive TME, composed of MDSC or Treg, is believed to play a key role in this process [239]. For instance, both PMN-MDSC and M-MDSC numbers were increased in stage IV melanoma patients and associated with poor overall survival and response to cancer vaccines [240]–[243]. Several

clinical studies that target different aspects of MDSC are ongoing [224]. These compounds induce, for instance, differentiation of M-MDSC into macrophages and DC in mouse models (ATRA, NCT02403778) [244], [245], block the CXCR1/2 chemokine receptor (SX-682, NCT03161431) [246], [247], or inhibit ROS and NO signaling (omaveloxolone, NCT02259231) [248].

Roadmap to future and objective of the project

In both mice and humans, MDSC accumulate under pathological conditions and suppress the functions of T-cells and NK cells. However, the road leading to specifically targeting MDSC in diseases like cancer remains long and demanding. The mechanisms behind how MDSC accumulate are still unclear. More importantly, identifying MDSC remains challenging due to the lack of unique markers. Finally, the suppressive mechanisms remain incompletely characterized, especially in humans where *in vivo* experiments are very limited. We propose perspectives and suggestions of methods to increase the knowledge surrounding MDSC and how this information may help to build better therapeutic approaches.

How to identify MDSC?

In both species, current M-MDSC markers are shared by monocytes and macrophages, while PMN-MDSC markers are also expressed by neutrophils. Currently, the distinction between MDSC and other myeloid cells relies on their cell density and/or a diminished expression of markers like HLA-DR or S100-family proteins [27], [249]. However, these criteria limit the accuracy of identifying tumor-infiltrating MDSC by immunohistochemistry.

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To identify potential markers that are exclusive to MDSC, we favor RNA sequencing approaches because they are unbiased and analyze all differentially expressed genes. RNA sequencing identified LOX-1 as a potential PMN-MDSC and CD84 as a potential pan-MDSC marker in mice and humans [50], [55]. While LOX-1 has gained wide-spread interest, its expression is often analyzed on whole neutrophil populations, without discerning between high-density and light-density neutrophils. Comparing LOX-1⁻ vs LOX-1⁺ light density neutrophils from the same patient could further validate this surface protein as a sufficient and unique marker to distinguish PMN-MDSC from light- and high-density neutrophils [42]. CD84 was identified by single-cell RNA sequencing from splenic murine CD11b⁺Gr-1⁺ cells. PMN-MDSC were identified as a cluster of arginase-2- and IL-1 β -positive light density PMN. The authors confirmed CD84 as a human pan-MDSC marker using *in vitro*-generated MDSC. However, because the data was not compared to pure or clinical samples, it is likely that CD84 is not exclusive to MDSC [25]. Nevertheless, approaches relying on bioinformatics are promising and performed against control cells. For instance, new MDSC biomarkers could be identified by comparing the transcriptome of MDSC and other myeloid cells from the same donor or from patients and healthy individuals.

Relevance of MDSC in the clinic – potential therapeutic targets?

Regardless of whether MDSC are a distinct subpopulation or rather represent a state of myeloid cell activation, they are attractive therapeutic targets as they are more frequent in patients with diverse conditions and are highly immunosuppressive.

To validate potential antibodies or drugs against MDSC, mouse models, especially humanized mice, hold great potential and will be crucial. Not only could

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they enable the study of potential immunotherapies targeted at MDSC, but they could also help understand how MDSC emerge in response to tumors and how tumors maintain their recruitment. Studying, for example, the role of PGE2 in the initial recruitment of MDSC to a tumor site or their maintenance in humanized mice could be interesting. However, human MDSC fail to develop in NSG mice because the mice lack cytokines required to maintain human myeloid cells (e.g. GM-CSF or M-CSF). The MITRG or MISTRG mice strains could support human myeloid cells because the mice express human GM-CSF, IL-3, M-CSF, thrombopoietin and SIRP α on a Rag2^{-/-}Il2rg^{-/-} background [250].

In parallel, clinical research on MDSC will benefit from the efforts to harmonize identification strategies, isolation methods and functional assays. Cassetta *et al.* (2020) demonstrated that small variations in processing of patient samples impact the frequency of MDSC that are observable and isolatable [28]. The most difficult aspects are functional assays that are used to assess immunosuppressive capacities of isolated MDSC. As protocols used in these assays are prone to high variations, comparison of results among different research groups might be challenging. We previously proposed a protocol to isolate MDSC and test their suppressive activity in proliferation assays with allogeneic T-cells that can be frozen and shared to reduce some variability inherent to these assays [68]. However, while T-cell proliferation assays remain the most common test to assess MDSC-mediated suppression of T-cell function, it is important to consider that *ex vivo* MDSC only survive for the first couple of days of the experiment.

Besides technical considerations, it is important to remember that most human MDSC are identified and isolated from blood. However, MDSC are thought to exert their immunosuppressive functions most at the site of disease, for example

within the tumor. MDSC samples from blood might phenotypically and functionally differ from MDSC in the TME. Thus, interesting potential markers or functions could be missed [12], [112], [251], [252]. Very few groups isolate MDSC directly from tumor samples. Immunohistochemistry of tumor-MDSC-T-cell interactions will provide much information, especially if MDSC-specific markers can be identified by RNA sequencing. Si *et al.* (2019) used immunohistochemistry of HN tumors to show that higher conjugations of LOX-1⁺ CD66b⁺-expressing PMN-MDSC and CD3⁺ T-cells in the TME correlated to reduced overall survival for patients [58].

Besides humanized mice, patient-derived organoids could be a creative model that accounts for longer time-courses and more diverse cell-cell interactions [253], [254]. However, *in vitro* organoids often aim to represent the TME only. This would disregard potential interactions with other compartments, e.g. lymph nodes [255]. Multi-compartment microfluidic chips that connect two *ex vivo* tissues might overcome this challenge and provide more information about MDSC and the wider TME [256].

Aim of the thesis

For the last two decades, MDSC have been studied extensively in cancer. However, most of their suppressive mechanisms were highlighted in mouse models, and thus information regarding human MDSC is still lacking. Currently, identification of M-MDSC and PMN-MDSC still remains challenging, and several groups think they might represent a pathological state of activated monocytes or neutrophils rather than distinct populations. Moreover, by the lack of harmonization regarding the pathologies studied, isolation strategies and protocols used to assess MDSC-mediated immunosuppression hinders inter-laboratory studies of MDSC. Finally,

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most studies on human MDSC focus on the phenotypes and functions of MDSC isolated from blood. However, studies that directly compare the phenotypes, functions and transcriptomes of MDSC isolated from blood and tumor are currently absent.

The purpose the thesis is to compare the presence, suppressive activity, and transcriptional signature of MDSC isolated from (i) different types of cancer and (ii) different tissues of the same patient. Moreover, and as suggested by Gabrilovich in his 2021 review, distinguishing MDSC from classical monocytes and neutrophils will be a critical step for the design of effective therapies targeting these suppressive myeloid cells [11]. Therefore, we decided to analyze the transcriptomic signatures of high-density neutrophils (HDN) and non-suppressive low-density neutrophils (LDN) and characterize their differences to PMN-MDSC.

We isolated the myeloid cells from blood and solid tumor from ovarian and lung cancer patients. We first defined the gating strategies and compared the frequency of M-MDSC and PMN-MDSC in the blood and tumor of the two cancer types. We then set up a T-cell proliferation assay to analyze the suppressive activity of the myeloid cells we isolated.

We extracted the RNA of paired samples of LDN and classical neutrophils and sequenced them. We focused our analysis on particular genes of interest such as gene involved in suppressive mechanisms reported in mice (e.g. ARG-1 and NOX-2), and on potential markers, chemokines or transcription factors that might be differentially expressed between PMN-MDSC and neutrophils.

Finally, tried to distinguish PMN-MDSC from non-suppressive LDN in terms of morphology, phenotype, suppressive activity, and transcriptomic approach.

Results

Results

Results

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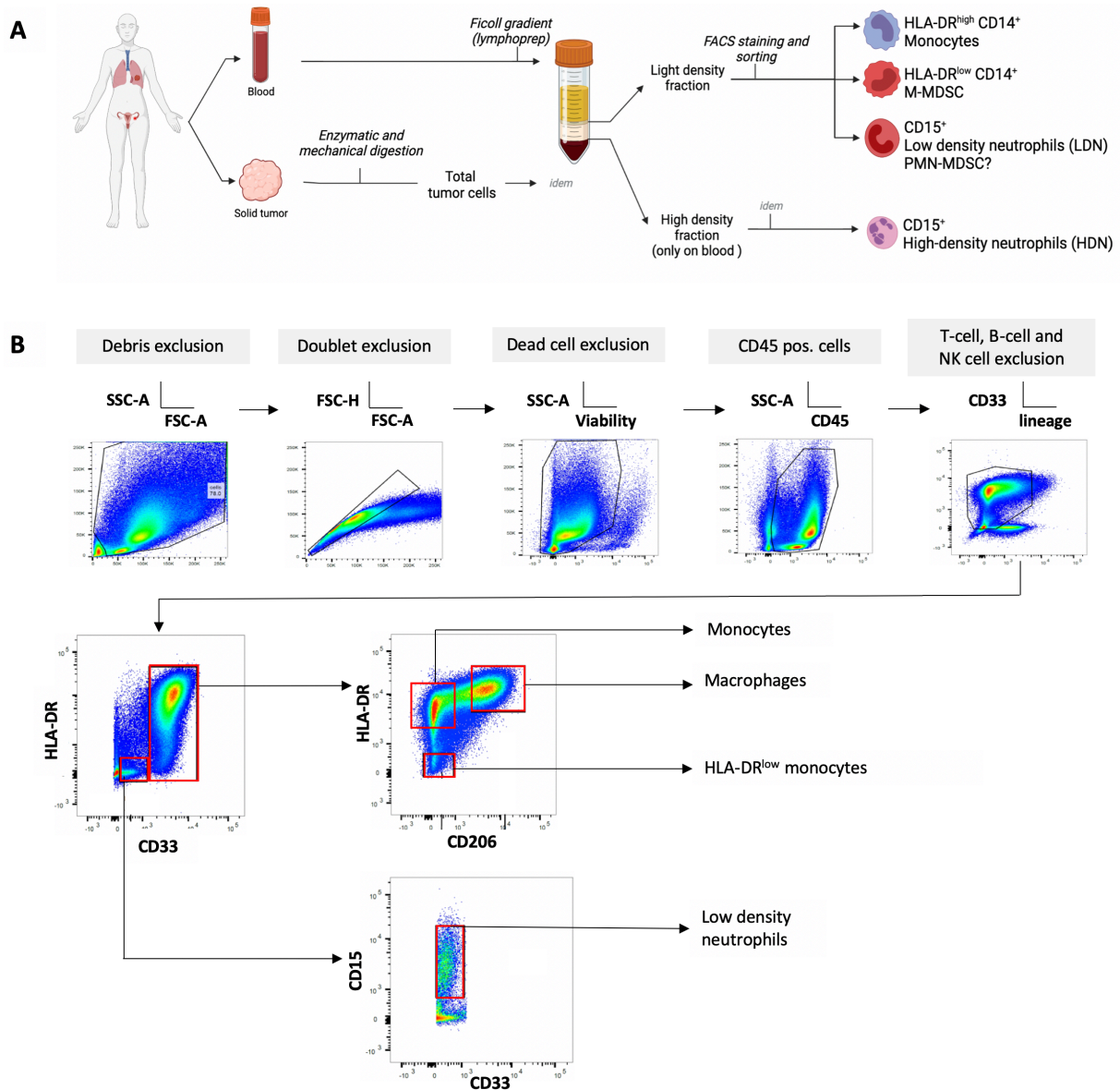


Figure 6. Overview of the procedure and gating strategy used to isolate myeloid cells from blood and tumors of cancer patients. **A.** Blood and tumor samples from lung and ovarian cancer patients are processed on the day of surgery. Tumor samples are dissociated and digested with liberase cocktail. Both single cell suspensions of tumors and PBMC and were obtained after density gradient separation. The light fraction was harvested and stained with the MDSC panel (see material and methods) and sorted by flow cytometry. High-density neutrophils (HDN) from blood were extracted from the high-density fraction with 1% polyvinyl alcohol and were stained and sorted following the same principle. **B.** Gating strategy for isolation of monocytes, macrophages, HLA-DR^{low} monocytes and low-density neutrophils (LDN). HDN are sorted from high-density fraction following identical gating strategy than LDN (not shown on figure). The cells' viability was assessed with the dye eFluor780. Gates were defined using FMO controls. Representative plot of one processed tumor sample is shown. In blood samples, anti-CD206 antibodies are replaced by anti-CD66b or anti-CD3 antibodies.

Part 1: Impact of cancer type or environment on the frequencies of myeloid cells

Currently, research groups usually work with immunosuppressive myeloid cells (IMC) from one cancer type, and with their own isolation protocols and strategies. Therefore, few studies directly compare the presence and clinical relevance of IMC in different cancer types. A notable exception is Cassetta *et al.*, 2020, which compared the frequencies of these myeloid cells in the blood of different cancers using a standardized protocol. However, the study focused on the effect of technical variations on the cells' frequencies (e.g. the anti-coagulant used).

We thus compared myeloid cells isolated from both blood and solid tumor samples from ovarian and lung cancer patients. To avoid confusion regarding terminology, we decided to keep the term “low-density neutrophils” (LDN) and HLA-DR^{low} monocytes to designate sorted cells, as it refers to a phenotype rather than a functional state, the latter being assessed only through specific assays. Only the LDN were isolated from tumors, hence the terminology “tumor LDN” instead of TAN. We recovered both the light-density (PBMC) and high-density (neutrophils and erythrocytes) fractions after density gradient separation to compare LDN against high-density neutrophils (HDN) (Figure 6A). Classical monocytes, HLA-DR^{low} monocytes, LDN and HDN were sorted by flow cytometry (see gating strategy Figure 6B).

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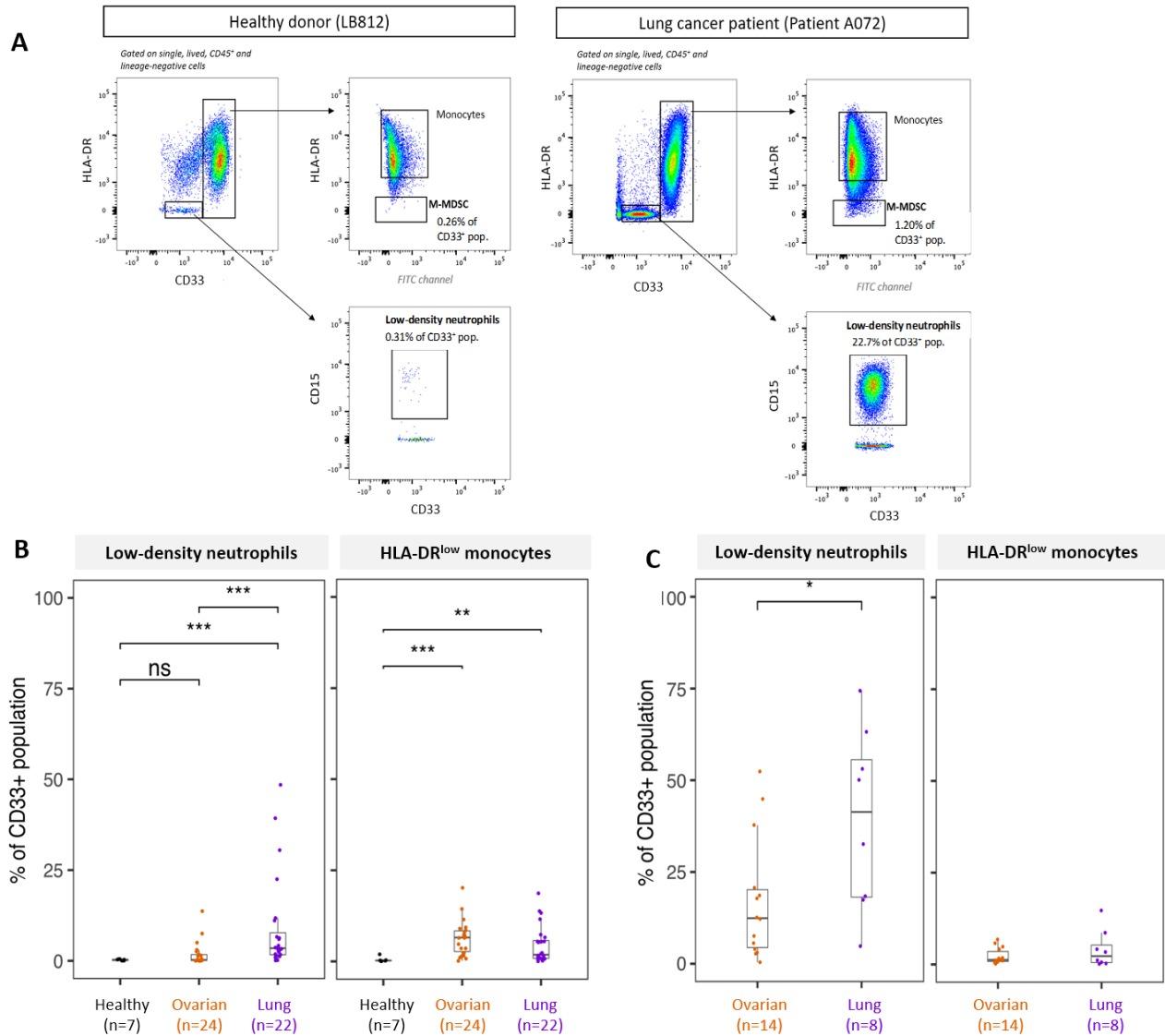


Figure 7. Presence of LDN and HLA-DR^{low} monocytes is increased in cancer patients, and their frequencies vary with cancer type. **A.** One representative plot of the frequencies of LDN and HLA-DR^{low} monocytes in the blood of one non-cancerous donor and one lung cancer patient. Plots represent live single CD45⁺ lineage⁻ cells, following the gating strategy described at Figure 6. PBMC were collected after density gradient separation and stained with MDSC-isolation antibody panel. Sorting gates were defined using cells stained with the MDSC-master mix without anti-CD15 and anti-HLA-DR antibodies. **B-C.** Frequency of LDN and HLA-DR^{low} monocytes among the total CD33⁺ myeloid population in (B) the blood and (C) tumors. Shown are data of non-cancerous donors, and chemotherapy-naïve ovarian cancer and lung cancer patients. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (Wilcoxon-Mann-Whitney test).

Frequency of HLA-DR^{low} monocytes and LDN in blood of cancer patients

As mentioned, PMN- and M-MDSC can be found at low frequency in healthy individuals but their numbers are elevated in cancer patients [224]. We thus compared the presence of LDN and HLA-DR^{low} monocytes in the blood of cancer patients (chemotherapy-naïve) and non-cancerous donors (Figure 7).

The frequency of LDN in non-cancerous donors never exceeded 1%. But, in the blood of some lung cancer patients, LDN represent more than 20% of the myeloid cells (Figure 7A). The overall frequency of LDN among the total CD33⁺ myeloid population was significantly higher in blood of lung cancer patients (mean of 8.9%, n = 22) compared to patients with ovarian cancer (mean of 1.8%, n = 23) or non-cancerous donors (mean of 0.27%, n = 7) (Figure 7B). Surprisingly, the frequency of LDN in the blood of ovarian cancer patients was not significantly higher than the one observed in the blood of non-cancerous donors. This difference cannot be explained by, e.g., neutropenia-inducing treatments as only chemotherapy-naïve patients were considered. Thus, it is likely that the differences in MDSC-frequencies observed between lung cancer and ovarian cancer patients are condition-dependent.

HLA-DR^{low} monocytes were found at higher frequencies in the blood of both ovarian (mean 6.4%, n = 23) and lung (mean 4.5%, n = 22) cancer patients compared to that of non-cancerous donors (mean 0.46%, n = 7) (Figure 7B). Unlike LDN, the frequencies of HLA-DR^{low} monocytes were not statically different between the two cancer types.

Results

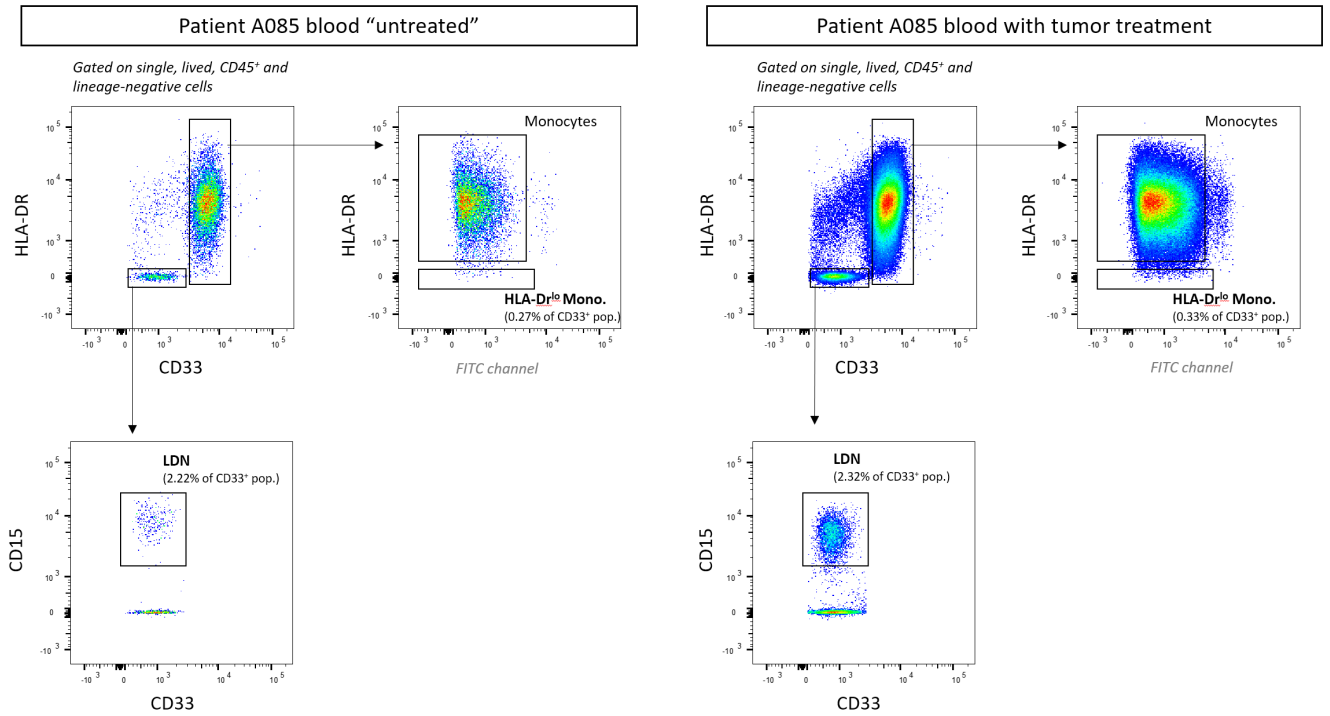


Figure 8. Mechanical and enzymatic digestion do not seem to alter the yields of LDN and HLA-DR^{low} monocytes from the blood of lung cancer patients. Blood of lung cancer patient A085 was collected and split into two 15 ml tubes. In the "tumor treatment" condition, total peripheral blood was mixed with liberase cocktail and DNase, mechanically disrupted with a MACS[®] dissociator and incubated for 45 minutes at 37°C. PBMC of both untouched total blood ("untreated") and tumor-treated blood were collected after density gradient separation and analyzed by flow cytometry. Plots represent cells gated on live single CD45⁺ lineage⁻ cells, following the gating strategy described at Figure 6.

Frequencies of HLA-DR^{low} monocytes and LDN in solid tumors

We also investigated the frequencies of LDN and HLA-DR^{low} monocytes in solid tumors of ovarian and lung cancer patients (Figure 7C). The presence of LDN in solid tumors shows high variability, ranging from less than 10% to 75% of the total tumor-infiltrating myeloid population. Similarly to the blood results, the frequency of LDN in solid tumors was significantly higher in lung cancer patients (mean 39.2%, n = 9) compared ovarian cancer patients (mean 17.1%, n = 14). In contrast, no significant difference was observed for HLA-DR^{low} monocytes between ovarian tumors (mean 2.3%, n = 14) and lung tumors (mean = 4.1%, n = 9) (Figure 7C).

We considered that the mechanical and enzymatical digestion required to obtain single cell suspensions of tumor cells could affect the frequencies of LDN or HLA-DR^{low} monocytes observed. We thus compared the frequencies of myeloid cells in blood of lung cancer patient A085 with or without mechanical and enzymatic digestion before density gradient separation (Figure 8). We observed that frequencies of both LDN (2.22% versus 2.32% of total CD33⁺ population) and HLA-DR^{low} monocytes (0.27% vs 0.33% of total CD33⁺ population) did not change after tumor digestion treatment.

Taken together, these observations suggest that the presence of PMN-MDSC (in the LDN population) varies between lung and ovarian cancers. As all patients presented were chemotherapy-naïve, we suspect disease-intrinsic reasons for the differences observed. These reasons are yet unknown as is the meaning of these differences for the choices of treatments for the patients.

Results

Part 2: Immunosuppressive potential of MDSC and LDN in lung and ovarian cancer patients

As mentioned before, phenotyping alone is insufficient to fully define MDSC because MDSC are characterized by their immunosuppressive capacities. However, most research groups do not assess suppression *in vitro* and rely solely on the phenotype and the expression of specific genes such as *ARG-1* to determine the presence of MDSC. Also, most studies only analyze MDSC in one type of cancer. Thus, direct comparisons of the suppressive activity of LDN or HLA-DR^{low} monocytes sorted from different cancer types or different environments are currently rare.

Suppression assay set-up

The lack of harmonization regarding sorting strategies and suppression assays protocols between research groups hinders the comparability of results [73]. We set up a proliferation assay protocol that measures the suppressive activity of LDN or HLA-DR^{low} monocytes T-cell proliferation, as this read-out is the most commonly use to assess MDSC functions [68].

Results

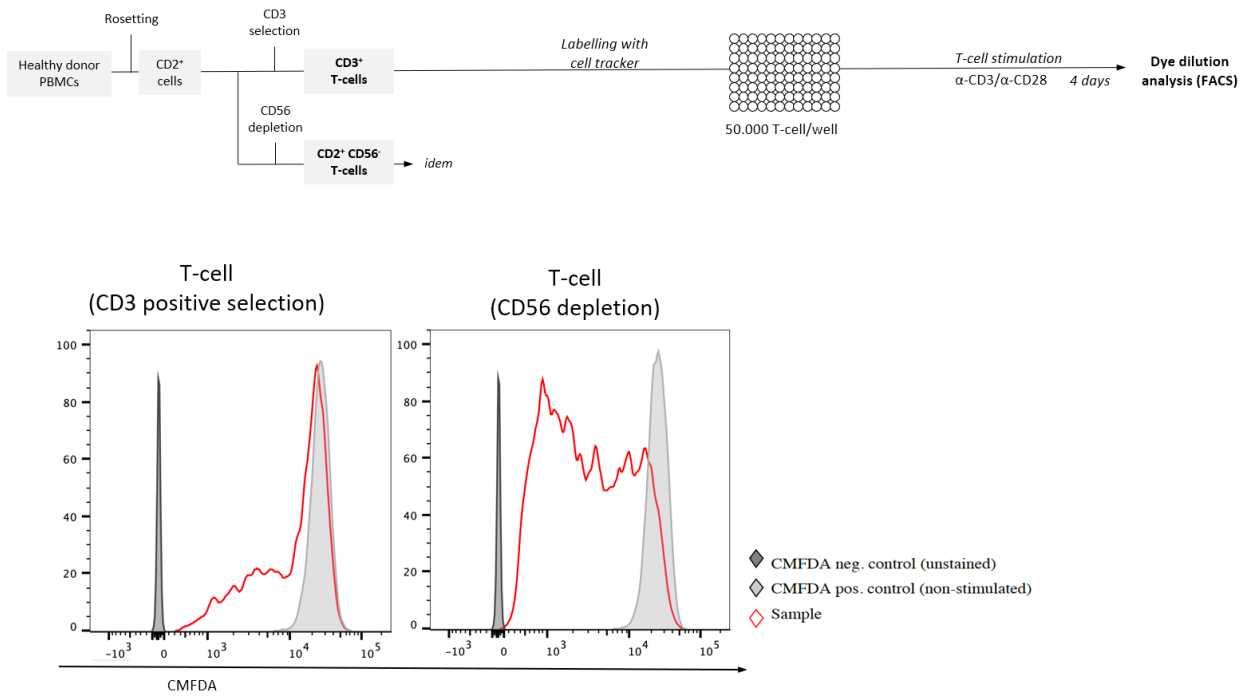


Figure 9. The protocol of T-cell isolation used impacts T-cell proliferation in suppression assays. PBMC were isolated from patient LB5859 by density gradient separation. CD2⁺ cells were isolated using rosetting with sheep erythrocytes. CD2⁺ cells were either mixed with anti-CD3 or anti-CD56 magnetic beads and were sorted by positive or negative (NK cell depletion) selection on autoMACS[®]. CD2⁺CD3⁺ and CD2⁺CD56⁻ T-cells were then frozen and thawed on another day before being stained with 1 μM CFDA. 50,000 T-cells were activated with coated anti-CD3 and soluble anti-CD28. Proliferation was monitored through CFDA dilution after 4 days of culture at 37°C, 5% CO₂. The dilution of the CFDA dye (in red) was analyzed on live CD3⁺ cells after doublet exclusion.

Results

Parameters influencing T-cell proliferation

We used frozen allogeneic T-cells isolated from non-cancerous (hemochromatosis) donors as responder cells. We first evaluated how the method of sorting T-cells impacts the cells' proliferative capabilities. We compared positively selecting T-cells using anti-CD3 magnetic beads against depleting NK cells from the CD2-positive fraction of PBMC (Figure 9). We observed that using anti-CD3 magnetic beads decreased the proliferation of the sorted T-cells, probably by masking the CD3 epitopes or causing low-level CD3 triggering during sorting.

We also observed that T-cell purity was a critical parameter, as cells with high purity (>99% CD3+) were unable to proliferate properly after thawing when they were stimulated with anti-CD3 and anti-CD28 antibodies (Figure 10). However, adding small amounts of myeloid cells (dendritic cells, MDSCs or monocytes) restored proliferative capacities (Figure 10 and data not shown). We therefore systematically added 1,000 mature DC autologous to the effector T-cells per well to avoid encountering poor proliferation, especially in the proliferation control wells.

Results

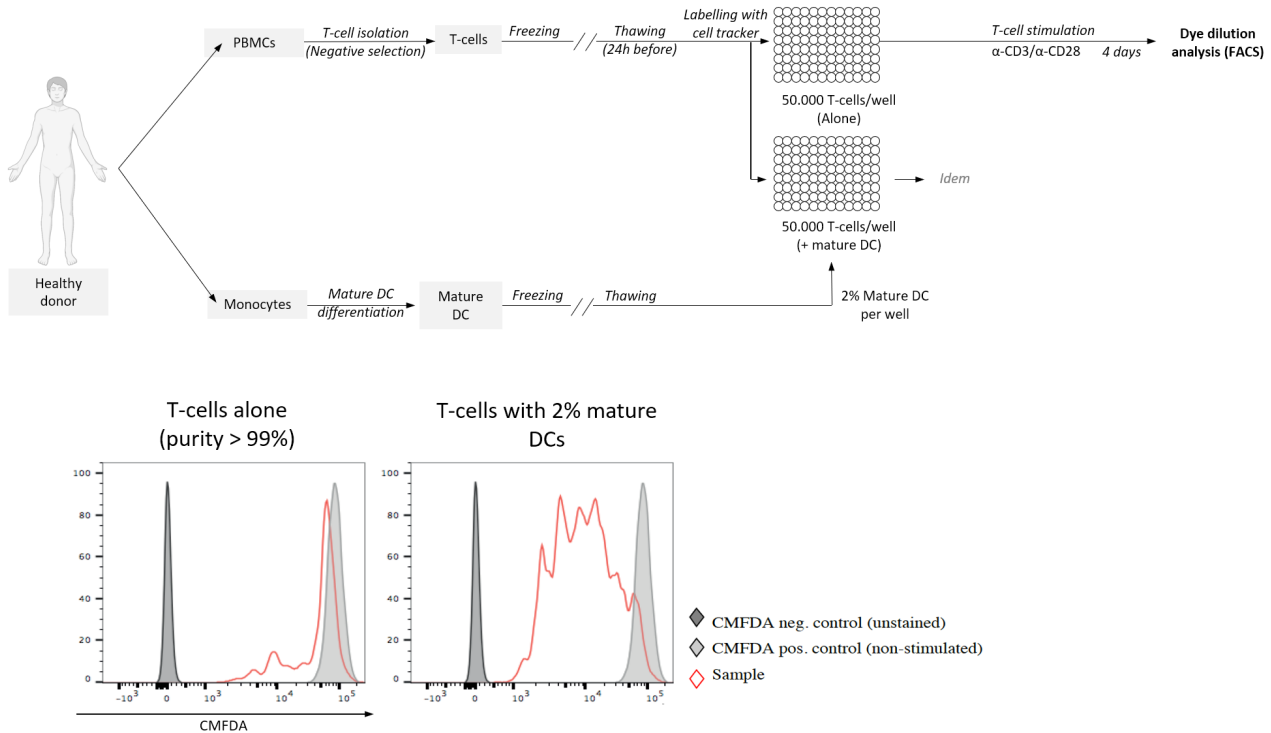


Figure 10. High purity of frozen T-cells impairs proliferation in suppression assay. PBMC were isolated from the blood of non-cancerous donor LB9229 by density gradient separation. $CD2^+$ and $CD2^-$ cells were separated using rosetting with sheep erythrocytes. Monocytes were isolated by positive selection with CD14 magnetic microbeads from the $CD2^-$ fraction and T-cells were isolated by negative selection with CD56 microbeads from the $CD2^+$ fraction. Monocytes were differentiated into DC with GM-CSF (100 ng/ml) and IL-4 (30 ng/ml) for 5 days followed by two days of DC maturation with $TNF-\alpha$ (10ng/ml) and Poly-IC (50 μ g/ml). Mature DC and T-cell were aliquoted and frozen for future assays. 30,000 thawed T-cell were activated with anti-CD3 (1 μ g/ml) and anti-CD28 (2 μ g/ml) antibodies alone or in the presence of 2% autologous thawed mature DC. Proliferation was monitored by CMFDA dilution after 4 days of culture at 37°C and 5% CO_2 . Dilution of the CMFDA dye (red line) was analyzed by flow cytometry on live $CD3^+$ cells after doublet exclusion.

Results

The choice of the stimulus is critical. We noticed that anti-CD3-anti-CD28 beads can be phagocytosed by myeloid cells (e.g. monocytes), which could then reduce the number of beads available to the T-cells, decreasing the proliferation rate and inducing bias into the experiment (Figure 11). We instead decided to use plate-bound anti-CD3 and soluble anti-CD28 antibodies to stimulate T-cells.

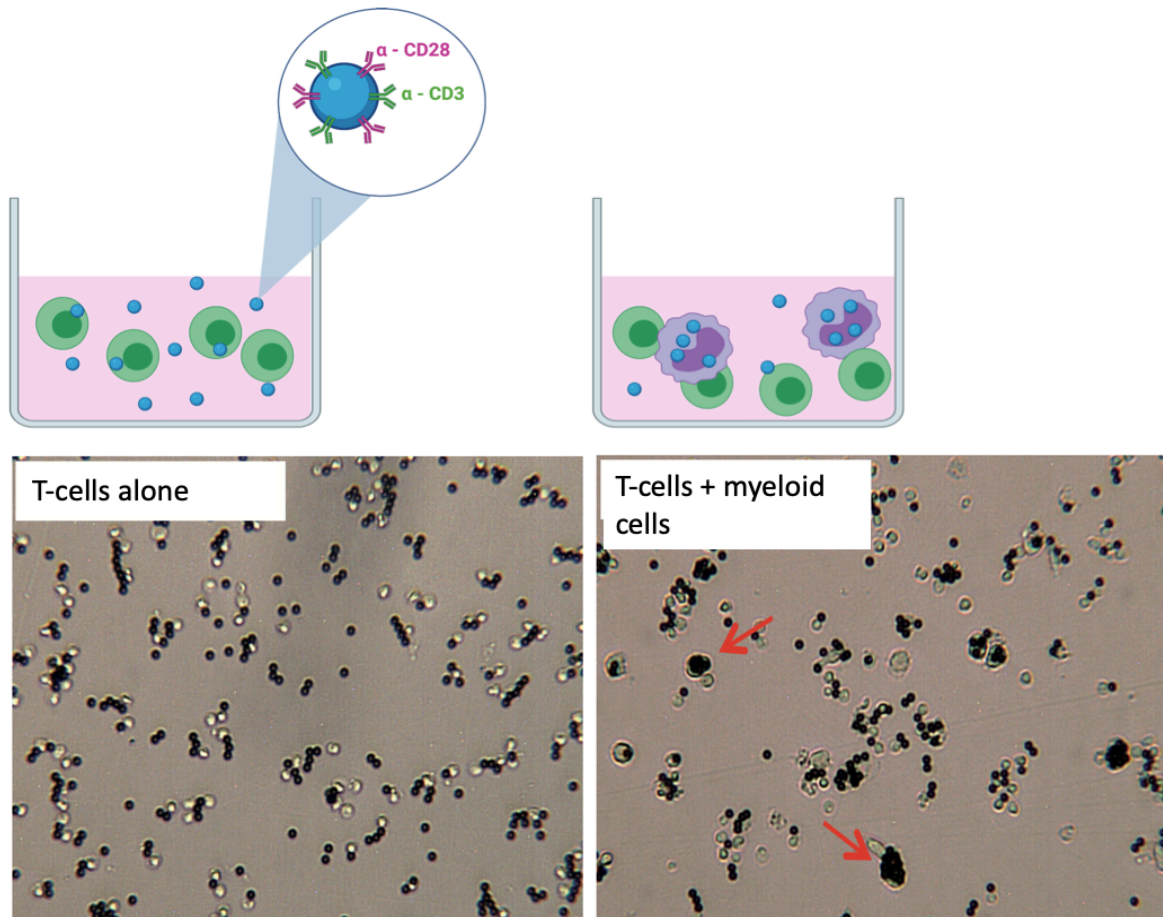


Figure 11. Myeloid-cells phagocytose anti-CD3-anti-CD28 microbeads during co-culture with T-cells. 50,000 T-cells per well were plated in a flat-bottom 96-well plate with or without 25,000 myeloid-cells (here: CD14⁺ HLA-DR^{low} monocytes from the ascites of cancer patients) and with 25,000 anti-CD3-anti-CD28 beads (Dynabeads®, Thermofisher). Cultures were checked after 4 days at 37°C, 5% CO₂ in RPMI with 10% HS. Red arrows highlight cells that phagocytosed Dynabeads® (40x magnification).

We adopted the parameters discussed in this chapter to assess the suppressive activity of LDN and HLA-DR^{low} monocytes sorted from ovarian and lung cancer patients.

Results

How to analyze T-cell proliferation?

During these set-ups, we noticed that technical variability, different batches of effector cells or changes of lot of the reagents cause differences in T-cell proliferation that are unrelated to any suppression by immune-regulatory myeloid cells (IMC) (personal observation). These test-intrinsic variations complicate comparisons of patients. We thus calculated the relative proliferation by normalizing the percentage of T-cells proliferating in presence of myeloid cells to the percentage of T-cells proliferating alone (control condition) (Figure 12). We also used different IMC-to-lymphocyte (mostly 1:3, 1:10 and 1:30) ratios in the co-culture to know whether the suppressive effect correlates with the presence of LDN in the well (Figure 12).

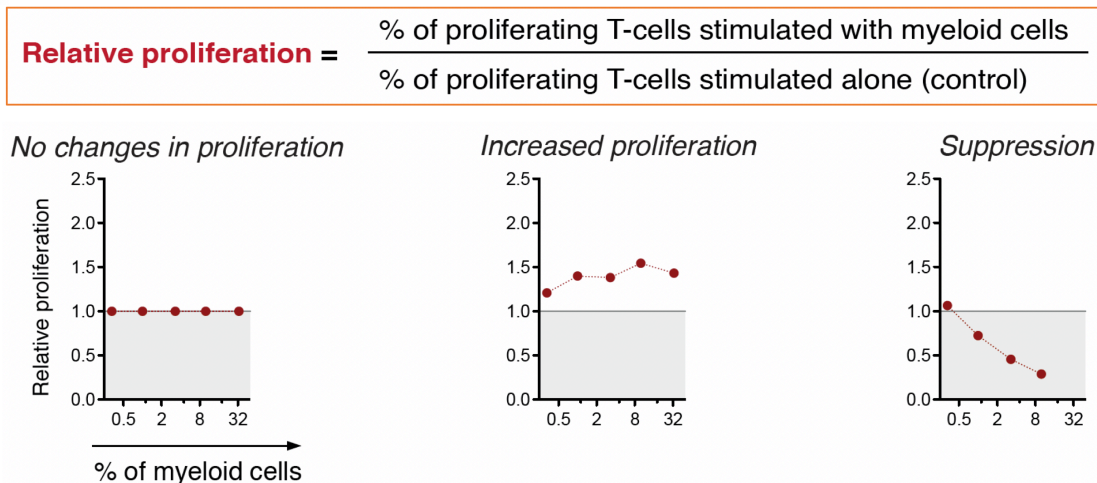


Figure 12. T-cell proliferation in the presence of myeloid cells is normalized to control conditions to allow comparison of different experiments. The percentage of T-cells that proliferate in the presence of myeloid cells (e.g., LDN or HLA-DR^{low} monocytes) is normalized to the number of T-cells that proliferate alone (in control condition) to calculate the relative proliferation value for the assay. Several ratios of IMC-to-lymphocytes are tested during the assay to see whether suppression of T-cell proliferation correlates with the presence of myeloid cells in the assay.

Proliferation assay with myeloid cells from different cancer types and tissues

Few assess the differences of MDSC in the blood against those in tumors. Thus, we analyzed the immunosuppressive functions of LDN and HLA-DR^{low} monocytes isolated from blood and tumors of lung and ovarian cancer patients in T-cell suppression assays.

Proliferation of T-cells co-cultured with LDN from cancer patients

We monitored the proliferation of CMFDA-labelled allogeneic T-cells that were activated in the presence of LDN isolated from the blood and the tumor of lung and ovarian cancer patients as previously described. We noticed that circulating LDN were more suppressive compared to tumor LDN in LB9182 ovarian cancer patient (Figure 13). However, we observed the opposite for lung cancer patient A062, where LDN isolated from tumor had higher suppressive activity compared to LDN isolated from the blood (Figure 13). These results suggest that intratumoral LDN have distinct suppressive capacities from blood LDN, even when they are isolated from the same patient.

Results

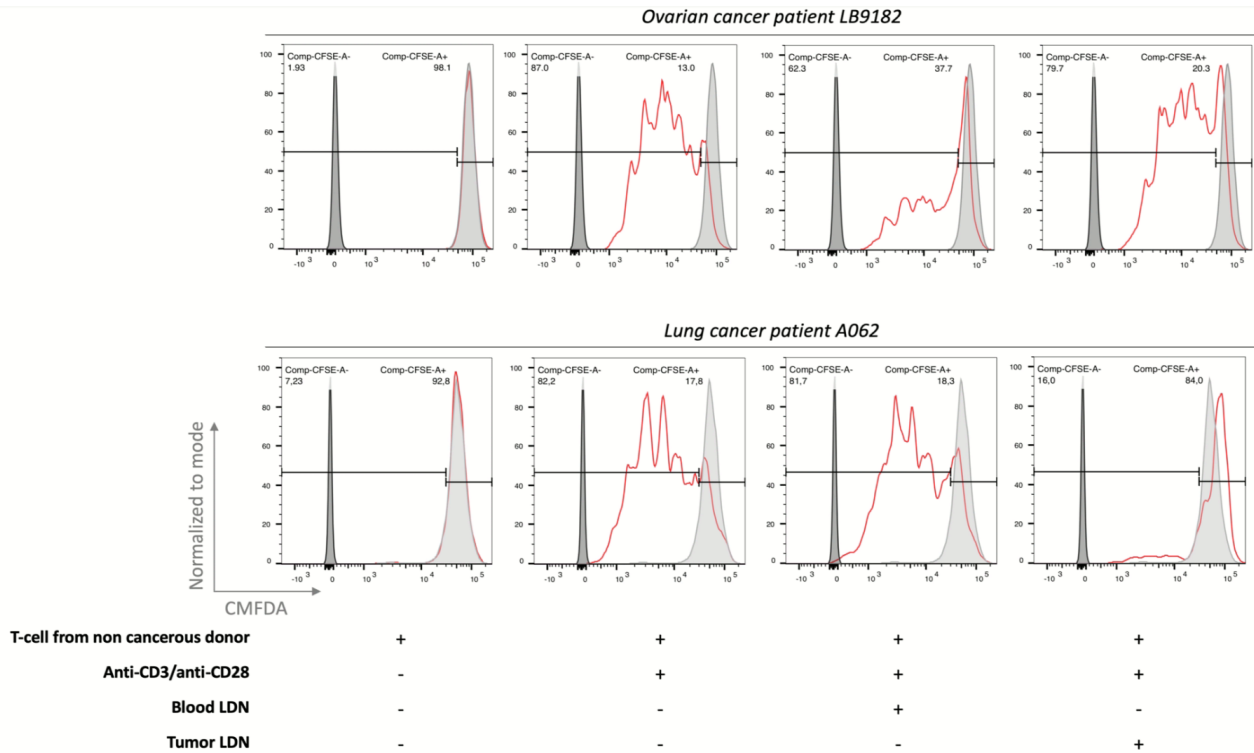


Figure 13. LDN isolated from ovarian and lung cancer patients show different suppressive capacities depending on the tissue from which they are isolated. 30,000 allogeneic CMFDA-labelled T-cells were stimulated with anti-CD3 and anti-CD28 antibodies alone or with 10,000 LDN isolated from blood and tumors of lung and ovarian cancer patients. To sustain proliferation, mature autologous DC were added to T-cells (1,000 cells/well) as accessory cells. After 4 days of co-culture at 37°C, 5% CO₂, the dilution of the CMFDA dye was analyzed by flow cytometry. The red curve represents the sample, the light grey peak the non-stimulated control and the dark grey peak the unstained control. Blood and tumor LDN were isolated and functionally tested on the same day.

Results

Impact of tumor digestion protocol on LDN suppressive activity

We hypothesized that the protocol to process tumor modulates the suppressive activity of LDN, leading to the differences we observed between circulating and tumor infiltrating LDN. To address this question, we isolated LDN from the blood of lung cancer patient A085 using either the usual protocol or the tumor processing protocol with mechanical and enzymatic digestion. We then co-cultivated the LDN isolated in the two different manners with allogeneic T-cells and assessed the T-cell proliferation (Figure 14). LDN and HDN that were treated following the tumor processing protocol reduced T-cell proliferation by 4.2% and 11.4% respectively, compared to control conditions. However, this change in proliferation was close to the variability we observed in control condition replicates, where the percentage of T-cells that proliferate at the onset of the assay ranged from 45.9 to 57.6% ($n = 6$ and $sd = 4\%$).

Based on this preliminary experiment, the tumor processing protocol is unlikely to induce strong immunosuppressive capacities, e.g. by activating LDN. Obviously, no statistical significance can be assessed without replicates and this control requires repetition.

Results

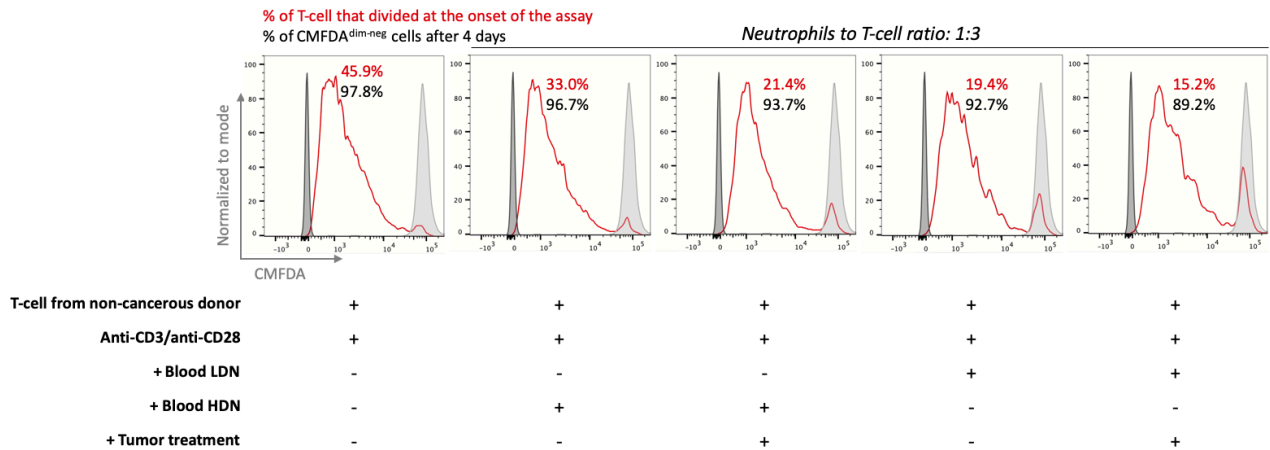


Figure 14. Mechanical and enzymatic digestion does not affect the suppression by LDN isolated from the blood of a lung cancer patient. The blood sample of lung cancer patient A085 was split into two. The PBMC of one sample were isolated directly by density gradient separation, as is standard procedure. The second sample was first treated with the tumor digestion protocol (mechanical separation of cells with a MACS[®] dissociator and enzymatic digestion with liberase) before density gradient separation. Both the light and heavy density fractions were retrieved from each sample. Both LDN and HDN were isolated by flow cytometry. Neutrophils were co-cultured with 30,000 CMFDA-labelled T-cells at a ratio of 1:3. T-cells were activated with anti-CD3 and anti-CD28 for 4 days at 37C and 5% CO₂. T-cell proliferation was assessed by the dilution of the CMFDA dye by flow cytometry (gated on single lived CD3⁺ cells). The red curve represents the sample, the light grey peak the non-stimulated control and the dark grey peak the unstained control. The red number represents the percentage of initial 30,000 T-cell that proliferated at onset of the assay and the black number represents the percentage of CMFDA-diluted (proliferating) cells after 4 days.

Results

Overall suppressive activity of LDN from lung and ovarian cancer patients

We examined the proliferation of T-cells co-cultured with LDN from multiple patients to compare the overall suppressive capacities of LDN from lung cancer and ovarian cancer patients. We also examined the suppressive activity of high-density neutrophils from the blood of cancer patients for comparison. We then calculated the mean of the relative proliferation with LDN and HDN from all patients to assess the cells' general suppressive activity (Figure 14).

Results

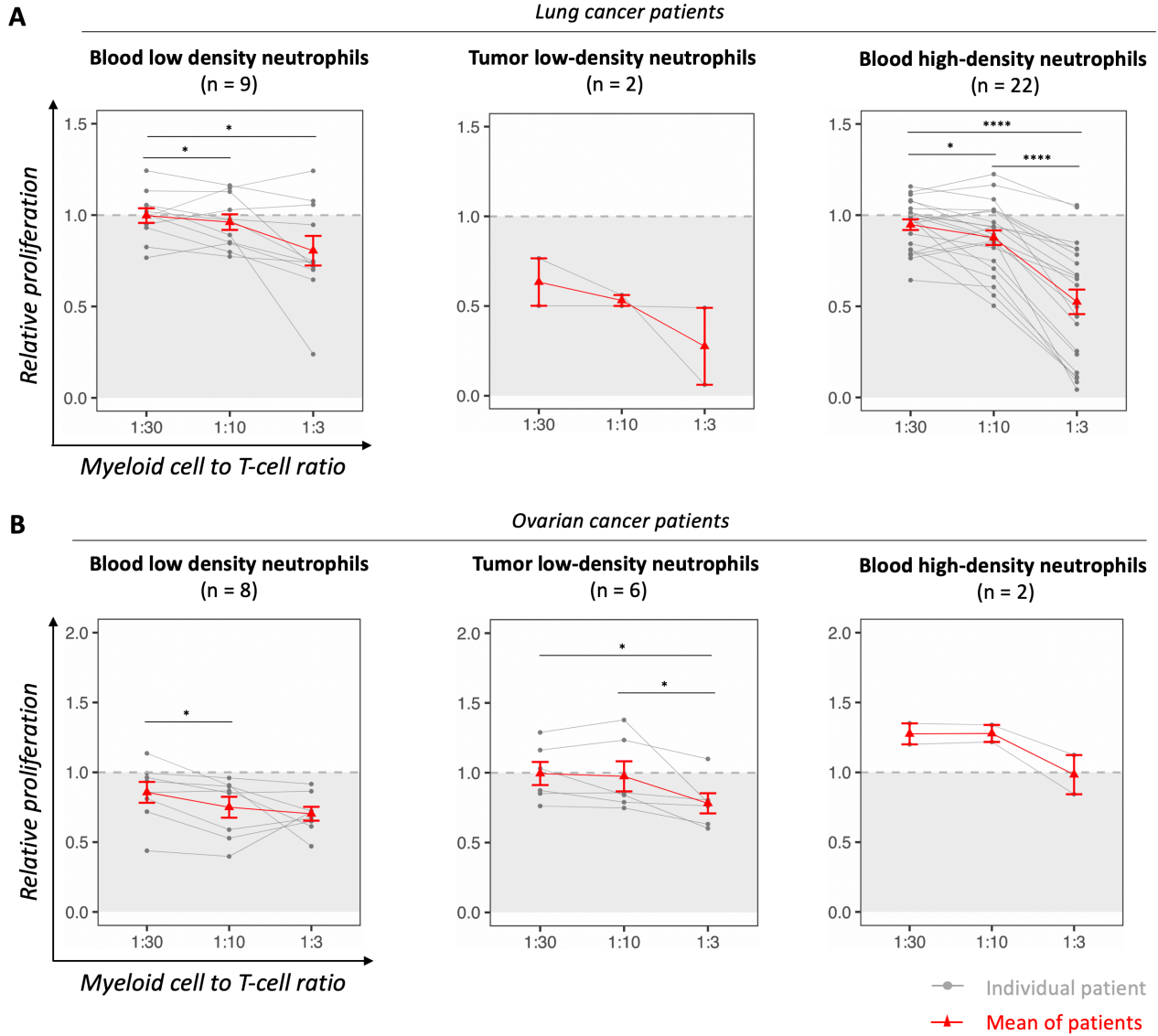


Figure 15. Overall suppressive capacities of LDN and HDN isolated from the blood and tumors of lung and ovarian cancer patients. Each value represents the proliferation data of 30,000 T-cells activated in the presence of 10,000 (1:3); 3,000 (1:10) or 1,000 (1:30) LDN or HDN isolated from one lung (A) or ovarian (B) cancer patients. Relative proliferation represents the percentage of T-cells that proliferated at the onset of the assay in the presence of myeloid cells normalized to the percentage of T-cells that proliferated alone (control condition). The grey curves represent individual patient, and the red curve represents the mean and SEM of all patients. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (Signed rank Wilcoxon test).

Results

Overall, LDN isolated from the blood of lung cancer patients were slightly suppressive (Figure 15A). However, the suppressive capacities of LDN were patient-dependent. In one third of the patients, circulating LDN suppressed T-cell proliferation at 1:10 and 1:30 LDN-to-T-cell ratios, while in another third, no suppression was observed, even at a 1:3 ratio (Figure 15A). Due to low yields, we only isolated and functionally tested LDN from two lung cancer tumors, which both show potent suppressive activity (Figure 15A). More surprisingly, HDN isolated from the blood of lung cancer patients show stronger suppression than blood LDN. Most of the HDN of the 22 patients suppressed T-cell proliferation even at a ratio of 1:10.

In ovarian cancer samples, LDN isolated from the blood showed stronger suppressive capacities compared to their siblings from lung cancer patients (Figure 15B). The overall inhibition of proliferation was also higher compared to the one of LDN isolated from ovarian cancer tumors, unlike what we observed in lung cancer tumors (Figure 15A). For practical reasons, the HDN from the blood of only two ovarian cancer patients were tested in suppression assays. They showed no clear inhibition of proliferation, unlike the HDN isolated from lung cancer patients (Figure 15B).

Together, these suppression assays highlight the potent inter-patient variability and the importance of a systematic functional validation of the isolated LDN to fully define them as PMN-MDSC. Also, these results suggest that both biological environment (blood or tumor) and cancer type shapes the suppressive activity of myeloid cells.

Results

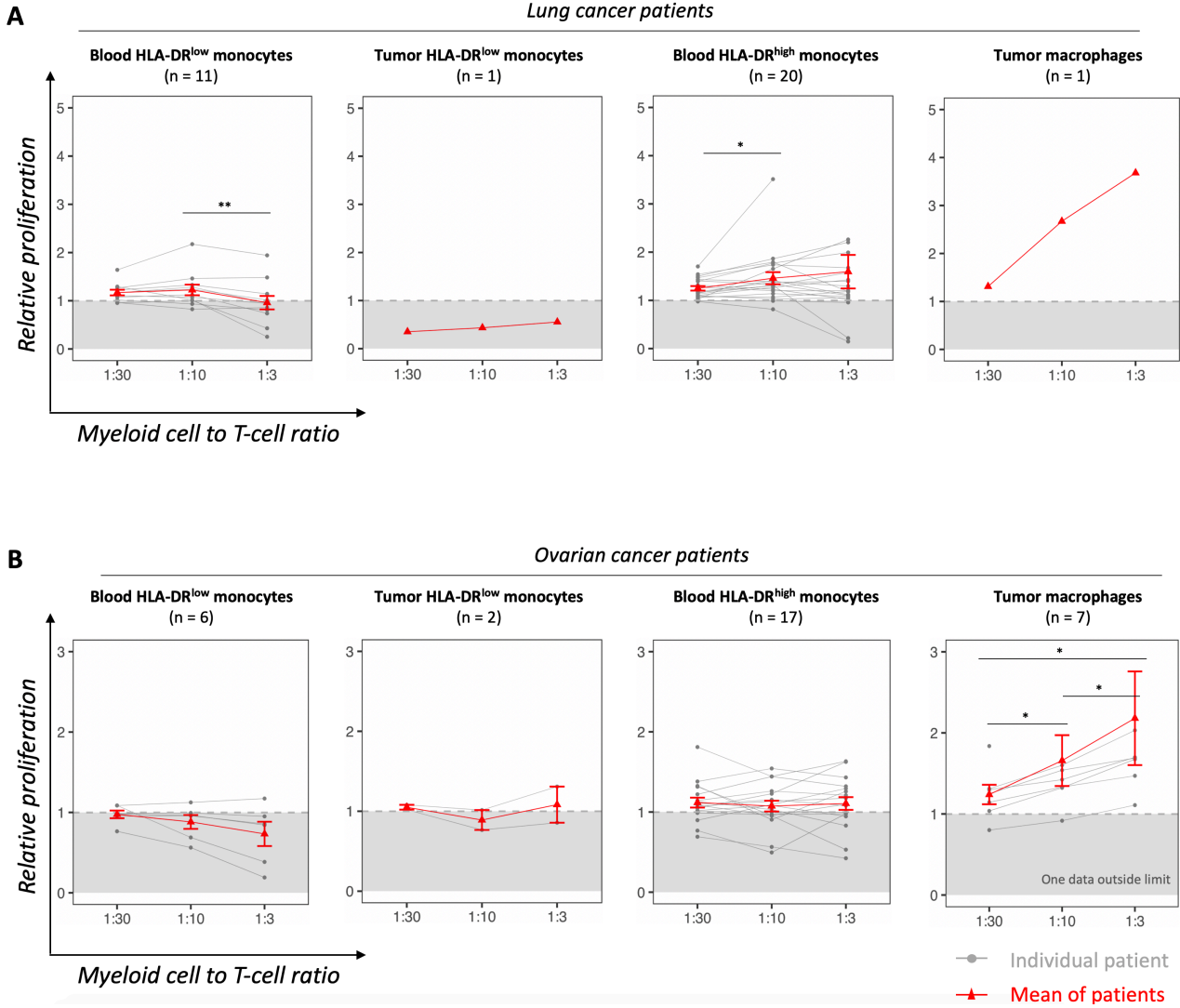


Figure 16. Overall suppressive capacities of HLA-DR^{low} monocytes isolated from blood and tumors of lung and ovarian cancer patients. Each value represents the proliferation data of 30,000 T-cells activated in the presence of 10,000 (1:3); 3,000 (1:10) or 1,000 (1:30) tumor-associated macrophages, HLA-DR^{high} or HLA-DR^{low} monocytes isolated from one lung (A) or ovarian (B) cancer patient. Relative proliferation represents the percentage of T-cells that proliferated in the presence of myeloid cells normalized to the percentage of T-cells that proliferated alone (control condition) at the onset of the assay. The grey curves represent individual patients and the red curve the mean and SEM of all patients. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (Signed rank Wilcoxon test).

Results

Overall suppressive activity of HLA-DR^{low} monocytes from lung and ovarian cancer patients

We isolated HLA-DR^{low} monocytes from the blood and tumors of patients with lung and ovarian cancer and assessed their suppressive activity as described before. The results were compared to the suppressive capacities of monocytes (isolated from blood) and macrophages (isolated from tumors) of the same lung and ovarian cancer patients (Figure 16).

HLA-DR^{low} monocytes isolated from the blood of lung cancer patients favored T-cell proliferation in some patients and inhibited it in others (Figure 16A). Overall, they were poorly suppressive. Once again, due to low yields, we isolated and tested HLA-DR^{low} monocytes from only one tumor sample, which was mildly suppressive (Figure 16A).

In ovarian cancer samples, blood HLA-DR^{low} monocytes showed a slight suppressive activity, while tumor HLA-DR^{low} monocytes of two patients did not inhibit T-cell proliferation (Figure 16B). Surprisingly, tumor-associated macrophages of both cancer types improved T-cell proliferation, which was unexpected (Figure 16).

As for LDN, these suppression assays highlight the inter-patient variability and remind us why it is important to functionally validate HLA-DR^{low} monocytes as M-MDSC. Moreover, the overall suppressive capacities of HLA-DR^{low} monocytes were lower compared to that observed in LDN from lung and ovarian cancers.

Part 2: Transcriptomes of LDN isolated from blood and tumors of lung and ovarian cancer patients

To better characterize the differences, we observed in functional assays, we compared the transcriptomes of myeloid cells isolated from lung and ovarian cancer patients. We decided to focus our analysis on LDN and HDN as (i) they represent a more distinct population without much phenotypic overlap (HLA-DR^{high} vs HLA-DR^{low}) and are therefore easier to sort, and (ii) show a stronger overall suppressive potential. We sorted LDN and HDN from the blood of 13 cancer patients (5 ovarian and 8 lung) and LDN from 6 tumors (4 ovarian and 2 lung).

Transcriptomic profiles of LDN and HDN from lung and ovarian cancer patients

Principal component analysis (PCA) showed distinct transcriptional programs between the LDN isolated from blood and those isolated from tumors (Figure 17). The biggest variance was found between HDN and most LDN isolated from blood. However, some blood LDN clustered with HDN, which indicate once again that LDN are a heterogenous population of cells. Surprisingly, the cancer type (lung or ovarian) did not seem to influence the transcriptomic profile of LDN or HDN.

Results

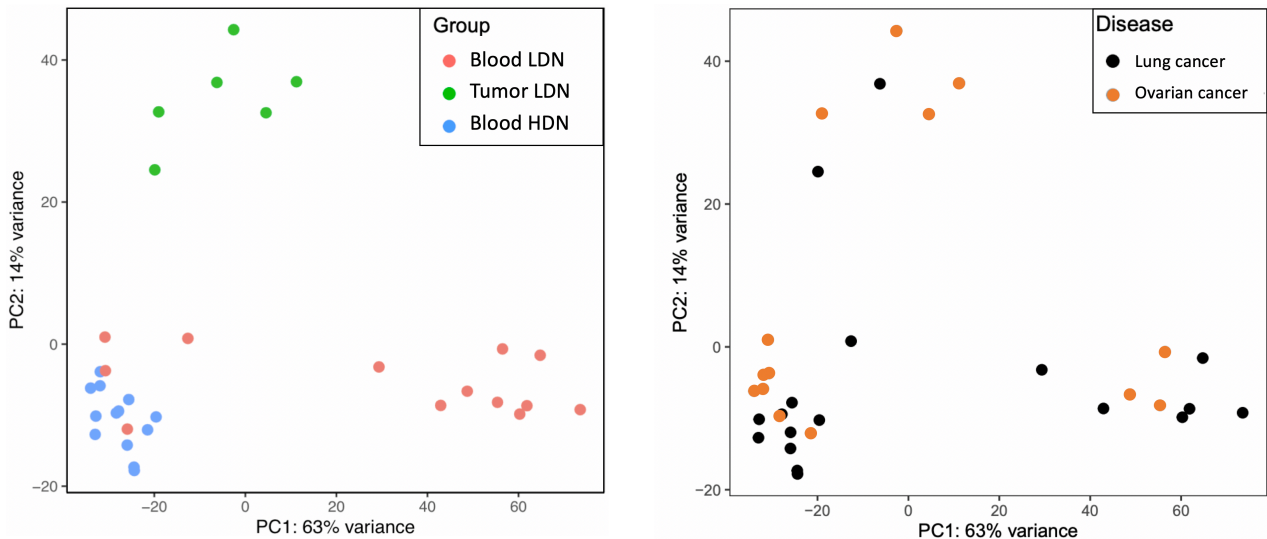


Figure 17. RNA-seq analysis showed distinct profiles for LDN and HDN according to the tissue from which they are isolated, but not according to cancer type. LDN and HDN were freshly isolated from the blood or tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly lysed in QIAzol Lysis Reagent and frozen at -80°C . RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data and principal component analysis were performed with DESeq2 (R). Each point represents one sample (bulk population). The two graphs illustrate the same data with different labelling (left: type of cell and tissue of origin, right: cancer type).

We first analyzed the differentially expressed genes (DEGs) between blood LDN and HDN using DESeq2 packages (Figure 18A). We found 7256 differentially expressed genes in LDN compared to HDN. We also compared the transcriptional profile of LDN isolated from blood and tumor (Figure 18B). We identified 1295 DEGs in LDN isolated from blood compared to tumors. Of these, 389 were differentially expressed with a minimum fold change of 2.

Results

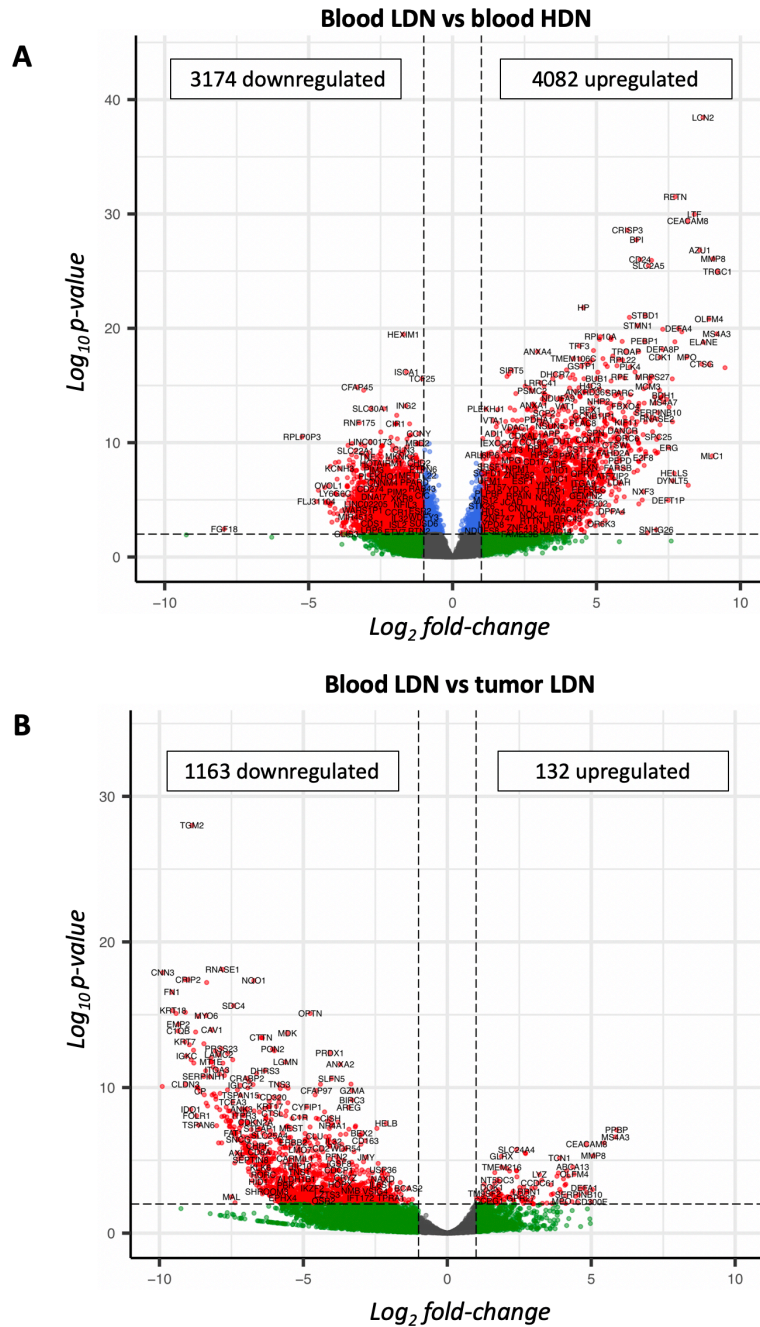


Figure 18. 7256 and 1295 genes are differentially expressed in blood LDN compared to blood HDN and tumor LDN respectively. LDN and HDN were freshly isolated from the blood or tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, the cells were directly lysed in QIAzol Lysis Reagent and frozen at -80°C . RNA was later extracted and sent for paired-end RNA-sequencing. Analysis of differentially expressed genes was performed using DESeq2 package (R). Adjusted p -value threshold was set to 0.01 with a log_2 fold-change cutoff. Volcano-plot and numbers represent genes that are down- or up-regulated in blood LDN samples compared to blood HDN (up) or tumor LDN (down).

Analysis of specific genes of interest

Genes potentially involved in suppressive mechanisms

We analyzed the expression of genes that were reported to be involved in suppressive mechanisms mediated by MDSC, including amino acid depletion, generation of oxidative stress, immunosuppressive cytokines and contact-dependent T-cell inhibition (PD-L1 and Fas-ligand) (Figure 19). We observed that the levels of *ARG-1* and *CYBB* (NOX2) expression in blood LDN varied but were higher compared to tumor LDN or blood HDN. Conversely, LDN isolated from tumors showed higher expression levels for *CD274* (PD-L1) and *IDO1* (IDO-1), which were poorly expressed in LDN isolated from blood. Finally, the expression of *IL10* (IL-10) and *FASLG* (Fas-ligand) were low in all cell types. We also found that LDN did not express *NOS1* or *NOS2*, and we did not observe any difference in the expression of *TGFB1* (TGF- β) between cell types (data not shown).

Results

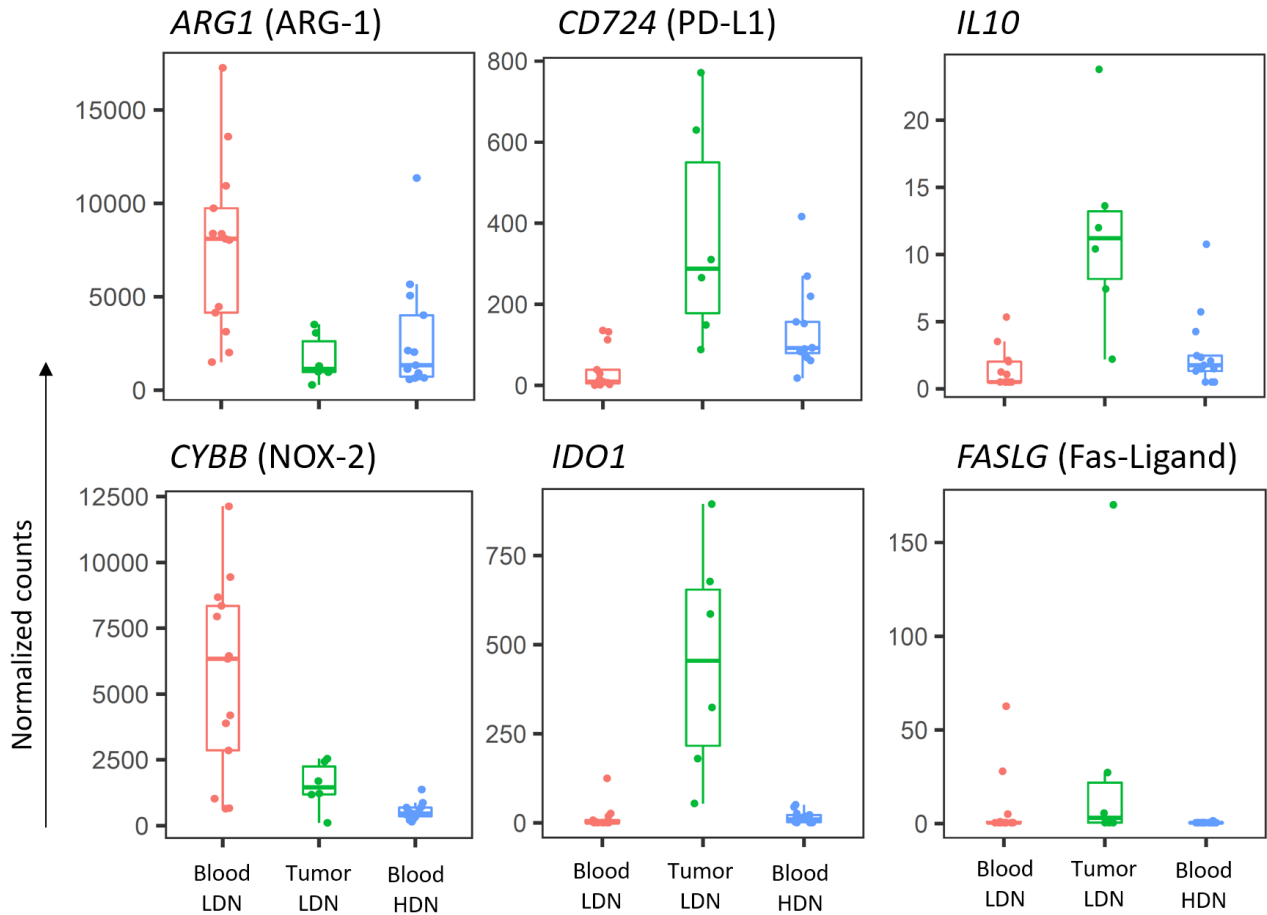


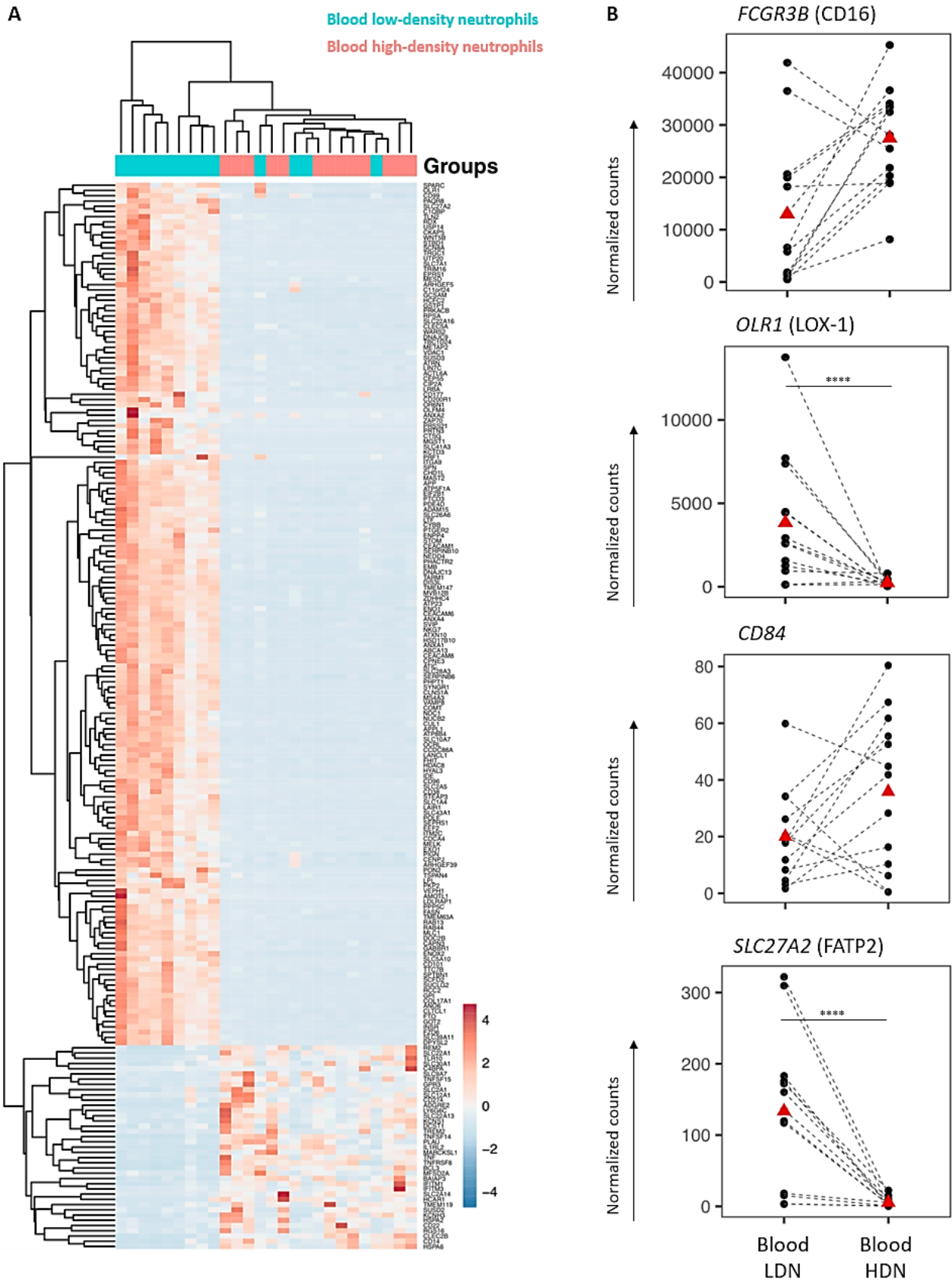
Figure 19. LDN and HDN from blood or tumors differentially express genes involved in MDSC-mediated immunosuppression. LDN and HDN were freshly isolated from the blood or the tumor of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly lysed in QIAzol Lysis Reagent and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data was performed with DESeq2 (R). Each point represents the gene expression (normalized counts) of one bulk population.

Results

Differentially expressed genes - potential surface markers

We examined potential surface markers that are differentially expressed in blood LDN compared to tumor LDN or blood HDN. We used gene ontology (GO) annotation to retrieve DEG that encode for surface protein (Figure 20A). We identified 204 potential surface markers that are differentially expressed with a minimum fold change of 2 between LDN and HDN isolated from the blood of ovarian and lung cancer patients (Figure 20A). We also analyzed the expression of surface markers that are reported as MDSC-specific in the literature (Figure 20B). We found *OLR1* (LOX-1) and *SCL27A2* (FATP2) were upregulated in LDN compared to HDN in blood (Figure 19B). In contrast, the neutrophil maturity marker CD16 (*FCGR3B* and *FCGR3A*) as well as *CD84* were not differentially expressed between the two cell types (Figure 20B and data not shown).

Results



Results

Figure 20. Several surface proteins are down- or upregulated in LDN compared to HDN in the blood of ovarian and lung cancer patients. LDN and HDN were freshly isolated from the blood or the tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly resuspended in QIAzol Lysis buffer and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data was performed with DESeq2 (R). **A.** Heatmap was generated only on surface markers between resting blood LDN and HDN using Pheatmap package (R). Potential surface markers were selected based on the following gene ontology annotations: GO:0005886, GO:0004872 and GO:0009897. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (DESeq2 paired sample analysis).

When we compared the potential surface markers differentially expressed between blood and tumor LDN, we found 190 genes that were differentially expressed with a minimum fold change of 2 (Suppl. Fig. 1). However, as between blood LDN and HDN blood, neither *FCGR3B* nor *OLR-1* nor *CD84* nor *SLC7A2* were significantly differentially expressed between tumor LDN and blood LDN.

The transcriptomic data revealed distinct profiles between LDN and HDN. Both populations were transcriptomically different from LDN isolated from tumors. Finally, LDN from the blood of cancer patients showed considerable variation in the expression of genes coding for protein involved in MDSC-suppressive mechanisms like *ARG-1* and *NOX2*. This indicates that LDN are a heterogeneous population and confirms high inter-patient variability.

Results

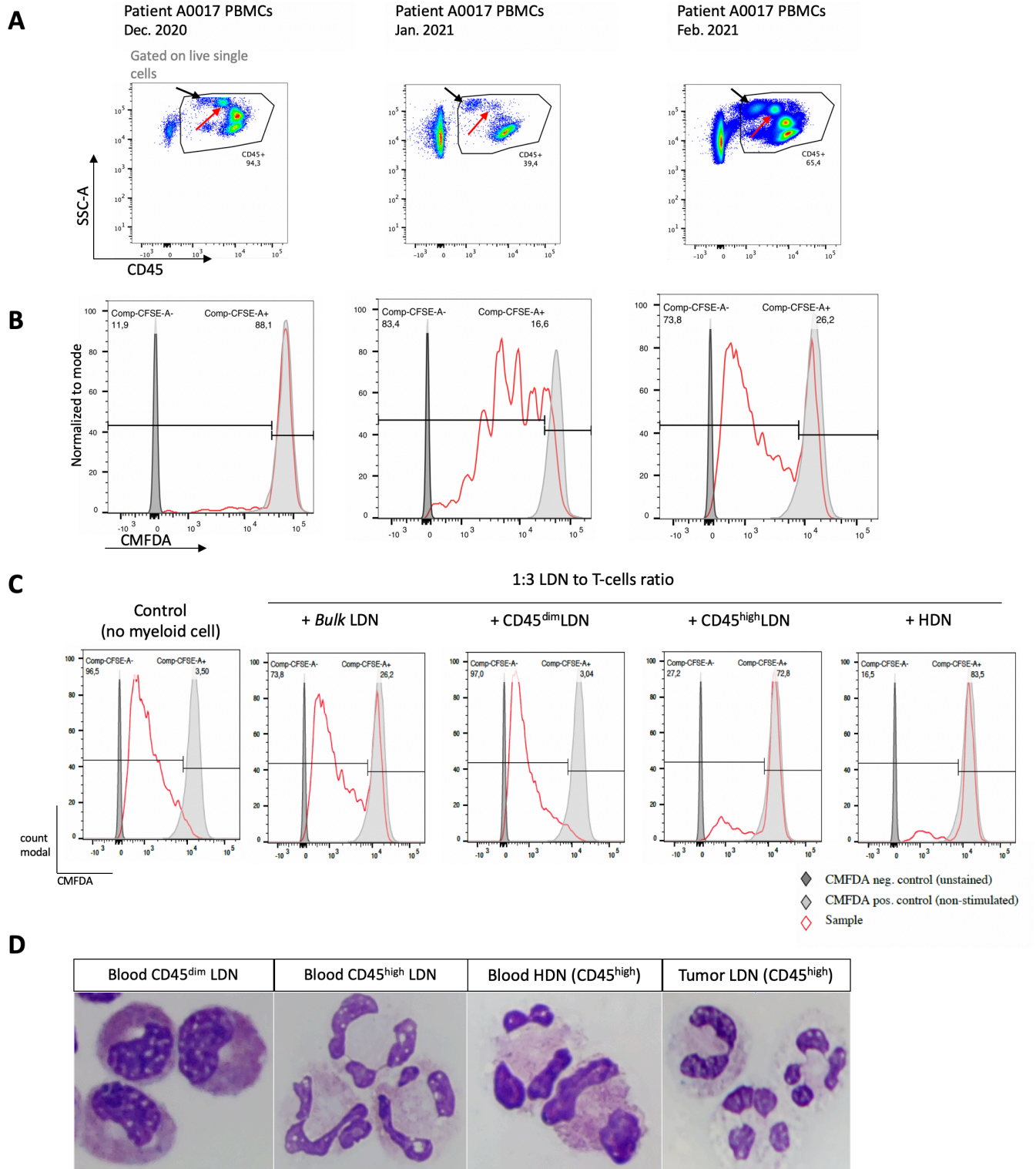


Figure 21. CD45 expression discriminates subsets of LDN in the blood of lung cancer patient A017 and the proportion of these subsets affects the suppressive capacity. PBMC of lung cancer patient A017 were harvested through density gradient separation at three time points (December 2020, January 2021 and February 2021). Cells were stained and isolated by flow cytometry as previously described. **A.** CD45 expression of PBMC after doublet and dead cell exclusion. Red arrows indicate

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CD45^{high} LDN and black arrows indicate the CD45^{dim} population. B. Proliferation assay of allogeneic T-cells activated in the presence of LDN isolated from the blood of lung cancer patient A017 at the three different time points. 30,000 CMFDA-labelled T-cells were activated with anti-CD3 and anti-CD28 for 4 days at 37C and 5% CO₂ with LDN at a ratio of 3:1 (T-cells:LDN). T cell proliferation was monitored by CMFDA dye dilution by flow cytometry (gated on live single CD3⁺ cells). The red curve represents the sample, the light grey peak the non-stimulated control and the dark grey peak the unstained control. C. Populations of CD33^{dim}CD15⁺CD45^{high} LDN and CD33^{dim}CD15⁺CD45^{dim} LDN were sorted from patient A017 in February 2021 by flow cytometry. CD45^{high} and CD45^{dim} LDN were co-cultured in the presence of 30,000 CMFDA-labelled T-cells that were activated with anti-CD3 and anti-CD28 antibodies for 4 days at 37C and 5% CO₂. T-cell proliferation was monitored by CMFDA dye dilution by flow cytometry. D. Neutrophil-like cells from lung cancer blood and tumors were resuspended in FBS and sent for cytospin analysis.

Part 3: Suppressive capacities of LDN subpopulations

Deciphering the heterogeneity of LDN in lung cancer patients

We observed that LDN isolated from the blood of ovarian and lung cancer patients varied in terms of frequency, suppressive potential, and even transcriptomic profile. To analyze the intra-patient heterogeneity, we compared the presence and the functional capacities of LDN from the blood of lung cancer patient A017 at three different time points (December 2020, January and February 2021) (Figure 21). Surprisingly, we noticed that the LDN contained two populations, with different levels of CD45 expression (Figure 21A). More interestingly, the ratio between CD45^{low} and CD45^{high} LDN in the blood was different at each time point and the frequency of CD45^{high} was correlated to the suppressive activity (Figure 21B).

Results

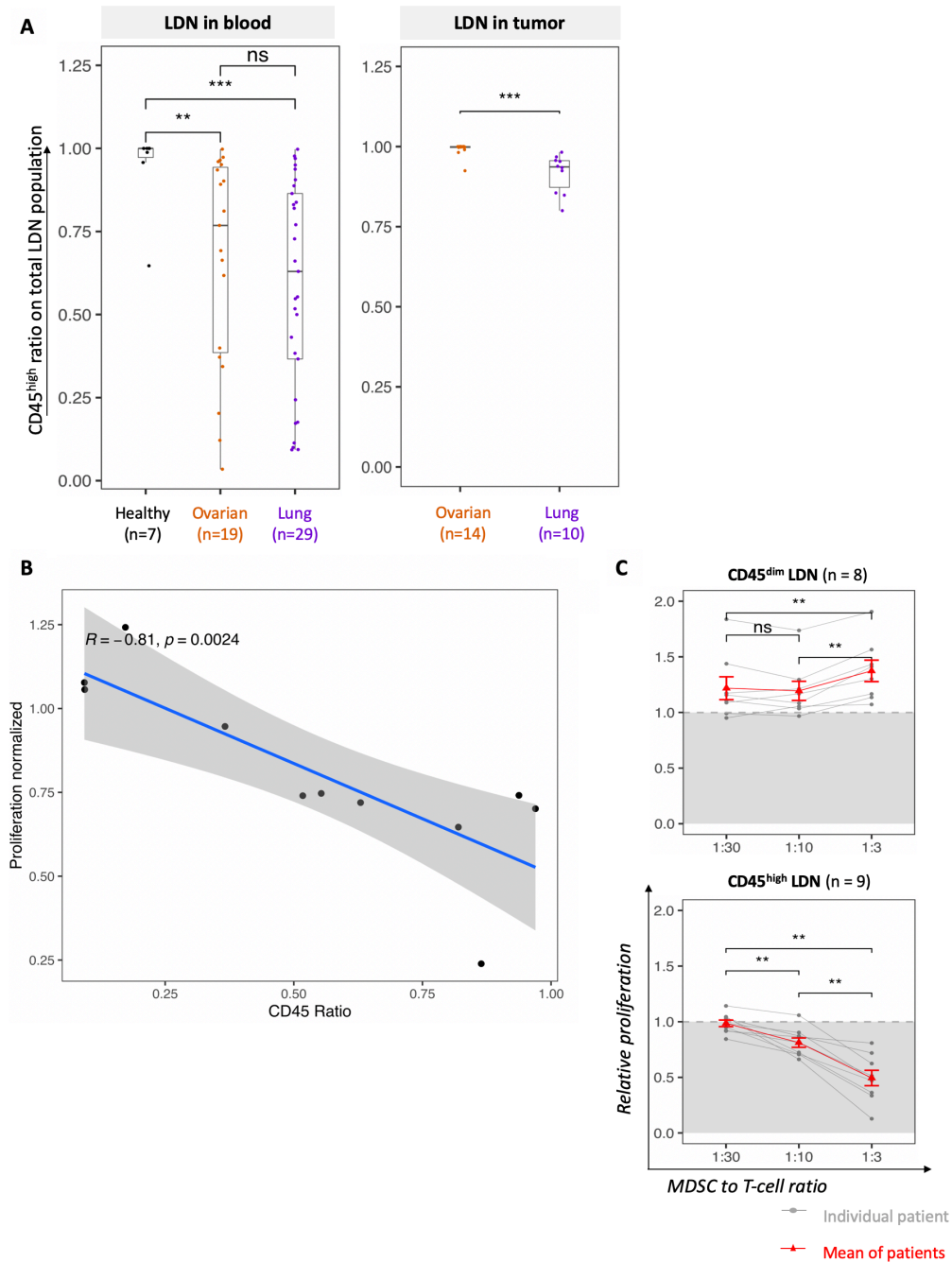


Figure 22. The frequency of the CD45^{high} LDN subset varies considerably in blood, but not in tumors, of ovarian and lung cancer patients and their presence correlates with suppressive activity. LDN were harvested from the blood and tumors of ovarian and lung cancer patients as previously described. **A.** The frequency of CD45^{high} LDN in blood (left) and tumor (right) was analyzed by flow cytometry on the live-cell population after doublet exclusion. The CD45^{high} ratio was calculated by dividing the percentage of CD45^{high} LDN by the whole LDN population. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (Wilcoxon-Mann-Whitney test). **B.** Correlation between the ratio of CD45^{high} LDN and relative T-cell proliferation during co-incubations of T-cells with blood LDN from lung cancer patients at a 3:1 ratio (see Fig. 13) (Pearson's product moment correlation coefficient). **C.** T-cell proliferation assay of allogeneic T-cells activated in the presence of 10,000

Results

(1:3); 3,000 (1:10) or 1,000 (1:30) CD45^{dim} or CD45^{high} LDN isolated from the blood of lung cancer patients. Relative proliferation represents the percentage of T-cells that proliferated at the onset of the assay in the presence of myeloid cells normalized to the percentage of T-cells that proliferated alone (control condition). The grey curves represent individual patients, and the red curve represents the mean and SEM of all patients. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (Signed rank Wilcoxon test).

Morphology and suppressive capacities of CD45^{high} and CD45^{dim} blood LDN

To confirm suppressive activity of CD45^{high} LDN in T-cell proliferation assays, we separately sorted CD45^{high} and CD45^{dim} LDN from the blood of lung cancer patient A017. We observed that CD45^{high} LDN were more suppressive than the “all” LDN population and inhibited T-cell proliferation at a similar level to HDN (Figure 21C). In contrast, blood CD45^{dim} LDN failed to inhibit T-cell proliferation in co-cultures. We analyzed the morphology of LDN (CD45^{dim} and CD45^{high}) and HDN isolated from blood and tumors by cytospin (Figure 21D). CD45^{high} LDN, both from blood and tumors, have several nuclear lobes and are classified as mature neutrophils, comparable to HDN. In contrast, CD45^{dim} LDN from the blood of cancer patients are immature cells similar to band cells or metamyelocytes.

To know whether this observation was unique to patient A017, we extracted the percentage of CD45^{high} LDN (from blood and tumors) from our previous analyses for both lung and ovarian cancer patients and compared it to the frequency found in the blood of healthy donors (Figure 22A). The frequency of CD45^{high} LDN among all blood LDN varied between 10 to 100% in the samples of both ovarian and lung cancer patients. In contrast, LDN isolated from tumors, as well as HDN from blood of non-cancerous donors, were over 75% CD45^{high} (Figure 22A and personal observation). When plotting the frequency of CD45^{high} LDN in the blood of lung cancer patients to the extent of the relative proliferation of T-cells in co-cultures

Results

with blood LDN, we discovered a direct inverse correlation (R of -0.81, p-value of 0.0024, Figure 22B).

We also specifically isolated CD45^{dim} and CD45^{high} LDN from lung cancer patients and confirmed that CD45^{high} LDN inhibited T-cell proliferation at a higher level than CD45^{dim} LDN (relative proliferation of 0.49 versus 1.37 at a ratio LDN-to-T-cell of 1:3) (Figure 22C).

Together, these observations highlight the heterogeneity of LDN in lung and ovarian cancer patients. Suppression of T-cell proliferation is caused by a subset of mature LDN with higher CD45 expression. These cells correspond to the definition of PMN-MDSC. However, LDN in the blood also contain immature and non-suppressive subsets, which needs to be considered in the design of experiments. Characterization of these CD45^{high} and CD45^{low} LDN subsets by RNA-seq might further define which genes identify PMN-MDSC or drive their suppressive functions.

Results

Transcriptomic analysis of CD45^{high} and CD45^{dim} LDN populations

We analyzed the transcriptome of CD45^{dim} and CD45^{high} LDN isolated from the blood and tumors of ovarian and lung cancer patients. We sorted CD45^{dim} and CD45^{high} LDN from the blood of 4 patients (3 lung cancer and 1 ovarian cancer). We also sorted tumor LDN from 2 patients (1 lung cancer and 1 ovarian cancer).

Surprisingly, the PCA analysis showed that CD45^{high} LDN were transcriptomically distinct from tumor LDN, despite their similar morphology, suppressive activity and level of CD45 expression (Figure 23A). In contrast, CD45^{dim} LDN differ considerably from their CD45^{high} siblings, even though the cells were isolated from the same tissue (blood). Paired-analysis revealed that CD45^{dim} differentially expressed 5946 genes (Figure 23B).

Results

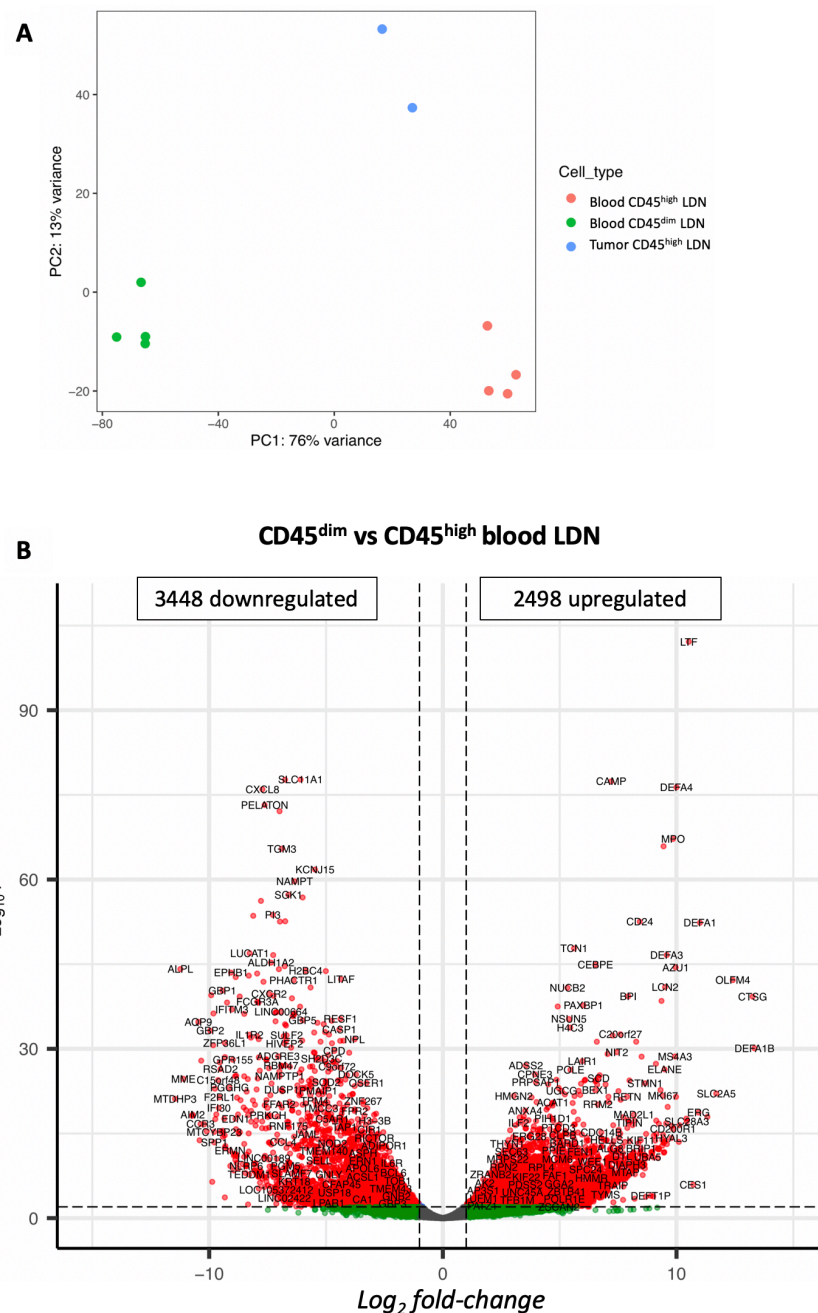


Figure 23. CD45^{high} and CD45^{dim} LDN from blood show different transcriptomic profiles, which are distinct from LDN isolated from tumors. CD45^{high} and CD45^{dim} LDN were freshly isolated from the blood or tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly lysed in QIAzol lysis reagent and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of reads was performed using DESeq2 package. **A.** Principal component analysis (PCA). Each point represents one sample (bulk population). **B.** Differential expression analysis between CD45^{high} and CD45^{dim} blood LDN was done with DESeq2 package (R). Adjusted p-value threshold was set to 0.01 with a log2 fold-change cutoff. Volcano-plot and numbers represent genes that are down- or up-regulated in CD45^{dim} LDN compared to CD45^{high} blood LDN.

Results

We compared the expression of genes reported to be involved in suppressive mechanisms by MDSC, such as *ARG1*, *IDO1*, *CYBB* (NOX2), or *CD274* (PD-L1) (Figure 24). Surprisingly, the expression of *ARG1* and *NOX2* were higher in CD45^{dim} LDN compared to CD45^{high} LDN, although the CD45^{high} LDN are the suppressive subset. However, CD45^{high} LDN expressed PD-L1 in contrast to non-suppressive CD45^{dim} LDN. The LDN isolated from the tumors of two cancer patients expressed PD-L1 comparably to the circulating CD45^{high} LDN and IDO at higher levels.

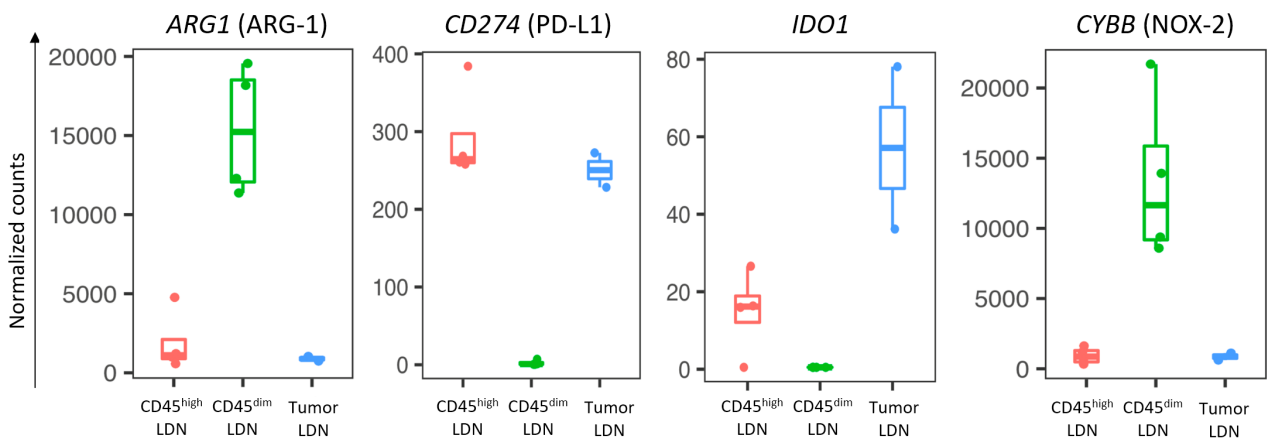
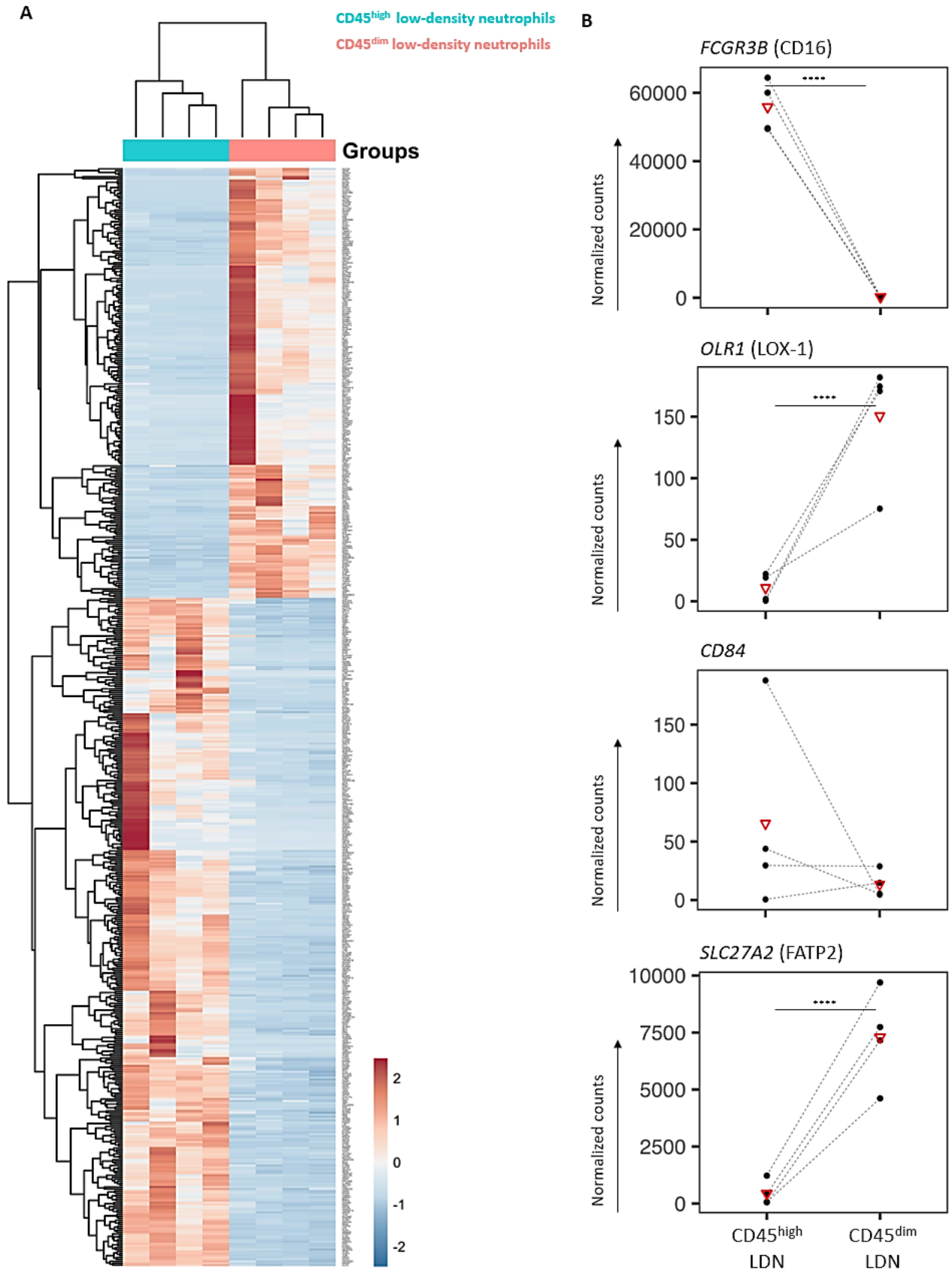


Figure 24. CD45^{dim} and CD45^{high} LDN from blood or tumors differentially express genes involved in MDSC-mediated immunosuppression. CD45^{high} and CD45^{dim} LDN were freshly isolated from the blood or tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly resuspended in QIAzol lysis reagent and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of reads was performed using DESeq2 package (R). Each point represents the gene expression (normalized counts) of one patient's LDN.

Results



Results

Figure 25. Several surface proteins are down- or up-regulated in CD45^{dim} LDN compared to CD45^{high} LDN. CD45^{high} and CD45^{dim} LDN were freshly isolated from the blood or tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly lysed in QIAzol lysis buffer and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data was performed with DESeq2 (R). **A.** The heatmap of surface markers between resting CD45^{high} and CD45^{dim} LDN was generated using Pheatmap package (R). Potential surface markers were selected based on the following gene ontology annotations: GO:0005886, GO:0004872 and GO:0009897. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (DESeq2 paired sample analysis).

We next examined which surface markers are differentially expressed in CD45^{high} compared to CD45^{dim} LDN isolated from blood (Figure 25). We found 990 differentially expressed genes corresponding to surface proteins and among them 562 were differentially expressed with a fold change of 2 or more (Figure 25A). We analyzed the expression of surface proteins that have been proposed as markers of PMN-MDSC (Figure 25B). For instance, LOX-1 is a low-density lipoprotein receptor that was identified as differentially expressed between PMN-MDSC and HDN isolated from head and neck and lung cancer patients [55]. We also analyzed FATP2 expression, which is involved in lipid metabolism and was described as unique to PMN-MDSC [51]. Surprisingly, these genes were expressed by the non-suppressive CD45^{dim} LDN but not (or at lower levels) by the CD45^{high} LDN. In contrast, CD45^{dim} LDN expressed lower levels of the CD16 compared to CD45^{high} LDN, both at transcriptomic and protein level (Figure 25B and Supp. Figure 4). CD16 is a surface marker expressed in the last stages of neutrophils differentiation/maturation and thus, these observations match the immature phenotype and morphology we observed in CD45^{dim} LDN. Finally, expression of CD84, a gene identified by single cell RNA-seq as a pan-MDSC marker, was low in most samples and not significantly different between CD45^{dim} and CD45^{high} LDN.

Results

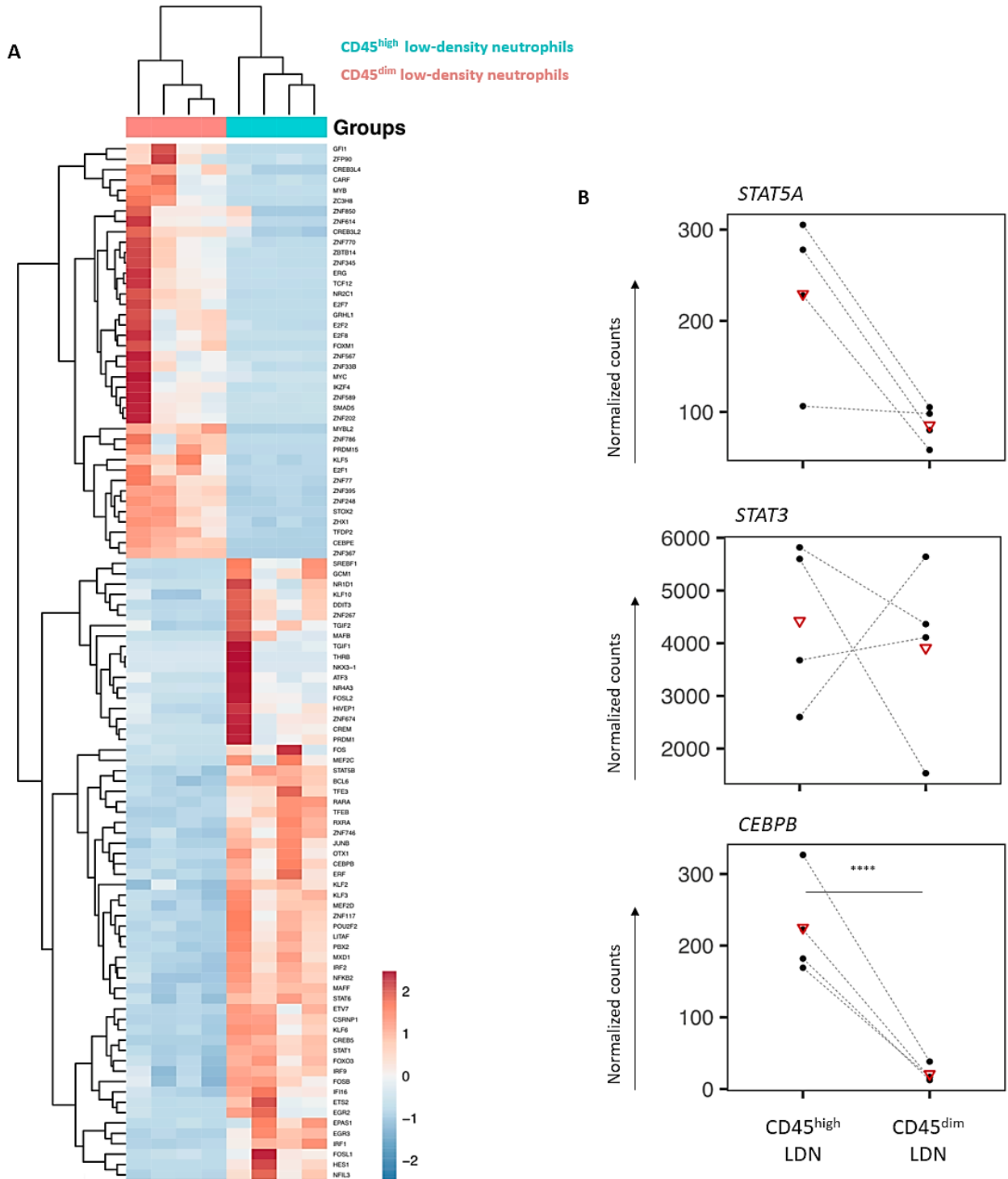


Figure 26. Transcription factors down- or up-regulated in CD45^{dim} LDN compared to CD45^{high} LDN. CD45^{high} and CD45^{dim} LDN were freshly isolated from the blood or tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly lysed in QIAzol lysis reagent and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data was performed with DESeq2 (R). **A.** A heatmap of transcription factors differentially expressed between resting CD45^{high} and CD45^{dim} LDN was generated using

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*Pheatmap package (R). Potential transcription factors were selected based on the following gene ontology annotations: GO:0003700, GO:0001228 and GO:0001227. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (DESeq2 paired sample analysis).*

We found 196 transcription factors that were differentially expressed in CD45^{high} compared to CD45^{dim} LDN (Figure 26A). We focused particularly on the expression of the transcription factors STAT3, STAT5 and C/EBP β because they are described as related to MDSC-functions [22], [25] (Figure 25B). STAT3 and STAT5 were not significantly differentially expressed between the two LDN groups, even though the expression of STAT5 was around 3-fold lower in CD45^{dim} compared to CD45^{high} LDN for 3 of 4 patients. C/EBP β is a positive regulator of MDSC immunosuppressive functions [22], [23], [257]. We observed that CD45^{high} LDN expressed C/EBP β unlike CD45^{dim} LDN, which might explain the difference in suppressive activities between the subsets.

Together, the transcriptomic data confirmed the distinct transcriptomic profiles of CD45^{dim} and CD45^{high} LDN. Furthermore, tumor LDN are transcriptomically distinct from either CD45^{high} or CD45^{dim} blood LDN. Functionally the CD45^{high} LDN correspond to the definition of PMN-MDSC. The expression of CD16 and PD-L1 is increased in CD45^{high} LDN, matching previous reports on PMN-MDSC [29], [112], [179]. CD45^{high} LDN also expressed higher levels of transcription factor C/EBP β , which was associated with MDSC-function and emergency granulopoiesis. However, CD45^{high} LDN express only low levels of LOX-1, CD84 and FATP2 which had been proposed as specific markers for PMN-MDSC [50], [51], [55]. Instead, these markers were upregulated in CD45^{dim} LDN compared to CD45^{high} LDN. Our observations and other reports differ possibly because other studies analyzed the transcriptomes of mixed LDN, and the data thus comprises of feature for both CD45^{high} and CD45^{dim} LDN.

Results

Discussion

Discussion

Discussion

Preamble

Neutrophils are plastic cells that have both pro- and anti-tumor functions [53], [166], [258], [259]. However, protocols and approaches used to study LDN in cancer are diverse and usually focused on the blood of one cancer type. Therefore, the field still lacks consistency. Our objective was to directly compare the suppressive functions of LDN isolated from both blood and tumors of the same patient. We also analyzed LDN from ovarian and lung cancer patients to know whether the type of cancer shapes the frequency, phenotype and suppressive activity of LDN.

We observed that the frequency of circulating LDN varies between ovarian and lung cancer, even though we applied the same procedures and gating strategies. This observation raises questions about how LDN expand, and more specifically, how these mechanisms differ between cancer patients and healthy donors.

We also showed that circulating LDN are composed of two subsets that differ both in maturity and suppressive activity. The relationship between these subsets and what drives suppression by mature LDN is currently unknown. We observed that circulating HDN in lung cancer patients suppressed T-cell proliferation *in vitro*, which was not observed in HDN from healthy donors.

Our transcriptomic analysis revealed that both subsets of circulating LDN were distinct from LDN from tumor. We believe that tumor-infiltrating LDN, and not just circulating LDN, have to be considered in studying immunosuppression within tumors.

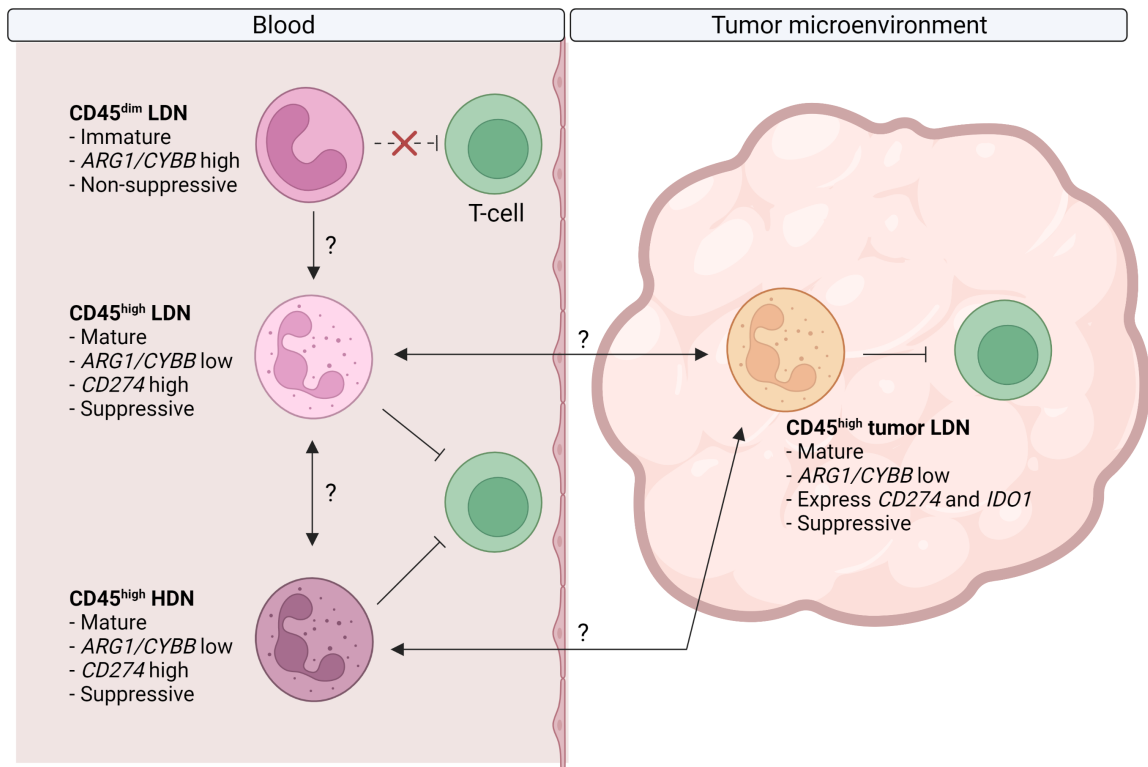


Figure 27. Graphical abstract of results. We identified two subsets of LDN in the blood of ovarian and lung cancer patients. Immature CD45^{dim} LDN express higher levels of ARG1 and CYBB but failed to suppress T-cell proliferation *in vitro*. In contrast, CD45^{high} LDN showed a mature morphology and were able to suppress T-cell proliferation despite expressing lower levels of ARG1 and CYBB. In lung cancer, CD45^{high} HDN were also potent suppressors of T-cell proliferation *in vitro*. Transcriptomically, all circulating LDN and HDN were distinct from tumor-infiltrating LDN, which expressed the highest levels of IDO1. CD274 (PD-L1) was upregulated in all CD45^{high} cells compared to CD45^{dim} LDN, making it as a potential suppressive mechanism.

On the frequency of LDN in the blood of cancer patients

What causes differences in the frequency of LDN in different cancers?

We wanted to know if the expansion of LDN in blood and tumors was universal to cancer pathologies or specific to types of cancers. Although there are comparisons between cancer types, these are only performed with blood samples and suffer from inter-lab variabilities [28]. Therefore, we analyzed the frequencies of LDN in blood and tumors of ovarian and lung cancer patients on the same day, using identical protocols. We found that the frequency of circulating LDN in ovarian cancer patients was statistically about 5-fold lower compared to that in lung cancer patients (24 vs 22 patients).

Increased inflammation, which drives the expansion of neutrophils, could explain these variations in LDN frequency. Highly immunogenic tumors with higher immune cell-infiltrations (often described as “hot” tumor) show increased signs of inflammation. Wang *et al.* assigned scores to the level of immune infiltration and the degree of expression of genes/proteins involved in the antigen processing and presentation machinery to classify immunogenicity of 32 cancer types [232]. They showed that lung squamous cell carcinoma and lung adenocarcinoma were more immunogenic than ovarian cancer. The hypothesis that highly immunogenic tumors are associated with an increase of circulating LDN should be confirmed by comparing samples from low immunogenicity cancers (testis cancer or pancreatic adenocarcinoma e.g.) and highly immunogenic cancers (melanoma or CRC for instance) [232], [233].

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When analyzing the frequencies of LDN in the blood, one needs to consider the treatment status of the cancer patient. Usually, ovarian cancer is treated with platinum-based (i.e. cis- or carboplatin) chemotherapy, which may cause neutropenia [260]. Indeed, we observed decreased LDN numbers in patients that have undergone three or six rounds of chemotherapy (data not shown).

Is there a link between the frequencies of LDN and the NLR?

We observed an increase in the frequency of LDN in the blood of lung cancer patients in comparison to healthy donors. The mean of percentage of LDN among CD33⁺ cells was also increased in the blood of ovarian cancer patients, but the change was not statistically significant.

Nevertheless, it is tempting to imagine that the expansion of circulating LDN correlates with an increased neutrophils-to-lymphocytes ratio (NLR) that is often observed in cancer patients [175]. However, we observe no correlation between the percentage of circulating LDN and the NLR or total circulating neutrophils numbers in lung cancer patients (Supp. Figure 3). Furthermore, of the 27 lung cancer patients tested for which neutrophil counts were provided, 21 of them showed neutrophil counts within the normal range (data not shown). This suggests that increases of circulating HDN do not systematically lead to an increase of circulating of LDN in lung cancer patients.

A trend, but no significant correlation, was previously found between NLR and the presence of LDN in lung cancer patients [261]. However, these patients had grade IIIb or IV NSCLC and some of them received treatment. Our samples derive from non-treated patients with stage II-IV lung cancer, which could explain the differences observed.

On LDN subsets in lung and ovarian cancer patients

Subsets of LDN and HDN in blood of lung and ovarian cancer patients

The suppressive activity of LDN isolated from blood of ovarian and lung cancer patient varied and was impossible to predict. We identified two circulating LDN subsets based on the level of CD45 expression: one was composed of immature granulocytes without suppressive activity (CD45^{dim}) and the other of mature granulocytes with suppressive activity (CD45^{high}). We observed that HDN also expressed high levels of CD45 and showed suppressive activity similar to CD45^{high} LDN. This heterogeneity in blood LDN is intriguing and raises questions about their origins. For instance, are CD45^{dim} LDN a transitional precursor of mature CD45^{high} LDN and/or HDN or do these subsets expand independently?

Box 3. CD45 expression in neutrophils

CD45, also known as Protein Tyrosine Phosphatase Receptor Type C (PTPRC), is a highly conserved receptor of 180-220 kDa that is expressed on all nucleated hematopoietic cells [289]. The cytoplasmic region of CD45 contains two phosphatase domains that are followed by a short transmembrane region. The extracellular domain of CD45 includes fibronectin repeats and cysteine-rich regions. The C-terminus of CD45 is composed of ectodomains that contain multiple sites for glycosylation [289], [290]. The three different ectodomains of CD45 (A, B and C) are encoded by the exons 4, 5 and 6, respectively. Differential splicing generates several CD45 isoforms, which can contain none (CD45RO), only one (CD45RA, RB or RC) or a combination (e.g. CD45 RABC) of the ectodomains [289].

Natural ligands of CD45 are either linked to specific conditions (infections or pregnancy) or are ubiquitous and known to interact with a large variety of molecules (including lectins) [289]. In T-cells CD45 plays a crucial role in the initiation of TCR signaling. However, information regarding its role in other cell types, including neutrophils, are limited. For instance, CD45 is involved in neutrophil migration in response to *S. aureus* infection in mice [291]. Also, using anti-CD45 antibodies as surrogate ligands enhanced phagocytosis and the secretion of IL-8 and TNF- α by human neutrophils [291]. Finally, mass spectrometry showed a correlation between higher levels of CD45 expression and neutrophil maturity in a non-human primate model of simian immunodeficiency virus [292].

Unfortunately, our data only represent a “snap-shot view” of LDN in cancer patients and cannot measure LDN dynamics overtime. Instead, LDN subsets in cancer should be further assessed by approaches such as single-cell genomics, which provides information on cell origin, development and response to stimuli [262]. Nevertheless, our transcriptomic data provides some clues.

First, CD45^{dim} LDN show an immature morphology, which indicates that they represent a population of progenitors. Alterations in granulopoiesis and the presence of early granulocytic progenitors (eNep) in the blood of cancer patients

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were already reported [263]–[266]. During normal granulopoiesis, progenitors express CXCR4, and thus they are retained in the bone marrow by stromal cells that express CXCL12. Mature granulocytes downregulate CXCR4 and join the circulation [166]. In our transcriptomic data, CD45^{dim} express low levels of CXCR4 (about 25-fold decrease compared to CD45^{high}), which could explain why they are not retained by stromal cells and leave the bone marrow early (Supplementary table 1). However, the fate of CD45^{dim} LDN once in circulation is unknown. Culturing CD45^{dim} LDN with inflammatory cytokines might provide information on their fate. However, culturing LDN *ex vivo* is challenging due to their short lifespan. Using humanized mice, like MITRG or MISTRG strains, represents an alternative for monitoring the fate of CD45^{dim} LDN in cancers.

Despite their maturity, CD45^{high} LDN highly express CXCR4 compared to CD45^{dim} LDN, and thus it is very unlikely that they just left the bone marrow after their maturation. The expression of CXCR4 is upregulated in aged neutrophils that are homing back to bone marrow to be phagocytosed by macrophages [166]. Therefore, it is likely that CD45^{high} LDN are cells that have been circulating for several hours.

We next hypothesized that CD45^{high} LDN derive from circulating mature neutrophils. Upon activation, HDN shift from the high to the light density fraction [43], [178], [267]. For instance, N-formyl-methionyl-leucyl-phenylalanine (fMLF) causes neutrophils to degranulate, and thus change their buoyancy, which increases the proportion of LDN [178]. Unfortunately, we did not analyze the transcriptome of HDN during the RNA-seq experiment we performed on CD45^{dim} and CD45^{high} LDN (Figure 22). However, we combined this data set with the transcriptome data of HDN and tumor LDN obtained during the first RNA-seq analysis (Figure 15). In doing so,

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we observed that blood CD45^{high} LDN and HDN cluster together, indicating that they are transcriptomically close (Supp. Figure 4). We also observed a slight increase of CD16 expression in CD45^{high} LDN compared to HDN, both at the transcriptomic and protein levels, which indicates that they are neutrophils that were recently activated and degranulated [267], [268] (Supp. Figure 5). Therefore, it is likely that ovarian and lung cancer patients have more activated neutrophils in the peripheral blood than healthy donors. However, how neutrophils in peripheral blood of cancer patients are activated and how this activation impact NLR value is still puzzling. Moreover, frequencies of CD45^{high} LDN in blood of ovarian and lung cancer patients vary considerably (15 to 95% of total LDN, Figure 22) meaning that activation of neutrophils in blood is highly patient-dependent. Paired analysis (FACS and RNA-seq) of CD45^{high} LDN and HDN isolated from the same cancer patients should be performed to fully decipher the expression patterns of activation markers with better statistical power.

In conclusion, we identified two different population of LDN with distinct maturation stages and with different levels of CD45 expression. These cells were not equally present in patients, and their origins and fate still require further investigation. In any case, if LDN are to become therapeutic markers, their heterogeneity must be considered. Currently, we are working with clinics to assess the value of CD45^{dim} and CD45^{high} LDN as markers that predict patient outcome or response to ICI.

Tumor LDN vs Blood LDN

Studies that directly compare circulating and tumor-infiltrating LDN in human cancer are rare. Thus, it is uncertain whether blood LDN are identical to tumor LDN. We

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observed that LDN from tumors have a mature morphology and express high levels of CD45 like CD45^{high} LDN in blood. But tumor LDN were transcriptomically distinct from blood LDN and HDN (Figure 15). Furthermore, the expression of IDO and PD-L1 was upregulated in LDN isolated from tumors of ovarian and lung cancer patients compared to total circulating LDN (Figure 17). These differences between circulating and tumor infiltrating LDN redefine how they should be studied in cancer.

Indeed, the vast majority of research groups focus entirely on circulating LDN - for practical reasons such as availability of samples - and assume that the suppressive mechanisms of circulating LDN *in vitro* assays are thus transferable to tumor LDN. Few analyze tumor LDN directly, especially in humans. For instance, Si *et al.* used immunohistochemistry and 3D light sheet microscopy to study CD66b⁺ TAN [58]. They found that TAN express ARG-1 and LOX-1 and interacted directly with intratumoral CD3⁺ T-cells to exert their suppressive functions. But the authors did not perform density gradient separation to specifically separate tumor LDN from HDN. Also, they did not compare TAN with blood LDN or HDN. When circulating and tumor LDN are directly compared, the studies are mostly conducted in mice and are limited to phenotypic and/or functional analysis [106], [112], [251]. Similarly to our transcriptomic data, Yamauchi *et al.* observed increased frequencies of PD-L1⁺ LDN in tumors compared to blood in lung cancer patients, (Figure 17) [112]. However, the authors studied only total circulating LDN without considering LDN subsets. To our knowledge, no group has yet compared the transcriptomes of blood LDN subpopulations with that of tumor-infiltrating LDN.

It is conceivable that these increases of PD-L1 and IDO expression are triggered by the release of IFN- γ and TNF- α by immune cells within TME [269]–[272]. However, PD-L1 expression was similar in circulating CD45^{high} LDN and tumor LDN,

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suggesting that the TME may not be the exclusive environment that drives PD-L1 expression in LDN. Additionally, tumor cells produce PGE-2, which leads to the acquisition of suppressive capacities in MDSC, and upregulate IDO expression in dendritic cells and fibroblasts [61], [63], [64], [273]–[276].

If both tumor and blood LDN expressed matrix metalloproteinase (MMP)9 at similar levels, MMP8 was expressed only in circulating CD45^{dim} LDN (data not shown). While matrix metalloproteinases like MMP9 facilitate the generation of pre-metastatic niches [277]–[279], the role of MMP8 in cancer remains controversial. In breast cancer and melanoma, MMP8 have anti-tumoral properties while higher levels of MMP8 correlated with poor prognosis in colorectal and liver cancers [280]–[282]. MMP8 also induces proliferation and migration of endothelial cells and thus favor angiogenesis in cancer [279]. The roles of LDN and MMP8 in angiogenesis or the establishment of pre-metastatic niches is currently unknown but could open the path to new investigations.

In conclusion, the differences in gene expression we saw in tumor LDN compared to circulating LDN are likely due to environmental factors (e.g. IFN- γ or PGE-2) that drive the expression of genes such as PD-L1 and IDO. Therefore, more attention should be placed on tumor LDN to decipher the suppressive mechanisms that occur specifically within the tumor microenvironment. However, whether circulating LDN are useful biomarkers, or they are involved in the generation of pre-metastatic niches should not be neglected [283], [284].

On suppressive aspects of neutrophils in lung and ovarian cancers

How do CD45^{high} LDN compare to MDSC described in the literature?

We identified mature CD45^{high} LDN, which suppressed T-cell proliferation *in vitro* and thus, match to the definition of PMN-MDSC. However, this subset expressed significantly lower levels of ARG-1 and NOX2 compared to non-suppressive CD45^{dim} LDN. Both genes are often described as key actors of MDSC-mediated suppressive mechanisms [285]. ARG-1 depletes L-Arginine while NOX-2 is involved in ROS generation. These results were unexpected and indicated that the sole expression of ARG-1 and/or NOX-2 is not sufficient to define PMN-MDSC.

We also analyzed the expression of surface markers usually associated with PMN-MDSC [285]. We observed that CD84 (a marker proposed as unique to pan-MDSC), was poorly expressed by all subsets, including circulating HDN [50]. We observed no difference in CD84 expression between CD45^{high} and CD45^{dim} subsets and thus, CD84 is a poor marker for identifying PMN-MDSC in lung and ovarian cancer patients (Figure 24).

We observed that bulk LDN isolated from the blood and tumors of ovarian and lung cancer patients express LOX-1. LOX-1 is a protein that was identified by Condamine *et al.* as differentially expressed between PMN-MDSC and HDN isolated from healthy donors, head and neck cancer patients and lung cancer patients [55]. In accordance, we showed that LOX-1 was absent in circulating HDN in cancer patients. However, the specific analysis of LOX-1 expression in CD45^{high} and CD45^{dim} LDN revealed that the marker was expressed only in the non-suppressive CD45^{dim}

subset (Figure 24). As LDN subsets were not considered in Condamine's paper, it is difficult to determine if LOX-1 is a marker of suppressive activity or simply a marker of immature LDN.

We also analyzed FATP2 expression, which is involved in lipid metabolism and was described as unique to PMN-MDSC [51]. Similar to LOX-1, FATP2 was not expressed by CD45^{high} LDN but expressed by CD45^{dim} LDN (Figure 24). Therefore, we think that LOX-1 and FATP2, rather than being specific markers of suppressive activity, might represent genes specific to immature LDN that are absent in mature LDN and HDN in ovarian and lung cancer. It is thus important to first characterize LDN subsets in cancer patients before trying to identify suppression-specific surface markers. We plan to increase the biological replicates in our transcriptomic analysis of CD45^{dim} and CD45^{high} LDN and then mine the data to identify differentially expressed surface markers that could be incorporated in our flow cytometry panel.

We further analyzed transcription factors associated to the functions of PMN-MDSC. The expression of C/EBP- β was around 4-fold higher in circulating CD45^{high} LDN compared to CD45^{dim} LDN (Figure 25). C/EBP- β is a positive regulator of MDSC immunosuppressive functions and associated with emergency granulopoiesis. Its expression is induced by IL-6, G-CSF, and GM-CSF through the JAK-STAT3 pathway. C/EBP- β is a transcriptional activator of genes like *ARG-1*, *PTGS2* (COX2) and *CSF3R* (G-CSF receptor) and is correlated with suppressive capacities of CD45^{high} LDN [22]–[25], [257].

However, we found no difference in expression of *STAT3*, *STAT5* and *IRF8* in CD45^{high} compared to CD45^{dim} LDN in blood of ovarian and lung cancer patients (Figure 25). In mice, STAT3 and STAT5 downregulate IFN- γ Regulatory Factor (IRF) 8 in the early stage of granulopoiesis, which leads to the accumulation of PMN-MDSC

[20]–[22]. There is an inversed correlation between IRF-8 expression and MDSC frequency [20]. While our transcriptomic data contradicts the mouse data, the activity of STAT3 and STAT5 should also be analyzed. This could be achieved with antibodies recognizing phosphorylated form of STAT (pSTAT) [286].

Mature suppressive LDN in the literature

We showed that mature CD45^{high} LDN suppress T-cell proliferation *in vitro*; unlike immature LDN. This contradicts the concept that MDSC are immature cells [30], [31]. However, this paradigm is a soft one as others also observed that mature circulating LDN exert suppressive activity and immature ones not [29], [179].

For instance, Lang *et al.* discriminated two LDN populations based on the expression of CD16, a marker of neutrophil maturation [29]. They notably observed that CD16⁺ LDN are mature and were more suppressive than immature CD16⁻ LDN in the blood of head and neck cancer patients. The proportion of CD16⁺ LDN of the total LDN population was directly correlated to the inhibition of T-cell proliferation in suppressive assays. We also observed that CD45^{high} LDN expressed higher levels of CD16 than CD45^{dim} LDN, both in transcriptomic and flow cytometry data (Figure 24 and Supp. Figure 5). This indicates that CD45^{high} LDN in the blood of lung and ovarian cancer patients are similar to the circulating CD16⁺ LDN observed in head and neck cancer patients. However, Lang *et al.* show a clear link between ARG-1 expression and suppressive abilities. These observations contradict our results: in lung cancer patients, non-suppressive CD45^{dim} LDN express higher levels of ARG-1 and CD45^{high} LDN express low levels of ARG-1. However, we analyzed the expression of ARG-1 transcriptomically while Lang *et al.* analyzed ARG-1 at the protein level and measured its activity in the supernatant of cultures. This difference in ARG-1

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expression at the transcriptomic and protein levels is puzzling. One reason could be that ARG-1 is first stored in granules, which are released upon contact with T-cells [123]. We plan to address this question in future by analyzing the expression of ARG-1 by intracellular flow cytometry.

Gondois-Rey *et al.* identified subsets of circulating LDN in melanoma patients. They discriminated the subsets according to the expression of CD16 and CD10 - the latter being a transmembrane glycoprotein expressed by mature neutrophils [287]. Similar to our research, they observed that HDN and mature LDN expressed higher levels of CD16 and CD10, and inhibited T-cell proliferation *in vitro*, unlike immature LDN [179]. Mature CD10^{high}CD16^{high} LDN also express the highest levels of *CXCR1*, *CD274* (PD-L1) and *SELL* (CD62L), while the immature CD10^{-/low}CD16^{-/low} LDN express more *CEACAM8* (CD66b). In our transcriptomic data, these genes are also differentially expressed between CD45^{high} and CD45^{dim} LDN (Figure 23 and Supp. Table 1). However, there is a notable difference in the expression of *OLR-1* (LOX-1), which is more expressed in circulating mature LDN compared to immature LDN in melanoma patients. Nevertheless, mature CD45^{high} and CD10^{high}CD16^{high} LDN are likely similar in melanoma and lung cancer patients, which indicates that the cells have broader applications.

In conclusion, CD45^{high} LDN match the definition of PMN-MDSC, even though they are mature cells. Others also showed suppressive activity by mature LDN in different cancer types. Our transcriptomic data indicate that CD45^{high} LDN are close to other suppressive mature LDN identified in melanoma or head and neck cancer patients. Still, our results have shown some contradictory results with what has been reported, especially about genes involved in suppressive mechanisms (e.g. *ARG-1* and *CYBB* (NOX-2)) or surface markers (e.g. *OLR-1*). However, contradicting results

are ubiquitous in the MDSC literature, notably regarding the “immature state” of these cells or the genes expressed by these cells and involved in their suppressive mechanisms [122], [285]. We think this means the characteristics of MDSC are difficult to generalize and are likely dependent on many parameters, like the method of isolation and analysis, the (stage of) pathology, the treatment, or the functional tests used. Hence, why in follow-up projects, we will look deeper at the functional properties, e.g. with blocking antibodies or with real-life imaging of cell-cell interactions.

Suppressive activity in HDN

In lung cancer patients, we observed potent suppressive activity in circulating HDN, which was unexpected as suppressive activity is usually linked to the presence of LDN. Their suppressive capacity was similar to the one observed in CD45^{high} LDN and these two populations were transcriptomically similar (Supp. Figure 3). However, circulating CD45^{high} LDN were less frequent (between 0.1% to 10% of PBMC in our analysis), while HDN are the major leucocyte population in the blood (60-70%).

In a preliminary analysis, we measured the suppressive activity of HDN isolated from three non-cancerous donors and we observed no suppression *in vitro* (data not shown). These observations were concordant with what is described in the literature, as HDN from healthy donors do not inhibit T-cell proliferation unless they have been activated [132], [286]. However, analyses of HDN isolated from cancer patients, especially in comparison to LDN, are currently rare. HDN from head and neck cancer patients were described as phenotypically and functionally different from those of healthy donors, notably by undergoing less apoptosis [177]. Also, HDN from melanoma patients suppress T-cell proliferation [179]. In contrast, HDN from

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non-metastatic HN, lung or mammary cancer patients failed to suppress T-cell proliferation [55], [141]. The results of this limited number of studies highlights the need of systematic evaluation of the inherent suppressive functions of HDN in cancer patients.

Furthermore, we think that HDN represent an essential control to LDN; notably to determine if any suppressive activity is a phenomenon specific to LDN or more likely a feature associated with all granulocytes in cancer patients. This approach will help to determine whether MDSC represent a unique and specific population or rather a state of pathological activation of neutrophils in cancer. We plan to further pursue the phenotyping and the functional characterization of HDN isolated from healthy donors. Also, we plan to do further RNA-seq analyses of HDN isolated from the blood of healthy donors and lung cancer patients to investigate whether these functional differences are embedded at the transcriptomic level. Finally, we plan to isolate HDN from tumors (equivalent of TAN) to characterize them against their circulating siblings.

Concluding remarks and perspectives

Our data suggest redefining the concept of PMN-MDSC. We have shown that circulating LDN in lung and ovarian cancer patients are composed of immature and mature cells that express different levels of CD45. Immature CD45^{dim} LDN do not suppress T-cell functions. Mature CD45^{high} LDN show potent immunosuppressive capacities, like tumoral LDN or circulating HDN in lung cancer patients. However, the transcriptomic profiles of circulating and tumor-infiltrating LDN were distinct, indicating that the environment shapes the phenotype and functions of LDN.

The heterogeneity of LDN needs to be further deciphered to define which neutrophil subsets mediate immunosuppression in different environments and/or pathologies. Approaches like single-cell RNA-seq and/or proteomics could further characterize the heterogeneity of neutrophils in cancer by identifying new subsets or states of activation [289]. Analyzing the frequency of these different subsets at different stages in cancer patients could turn them to biomarker that predict, e.g. the response to ICI. Humanized mice may also shed light on the fate of neutrophils progenitors in the bone marrow and the circulation (e.g. CD45^{dim} LDN) in malignant disease, leading to a deeper understanding of the subsets relationship, life cycles and fates.

Higher expression of ARG-1 and NOX-2, whose sole expression by LDN is often used to define PMN-MDSC, was not correlated with more potent suppressive capacities in our data. We think suppressive mechanisms could be studied with a higher “T-cell focus”, e.g. by analyzing the transcriptomes of CD4⁺ and CD8⁺ T-cells co-cultured with suppressive and non-suppressive LDN or HDN. This approach could have the advantage of identifying inhibitory mechanisms that might be more specifically targeted than those identified on LDN, which are shared with other

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myeloid cells. Lastly, *CD274* (PD-L1) was upregulated in all suppressive subsets (CD45^{high} LDN from both blood and tumor as well as HDN) compared to CD45^{dim} LDN in lung cancer patients. Thus PD-L1/PD-1 interactions represent a potential suppressive mechanism mediated by neutrophils in cancer and should be studied by, e.g., adding anti-PD-L1 antibody to proliferation assays.

Material and Methods

Material and Methods

Isolation of allogeneic T-cells

Extraction of peripheral blood mononuclear cells (PBMC)

Blood bags of approximately 500 ml from non-cancerous donor, i.e. hemochromatosis patients, were collected at Cliniques Universitaires St-Luc Hospitals (CUSL) and centrifuged at 4000 rpm for 20 minutes. The layer corresponding to the buffy coat that contains the white blood cells was retrieved and diluted 1:2 in cold PBS (in-house) supplemented with 1 mM of EDTA (Invitrogen, 15575-038). The cell suspension was layered on Lymphoprep® (Alere Technologies) for density gradient separation at room temperature (RT) and centrifuged at 910g for 20 minutes with lowest acceleration and brake. Mononuclear cells, contained in the ring above the Lymphoprep solution, were retrieved and washed four times with PBS 1mM EDTA to remove residual serum, Lymphoprep and platelets (centrifugation: 3 x 400g 10 minutes, 1 x 320g 6 minutes). The PBMC were resuspended at 10^7 cells/ml in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies, 52400).

Selection of CD2-positive and CD2-negative cells by rosetting

Sheep-red blood cells (Labor Dr Merck, E-410) (shRBC) are pre-treated with 2-Aminoethylisothiuronium bromide or AET (Sigma) for 15 minutes at 37°C as previously described [68]. These sheep red blood cells are mixed with PBMC (1 ml shRBC per 10^8 PBMC) for 2 to 3 minutes at RT. Then a density gradient density separation is performed with Lymphoprep as described earlier. The lighter fraction, containing CD2-negative cells, i.e. monocytes, was collected and wash three times with PBS 1 mM EDTA 1% Human Serum (HS) (Ludwig Institute for Cancer Research,

Brussel Branch) at 400g for 10 minutes and used to generate mature DC autologous to the T-cells (see infra). The pellet contains the shRBC and CD2-positive cells. The shRBC are lysed (80% 160 mM NH₄Cl, 10% RPMI 1640 and 10% FBS) for 1 minute and then immediately centrifuged at 510g for 2 minutes. The pelleted CD2-positive cells were then resuspended at 10⁷ cells/60μl in PBS 1 mM EDTA 1% HS.

Magnetic cell sorting of T-cells by negative selection

CD2-positive cells are mixed with Fc-Receptor blocking reagent (FcR Block, Miltenyi, 130-059-901) and anti-CD56 magnetic beads (Miltenyi, 130-050-401) at 20μl/10⁷ cells each for 15 minutes at 4°C. Cells are sorted the pos_sel program on the AutoMACS® magnetic cell sorter (Miltenyi). T-cells (CD2⁺CD56⁻) are collected in the negative fraction. T-cells were frozen in RPMI 1640 40% HS and 10% Dimethylsulfoxide (DMSO, Sigma, 472-301) at -80°C.

Generation of autologous dendritic cells

Monocytes were sorted from the CD2-negative fraction using anti-CD14 beads (Miltenyi) and a magnetic cell sorter (AutoMACS, Miltenyi) according to protocols provided by the manufacturer and above protocol. Monocytes were resuspended RPMI 1640 complemented with 10% FBS, 200 nM L-alanyl-L-glutamine dipeptide (GlutaMAX, Gibco, 35050-038), 100 U/ml Penicillin + 100 μg/ml Streptomycin (Sigma Aldrich, St Louis, Missouri, P4333), 100 ng/ml of GM-CSF and 30 ng/ml of IL-4 (which will be summarized as *DC medium*) at 5 x 10⁵/ml. Monocytes were plated in a 24 well plate (10⁶/well) and incubated for 5 days at 37°C 5% CO₂. Dendritic cells were then matured with 50 μg/ml Poly-IC (Sigma, P1530) and 10 ng/ml TNF-α (Peprotech,

300-01A) in DC medium for another two days at 37°C 5% CO₂. Cells were harvested and checked by flow cytometry to confirm their correct mature DC phenotype (CD3⁻, CD19⁻, CD14⁻, CD11c⁺, CD1a⁺, CD83⁺) (Panel 2). Mature DC are then frozen in RPMI 40% FBS and 10% DMSO.

Isolation of myeloid cells from cancerous patient's samples

Samples of blood and solid tumor from lung and ovarian cancer patients were collected after approval by the ethical committee of CUSL. Blood was diluted at 1:1 with PBS 1 mM EDTA at RT. Solid tumors were mechanically dissociated with MACS dissociator (Miltenyi) and enzymatically digested for 45 minutes at 37°C with 5 U/ml of DNase 1 (Worthington, DCLS), and Liberase™ TL and DL (thermolysin-dispase-low, both Sigma and Roche, catalog #05401020001 and LIBDL-RO) at 0.5 and 0.26U/mL, respectively. The tumor suspension is then washed with PBS 1mM EDTA passed through 70 µm and 40 µm filters. Density gradient separation was performed on both blood and tumor suspensions as described before. LDN were separated from the PBMC section. Heavy granulocytes were harvested from the layer just above red blood cells by diluting cells in 1:2 RPMI and 1% polyvinylalcohol (PVA). The separation occurred over 30 minutes at RT (by gravity). HDN were isolated from the upper fraction, washed with RPMI (380g, 10 minutes). Remaining RBC were lysed as described before. HDN were washed further 2 times with RPMI (380g, 10 minutes). LDN and HDN were isolated next by flow cytometry.

Flow cytometry

Analysis of purity, phenotype and T-cell proliferation

Cells were resuspended in 50 µl of PBS 1mM EDTA 1% HS 20% FcR Block reagent in a 96-well round bottom plate (Falcon, 353910). 50 µl of antibodies mix (See panel 1-2) were added to the well and cells were incubated at RT and in darkness for 15 minutes. Cells were then washed three times with PBS 1mM EDTA 1% HS. Data acquisition was performed on FACS Fortessa (BD). Analysis of flow cytometry data was performed with Flowjo software (BD). For proliferation assay, 2,500 rainbow calibration beads (RCB, BD, 559123) per samples were added in proliferation assay to standardize cell counting in replicates.

Panel 1. Proliferation assay reading

Target	Fluorochrome	Clone	Dilution (final)	Manufacturer
CD3	PE	UCHT1	1/100	BD, 555333
CD8	APC	RPA-T8	1/50	BD, 555369
Viability	eF780	/	1/1000	Affimetrix
Sphero™ Rainbow Calibration Particles	/	/	2,500/well	BD, 559123

Panel 2. Phenotyping of mature DC

Target	Fluorochrome	Clone	Dilution (final)	Manufacturer
CD19	BV510	SJ25C1	1/50	BD, 562947
CD3	Alexa Fluor 488	HIT3a	1/50	BL, 300319
CD11b	PE	ICRF44	1/20	BD, 555388
CD1a	BV421	HI149	1/200	BD, 555808
CD83	APC	HB15a	1/20	BC, IM2218
CD14	APC-Cy7	HCD14	1/40	BL, 325619

Sorting of myeloid cells from cancerous patients

Total mononuclear cells or heavy granulocytes from blood and tumor were resuspended at 10^8 cells/ml in PBS 1 mM EDTA 1% HS 20% FcR Block reagent at room temperature. 100 μ l of antibodies mix (Panel 2 and 3) for 15 minutes at room temperature. Cells were washed two times in PBS 1mM EDTA 1% HS and passed through 40 μ m filter. Cell sorting was performed on Aria III (BD) (Panel 3). From blood samples, monocytes ($CD45^+$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{high}$, $HLA-DR^{high}$, $CD14^+$), $HLA-DR^{low}$ ($CD45^+$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{high}$, $HLA-DR^{low}$, $CD14^+$, $CD15^-$), LDN ($CD45^+$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{dim}$, $HLA-DR^-$, $CD14^-$, $CD15^+$), autologous T-cells ($CD45^+$, $CD3^+$, $CD19^-$, $CD56^-$, $CD33^-$), $CD45^{dim}$ LDN ($CD45^{dim}$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{dim}$, $HLA-DR^-$, $CD14^-$, $CD15^+$, $CD16^{-/low}$), $CD45^{high}$ LDN ($CD45^{high}$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{dim}$, $HLA-DR^-$, $CD14^-$, $CD15^+$, $CD16^+$) and HDN ($CD45^{high}$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{dim}$, $HLA-DR^-$, $CD14^-$, $CD15^+$, $CD16^+$) were sorted. From solid tumor, monocytes, macrophages ($CD45^+$, $CD3^-$, $CD19^-$, $CD56^-$, $CD33^{high}$, $HLA-DR^{high}$, $CD14^+$, $CD206^+$), $HLA-DR^{low}$ monocytes and LDN (bulk or subpopulations) were sorted. Cells were collected in 5 ml FACS tubes (Falcon, 352058) filled with 1 ml of FBS and were directly centrifuged and resuspended in appropriate medium. Gates for $CD15$, $CD14$, $CD206$, $HLA-DR^{low}$, $LOX-1$ and $CD45^{high}$ expression were set with a control of myeloid cells stained with only anti-lineage ($CD3$, $CD19$, $CD56$), anti- $CD45$, anti- $CD33$ antibodies and viability dye.

Material and Methods

Panel 3. Sorting of myeloid cells (blood and tumor)

Target	Fluorochrome	Clone	Dilution (final)	Manufacturer
CD3	Alexa Fluor 488	HIT3a	1/100	BL, 300319
CD19	BV510	SJ25C1	1/100	BD, 562947
CD56	BV510	NCAM16.2	1/100	BD, 563041
CD45	Alexa Fluor 700	HI30	1/40	BD, 304024
CD33	BV711	WM53	1/40	BD, 563171
HLA-DR	Pe-Cy-7	L243	1/400	BL, 307616
CD15	PE	HI98	1/30	BD, 555402
CD14	PerCP	MφP9	1/40	BD, 345786
CD16	BV421	3G8	1/100	BD, 562876
CD66b (blood only)	FITC	80H3	1/40	BC, IM0531U
CD206 (tumor only)	FITC	19.2	1/30	BD, 551135
Viability	eF780	/	1/400	Affimetrix
LOX-1	APC	15C4	1/100	BD, 556288

T-cell proliferation assay

Set up of assay

Allogeneic T-cells and mature DC were thawed in RPMI 1640 medium (Life Technology) complemented with 5% human serum (HS), 200 nM L-alanyl-L-glutamine dipeptide, 100 U/ml Penicillin and 100 µg/ml Streptomycin (Pen/Strep) and 5 U/ml human recombinant IL-2 (Novartis, CLB-P-476-700-1447) (T-cell proliferation medium) complemented with 5 U/ml of DNase I for minimum 4 hours at 37°C 5% CO₂. In the meantime, a 96 flat-bottom wells plate (Corning Co-star, 7007) is coated with 100µl of anti-CD3 antibody (Biolegend, clone OKT3, 317326) 1 µg/ml for minimum 4 hours at 37°C. T-cells from donors and cancer patients were labelled with 0.5 µM of 5-Chloromethylfluorescein diacetate (CMFDA, Thermofisher Scientific, C2925) in RPMI 1640 without serum for 20 minutes at 37°C 5% CO₂ and then washed one time with RPMI 1640. Labelled T-cells are resuspended at 0.6 x 10⁶

Material and Methods

cells/ml in T-cell proliferation medium. Mature DC are resuspended at 10^5 cells/ml in T-cell proliferation medium.

The coating solution of the 96 flat-bottom well plate is carefully removed. Each well is filled with 50 μ l of mature DC suspension (for allogeneic T-cell condition) or proliferation medium (for autologous T-cell conditions). Another 50 μ l of anti-CD28 at 8 μ g/ml is added to the wells coated with anti-CD3. Sorted myeloid cells are added to the plates in a volume of 50 μ l, with different concentrations as indicated (usually 10,000; 3,000 and 1,000 cells per well). Finally, 50 μ l of labelled T-cells are added in each well (30,000 cells/well). The 96-well plate is then incubated for 4 days at 37°C 5% CO₂. T-cell proliferation is monitored by flow cytometry (Panel 1).

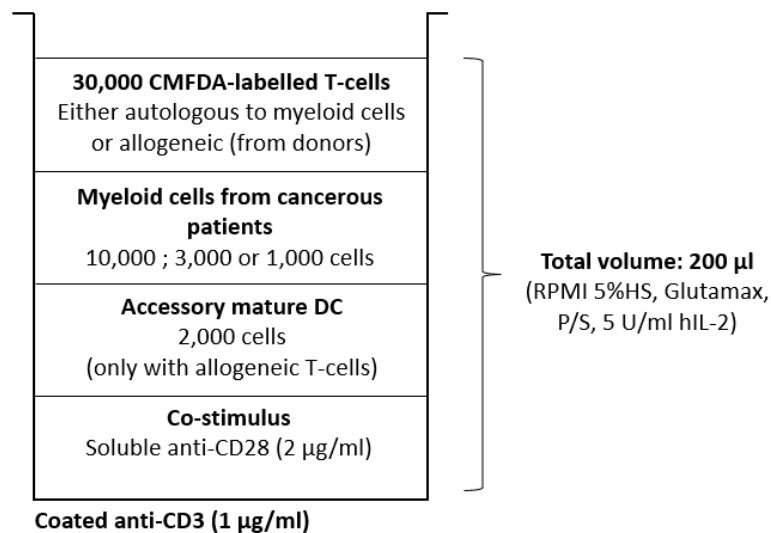


Figure 28. Scheme representing composition of well (96-well flat-bottom plate) for proliferation assay.

Analysis of cell morphology by cytopsin

After isolation by flow cytometry, LDN (CD45^{dim} or CD45^{high}) from tumors and blood, and heavy-density neutrophils from blood were centrifuged and resuspended at >50,000 cells/ml in a minimum volume of 100 μ l of FBS and were sent to the

Hematology service within the 6 hours (Franklin Tower, St-Luc hospital) for cytospin analysis.

RNA extraction for transcriptome analysis

5,000 to 250,000 myeloid cells sorted from blood and tumors of cancer patients were centrifuged and resuspended in 200 µl of PBS. The suspension was then mixed with 900 µl of Qiazol™ lysis solution (Qiagen, 79306), transferred to RNase-free (RF) 1.5 ml low-binding Eppendorfs (Ambion, AM12450) and then vortexed for 1'. Lysate were kept at -80°C until RNA extraction with miRNeasy kit (Qiagen, 217084) according to manufacturer protocol. RNA was eluted in 15 µl of RF water and precipitated with 0,1 volume of sodium acetate 3M (Thermo Fischer Scientific, AM9740), 3 volumes of ice-cold ethanol 100% (Anafor-Normapur, 20821.310) and 1µg glycogen (Thermo Fisher Scientific, R0551). After overnight incubation at -20°C, sample were centrifuged at 14000g for 30 minutes at 4°C and washed two times with ice-cold ethanol 75%. Supernatant was removed and samples were left to dry at RT before being resuspended in 4.5 µl of RF water.

RNA sequencing and transcriptome analysis

RNA-seq library construction was done using the Nextera XT DNA Library Preparation Kit (Illumina, FC-131-1096) in combination with Nextera XT Index Kit v2 Set D index kits (Illumina, FC-131-2004) and was unstranded and poly-A-based. RNA-library were sent for ultra-low input RNA-sequencing (Genewiz, Leipzig, Germany). Between 20 and 30 million paired-reads were sequenced per sample on an Illumina HiSeq 2x150 platform. Quality control of reads was done through FastQC (Babraham

Bioinformatics) before and after trimming with Trimmomatic (0.38 version, Bolger, Lohse, and Usadel 2014). Alignment of reads on human genome GRCh38 was done using HISAT2 program (Kim et al. 2019) and samtools was used for indexation in .bam files. Count of aligned reads was made with the FeatureCounts program from the Subread package [288], which gave similar results than htseq-count but with shorter amount of time (personal observations). Normalization, principal-component analysis (PCA) and differential expression analysis was realized on R using the DESeq2 package (Love et al 2014). Pre-filtering was set to keep all genes with >10 reads in at least three different samples. For differentially expressed gene (DEG), log-2-fold change threshold was usually set to > 0.5 (min. 2-fold) with a p-value below 0.01. Volcano plots were drawn using the Enhanced Volcano plot package (R).

Potential differentially expressed cell-surface proteins were selected based on the following gene ontology annotations: GO:0005886, GO:0004872 and GO:0009897. For transcription factors that are differentially expressed, the selection was based on the following gene ontology annotations: GO:0003700, GO:0001228 and GO:0001227.

Statistical analysis

The frequency of M-MDSC and LDN among total myeloid population (CD33⁺) or hematopoietic cells (CD45⁺) and proportion of CD45^{high} LDN was for tumor and/or blood of each sample was determined using FlowJo program. All data were imported into R to be plotted and statistically analyzed. To know whether parametric or non-parametric tests were required, normality of distribution was assessed by Shapiro-Wilk test on R. For proliferation assays, number of cell-cycles and the number of cells

Material and Methods

that undergo division at day 0 were determined using the MFI of CMFDA as previously described [68]. All statistic tests were made on R and were indicated in figure legends.

Figures

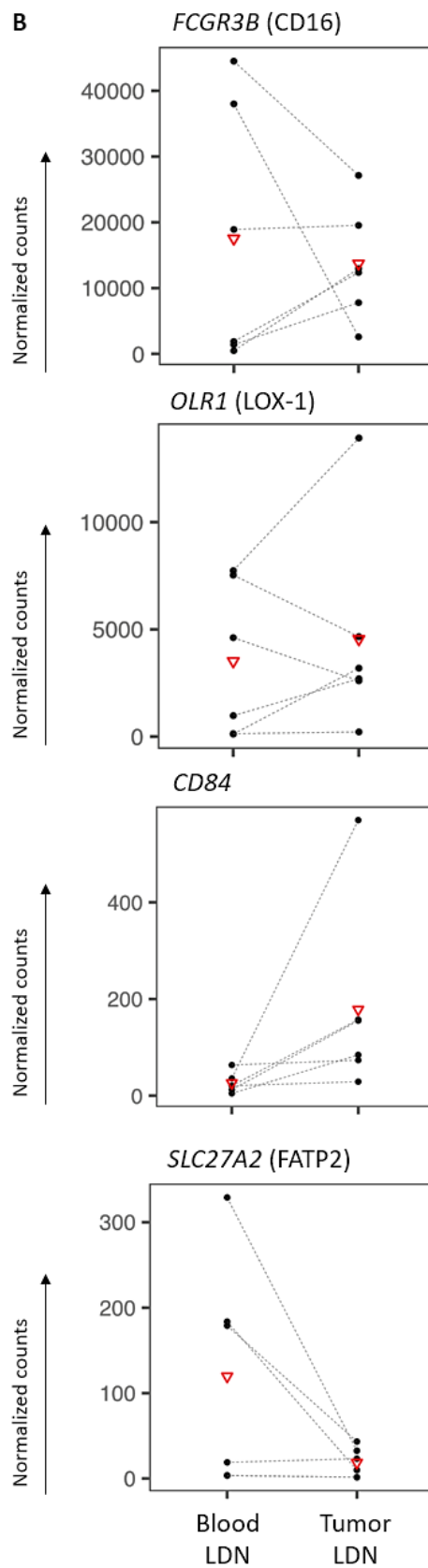
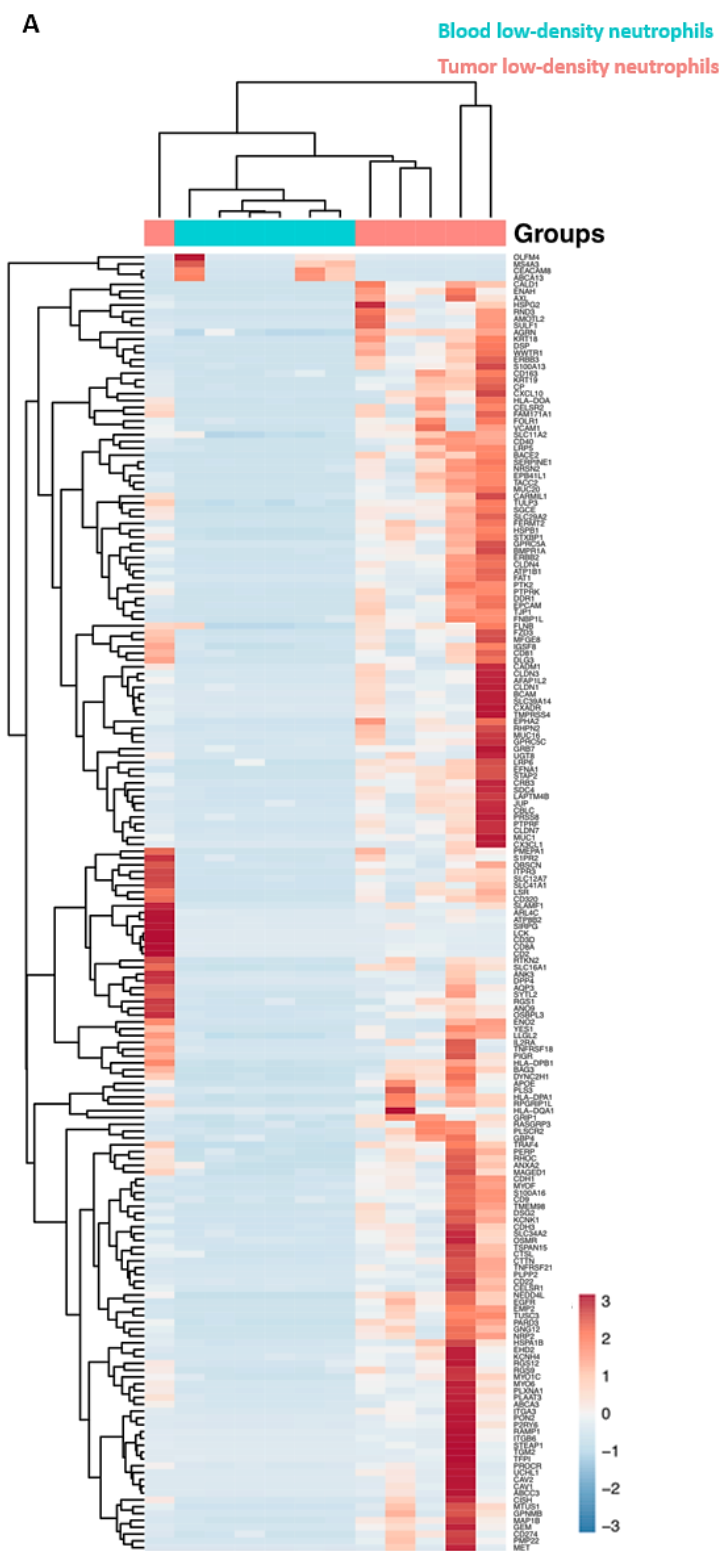
Figures and scheme used in introduction were created by Christophe Vanhaver using BioRender.com

Supplementary data

Supplementary data

Supplementary data

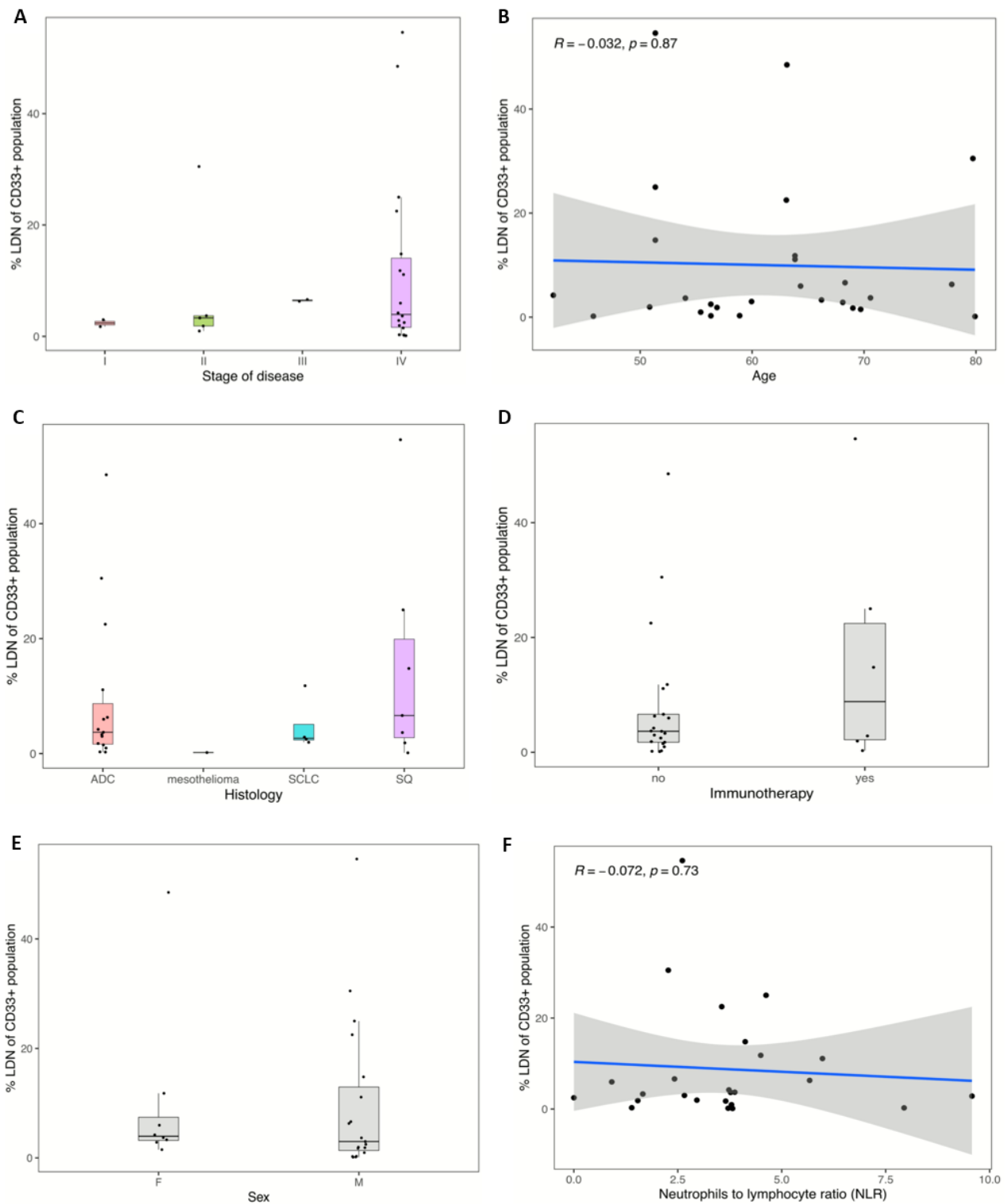
Supplementary data



Supplementary data

Supplementary Figure 1. Several surface proteins are down- or upregulated in circulating LDN compared to tumor LDN in ovarian and lung cancer patients. LDN were freshly isolated from the blood or the tumors of patients with ovarian or lung cancer by flow cytometry as previously described. After sorting, cells were directly resuspended in QIAzol Lysis buffer and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data was performed with DESeq2 (R). **A.** Heatmap was generated only on surface markers between resting blood LDN tumor LDN using Pheatmap package (R). Potential surface markers were selected based on the following gene ontology annotations: GO:0005886, GO:0004872 and GO:0009897. P-values * = < 0.05, ** = < 0.01, *** = < 0.001 (DESeq2 paired sample analysis).

Supplementary data



Supplementary Figure 2. Frequency of circulating LDN is not impacted by patient parameters such as age, sex, immunotherapy treatment or NLR. PBMC were collected after density gradient separation and stained with MDSC-isolation antibody panel. LDN were defined following the gating

Supplementary data

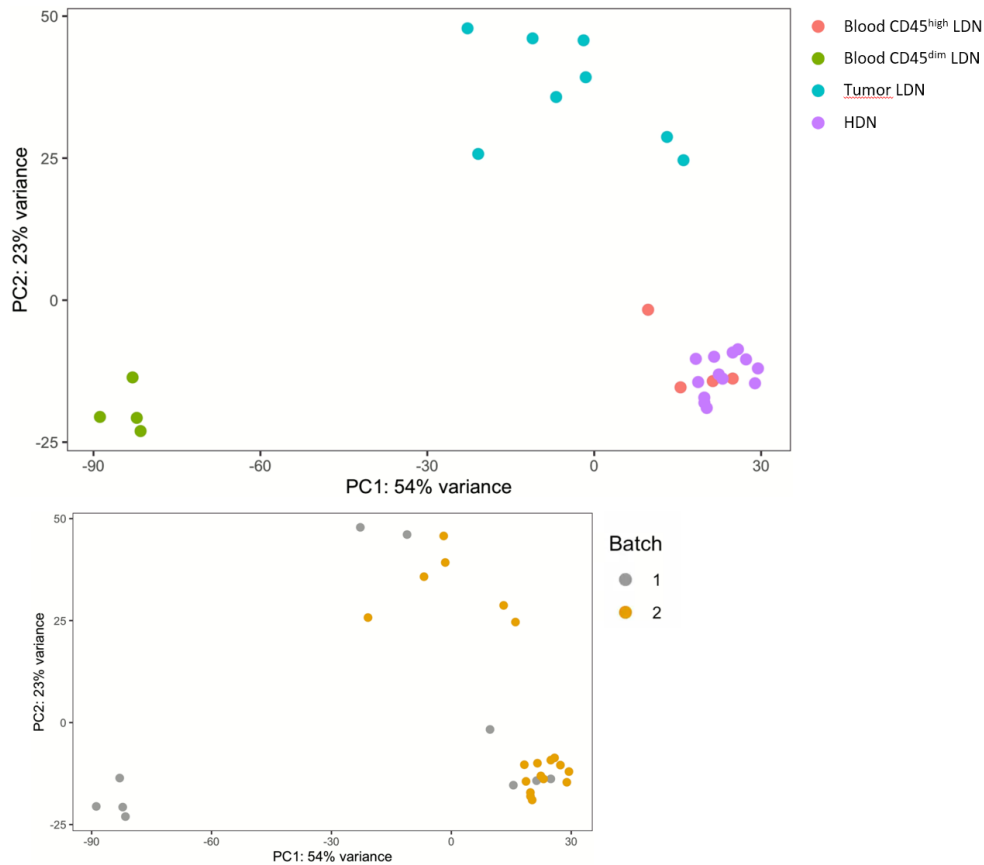
*strategy described at Figure 6. Sorting gates were defined using cells stained with the MDSC-master mix without anti-CD15 and anti-HLA-DR antibodies. **A.** Frequency of LDN among myeloid cells according to stages of disease in lung cancer patients. **B.** Correlation between frequency of LDN and age of lung cancer patients (Pearson's product moment correlation coefficient). **C-E.** Frequency of LDN among myeloid cells according to tumor histology classification (C), immunotherapy treatment (D) and sex of lung cancer patients (E). **F.** Correlation between frequency of LDN and NLR in lung cancer patients (Pearson's product moment correlation coefficient). ADC: Adenocarcinoma, SCLC: Small Cell Lung Cancer, SQ: Squamous Cell Carcinoma.*

Supplementary data

Supp. Table 1. Expression value (RNA-seq) and statistical test for differential expression of gene linked to neutrophils and PMN-MDSC in CD45^{high} and CD45^{dim} LDN in cancer patient. First values represent mean of normalized counts for each group. Log₂ FC (Fold-change) represent fold-change in CD45^{dim} group compared to CD45^{high} LDN. Adjusted p-value was determined through DESeq2 analysis (R). Bold and italic value represent statistically significant result (**, p-value < 0.0001).**

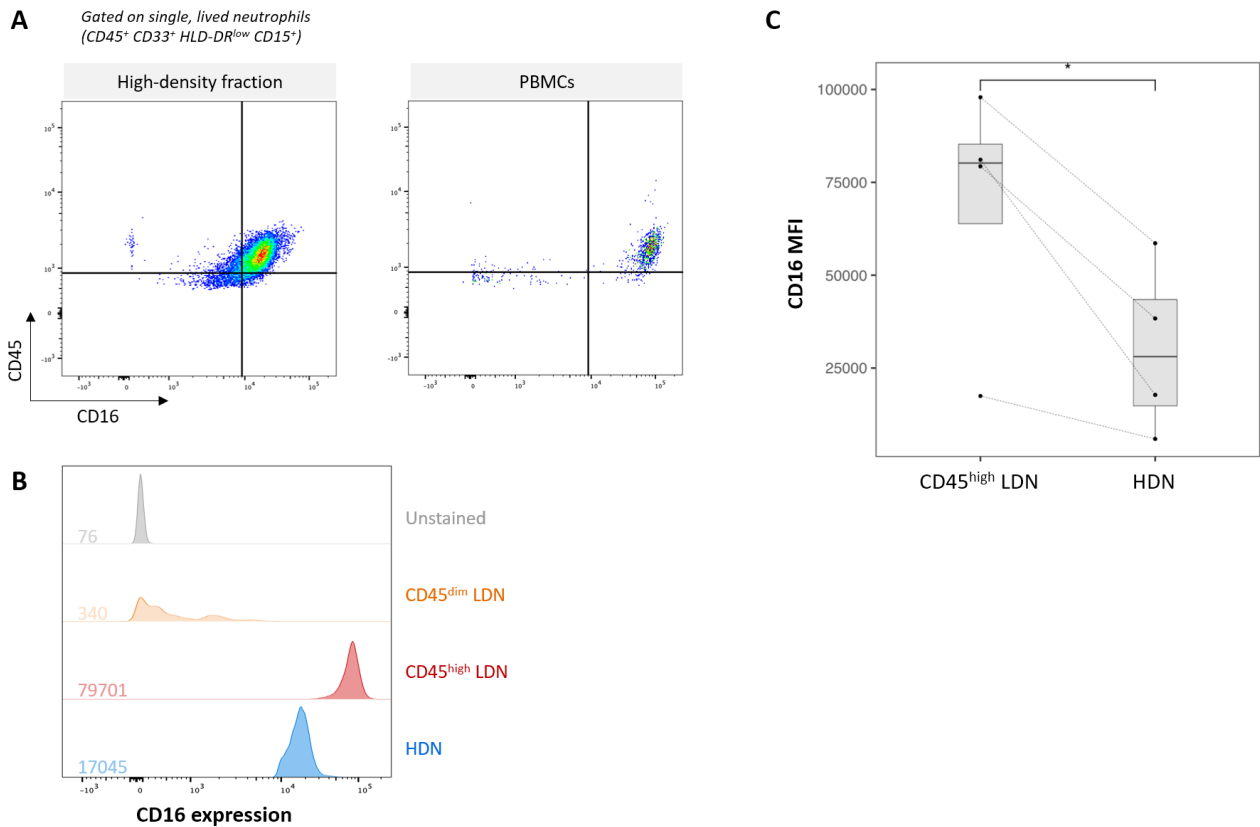
Gene ID	CD45 ^{high} LDN	CD45 ^{dim} LDN	Log ₂ FC	p-value	Symbol	Gene name
ENSG00000090339	4,59E+03	1,93E+02	-6,08	<i>3,34E-11</i>	ICAM1	intercellular adhesion molecule 1 (MAC-1 ligand)
ENSG00000100985	5,71E+03	2,56E+03	-0.871	>0.05	MMP9	matrix metalloproteinase 9
ENSG00000118113	8,66E+01	4,56E+04	9,45	<i>5,24E-53</i>	MMP8	matrix metalloproteinase 8
ENSG00000119535	7,03E+04	4,86E+03	-3,86	<i>2,93E-08</i>	CSF3R	colony stimulating factor 3 receptor
ENSG00000121966	8,55E+04	8,68E+03	-3,26	<i>4,69E-07</i>	CXCR4	C-X-C motif chemokine receptor 4
ENSG00000163464	1,87E+04	5,52E+02	-5,09	<i>8,00E-12</i>	CXCR1	C-X-C motif chemokine receptor 1
ENSG00000169896	2,04E+03	4,60E+03	1,06	> 0.05	ITGAM	integrin subunit alpha M
ENSG00000180871	3,36E+04	2,06E+02	-7,44	<i>3,73E-30</i>	CXCR2	C-X-C motif chemokine receptor 2
ENSG00000188404	6,87E+04	1,87E+03	-5,31	<i>2,86E-07</i>	SELL	selectin L (CD62L)
ENSG00000124469	1,17E+02	4,86E+05	9.36	<i>4.35E-31</i>	CEACAM8	CEA cell adhesion molecule 8 (CD66b)
ENSG00000196549	6,71E+03	1.57E+01	-11.1	<i>7.36E-21</i>	MME	Membrane metalloendopeptidase (CD10)

Supplementary data



Supplementary Figure 3. RNA-seq analysis showed similar profile between circulating CD45^{high} LDN and HDN in blood of cancer patients. LDN and HDN were freshly isolated from the blood or the tumor of patient with ovarian or lung cancer by flow cytometry as previously indicated. After sorting, cells were directly lyzed in appropriate buffer and frozen at -80°C. RNA was later extracted and sent for paired-end RNA-sequencing. Normalization of data and principal component analysis were performed with DESeq2 (R). This plot represents the combination of two batch of RNA-seq analysis. Each point represents one sample (bulk population), and the two graphs illustrate the same data with a different labelling (up: type of cell and tissue of origin, down: batch of RNA-seq analysis).

Supplementary data



Supplementary Figure 4. Circulating CD45^{high} LDN have higher expression of CD16 than circulating CD45^{dim} LDN or HDN. PBMC are isolated from the blood of lung cancer patient after density gradient separation. Cells are stained with MDSC-mix antibody cocktail (see methods) and LDN are gated as CD45⁺, CD33^{dim}, HLA-DR^{low}, CD15⁺ cells. **A-B.** Representative plots of LDN heterogeneity for one lung cancer patient, showing CD16 expression of total CD15⁺ population (A) or HDN and LDN subsets (B). **C.** Comparison of CD16 expression (MFI) in CD45^{high} LDN and HDN in 4 lung cancer patients. P-values * = < 0.05 (paired t-test).

Supplementary data

Abbreviations

Abbreviations

Abbreviations

ADC	Adenocarcinoma
ADCC	Antigen-dependent Cell Cytotoxicity
Ado	Adenosine
ALK	Anaplastic Lymphoma Kinase
AMP	Adenosine Mono-phosphate
ARG-1	Arginase-1
ASC	Alanine-serine-cysteine
ATP	Adenosine Tri-phosphate
BM	Bone Marrow
BRAF	B-Raf Proto-Oncogene, Serine/Threonine Kinase
BRCA1 & BRCA2	Breast Cancer 1 & 2
CAF	Cancer Associated Fibroblast
CAR T-cell	Chimeric Antigen Receptor T-cell
CCL & CXCL	C-C & C-X-C Motif Chemokine Ligand
CCR & CXCR	C-C & C-X-C Motif chemokine receptor
CD	Cluster of Differentiation
CD40L	CD40-Ligand
CEACAM8	CEA Cell Adhesion Molecule 8 (CD66b)
CMFDA	5-chloromethylfluorescein diacetate
COX2	Cyclooxygenase 2
CRC	Colorectal Carcinoma
CSF3R	Colony-stimulating Factor 3 receptor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CUSL	Cliniques Universitaires Saint-Luc
CYBB	Cytochrome B-245 Beta Chain
DC	Dendritic Cell
DEG	Differentially expressed gene
DL	Dispase-low
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetra-acetic acid
EGFR	Epithelial Growth Factor
eIF2a	Eukaryotic Translation Initiation Factor 2
ELISA	Enzyme-Linked Immunosorbent Assay
eNep	Early Granulocytic Progenitor
ER	Estrogenic Receptor
FACS	Fluorescence-activated cell sorting
Fas	Fas Cell Surface Death Receptor
FasL	Fas Ligand
FATP2	Fatty Acid Transporter Protein 2
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
FcR	Fc Receptor
fMLF	N-formyl-methionyl-leucyl-phenylalanine
FMO	Fluorescence Minus One
Foxp3	Forkhead Box P3
G -	Granulocytic
G-CSF	Granulocyte Colony Stimulating Factor
Gal-9	Galectin-9
GCN2	General-Control Nonderepressible-2 Kinase

Abbreviations

GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GO	Gene Ontology
Gr-1	Granulocyte Receptor-1 Antigen
HD	Healthy Donor
HDN	High-density Neutrophil
HER2	Human Epidermal Growth Factor Receptor
HLA	Human Leukocyte Antigen
HN	Head and Neck
HS	Human Serum
ICI	Immune Checkpoint Inhibition
IDO	Indoleamine 2,3 Dioxygenase
IFN- γ	Interferon Gamma
IL	Interleukine
IMC	Immunosuppressive myeloid cell
iNOS	Inducible Nitric Oxide Synthase
IRF-8	IFN- γ Regulatory Factor 8
JAK	Janus Kinases
KRAS	KRAS Proto-oncogene
L-NIL	N6-(1-Iminoethyl)-L-lysine hydrochloride (iNOS inhibitor)
L-NMMA	N-Methyl-L-Arginine (ARG-1 inhibitor)
LCK	Lymphocyte-specific protein Tyrosine Kinase
LCLC	Large-cell Lung Carcinoma
LDN	Low-density Neutrophils
LOX-1	Lectin-type Oxidized LDL Receptor 1
Ly6	Lymphocyte Antigen 6
M -	Monocytic
M-CSF	Macrophage Colony Stimulating Factor
MDSC	Myeloid Derived Suppressor Cell
MET	MET Proto-Oncogene, Receptor Tyrosine Kinase
MFI	Median of Fluorescence
MHC	Major Histocompatibility Complex
MMP	Matrix Metalloproteinase
mTOR	Mammalian Target of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBSGW	NOD.Cg-Kit ^{W-41J} Tyr ⁺ Prkdc ^{scid} Il2rg ^{tm1Wjl}
NCT...	National Clinical Trial
NK cell	Natural Killer Cell
NLR	Neutrophils to Lymphocytes Ratio
NO	Nitric Oxide
NOX	NADPH Oxidase
NSCLC	Non-Small Cell Lung Carcinoma
NSG	NOD Scid Gamma
NTRK	Neurotrophic tyrosine Receptor Kinase
OLR-1	Oxidized Low-Density Lipoprotein Receptor 1
ONOO	Peroxynitrite
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphobuffered saline

Abbreviations

PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PD-1	Programmed-death 1
PD-L1	Programmed-Death Ligand 1
Pen/Strep	Penicillin & Streptomycin
PGE2	Prostaglandin-E2
PMA	Phorbol 12-myristate 13-acetate
pMHC	Peptide-MHC complex
PMN	Polymorphonuclear
Poly-IC	Polyinosinic:polycytidylic acid
PR	Progesterone Receptor
pSTAT	phosphorylated STAT
PTGS2	Prostaglandin-endoperoxide Synthase 2
PVA	Polyvinil Alcohol
qRT-PCR	Quantitative Retro-transcriptase PCR
RAG	Recombinant-activating Gene
RCB	Rainbow Calibration Beads
RET	RET Proto-Oncogene
RF	RNase Free
RNA	Ribonucleic Acid
RNA-seq	RNA sequencing
ROS	Reactive-Oxygen Species
RPM	Rotation per minute
RPMI	Roswell Park Memorial Institute Medium
RT	Room Temperature
S100A9	S100 Calcium Binding Protein A9
SCLC	Small Cell Lung Carcinoma
SELL	Selectin L (CD62L)
SEM	Standard error of the mean
shRBC	Sheep Red Blood Cells
SIRP	Signal Regulatory Protein
SLC...	Solute Carrier Family...
SOC	Standard of Care
SOD	Superoxide Dismutase
SqCQ	Squamous Cell Carcinoma
STAT	Signal Transducer and Activator of Transcription
STK	Serine-Threonine Kinase
T cell	T-lymphocyte
TAM	Tumor associated macrophage
TAN	Tumor associated neutrophil
TCR	T-cell Receptor
TDO	Tryptophan 2,3-dioxygenase
TGF- β	Transforming Growth Factor Beta
TIM-3	T-cell immunoglobulin and mucin-domain containing 3
TL	Thermolysin-Low
TME	Tumor Microenvironment
TNF- α	Tumor Necrosis Factor Alpha
TP53	Tumor Repressor 53
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T-cell
tRNA	Transfer RNA

Abbreviations

WB	Western Blot
WT	Wild Type

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