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PlexinB1 deficiency in the microenvironment inhibits tumor growth and metastatic dissemination in a murine breast cancer model

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1. Abstract

Semaphorin and Plexin signaling, initially characterized for its role in axonal navigation, has been implicated in a number of diseases and plays a major role in the tumor context as well. Semaphorin4D (SEMA4D), in particular, has been shown to affect tumor growth and the tumor microenvironment (TME). However, at present the role of the SEMA4D high-affinity receptor PlexinB1 (PLXNB1) in the TME has been poorly characterized. The aim of this thesis project has been to study the role of PLXNB1 in the TME in the metastatic triple-negative murine breast carcinoma 4T1 model and its contribution to tumor progression. We found that primary tumor growth and metastatic dissemination were strongly reduced when 4T1 tumor cells were injected orthotopically in PLXNB1 deficient mice. PLXNB1 deficiency in the TME did not affect tumor vessel density but enhanced vessel normalization by increasing pericyte coverage and reducing intra-tumoral hypoxia. Notably, in PLXNB1 deficient mice we observed an increased infiltration of $CD8^+$ T lymphocytes, $CD11c^+$ antigen presenting cells and a reduction of myeloid derived suppressor cells. In addition, in *Plxinb1*$^{-/-}$ mice as compared with WT animals, we detected a switch in the polarization status of tumor-associated macrophages (TAMs) towards a pro-inflammatory M1 phenotype. Interestingly, treatment of *Plxnb1*$^{-/-}$ mice with a CD8 blocking antibody enhanced cancer growth and metastasis number, indicating that $CD8^+$ T-cell recruitment contributes to the anti-tumor effect observed in *Plxnb1*$^{-/-}$ mice. In line with these observations, in tumors grown in *Plxnb1*$^{-/-}$ mice we detected enhanced expression of factors shown to mediate the activity of infiltrated lymphocytes, such as IFNγ and Chemokine (C-X-C motif) ligand 9 (CXCL9). On the basis of the strong impact of PLXNB1 deficiency in re-programming the TME, leading to a more suppressive tumor milieu, we tested its therapeutic use in combination with immunotherapy. Remarkably, we found that PLXNB1 deficiency strongly enhanced the efficacy of anti-PD-1 blocking antibody, efficiently reducing tumor growth and distal metastasis. Altogether, these data suggest that PLXNB1 is a promising therapeutic target for metastatic breast tumors and that its blockade can improve immunomodulatory therapies.

2. Introduction

2.1. Breast Cancer

Breast cancer (BC), along with lung and colon cancer, is one of the most frequent tumor types and the most common malignancy in women, with one in eight estimated to develop the disease during their lifetime[1,2].

In the clinic, patients are routinely classified and treated based on the presence or absence of three molecular markers: the estrogen (ER) and the progesterone receptors (PgR), and the HER2 tyrosine kinase. Hormone receptor positive/HER2 negative tumors are found in approximately 70% of patients, HER2-positive account for 15%-20%, whereas 15% of patients lack all the former molecular markers and are referred to as triple-negative tumors (TNBCs). The latter tumor subtype is more likely to recur and has worse prognosis; moreover, chemotherapy along with surgical resection of non-metastatic lesions are the only therapeutic options[3]. More recently, gene-expression profiling studies enabled the definition of five different major molecular subtypes; Luminal A, Luminal B, HER2 enriched, Basal-like and Claudin-low[4].

Surgical intervention is the primary therapeutic option for local and regional breast cancer. Tumor features and staging determine the need for systemic therapy, which can be constituted by chemotherapy, endocrine therapy, or HER2-targeted therapy. Chemotherapy is recommended in early-stage breast cancer depending on ER, PgR and HER2 status (ER^-, PgR^-, and HER2-negative; HER2-positive), lymph node involvement and tumor size; whereas for stage IV disease, the receptor status and metastasis localization are the main discriminating factors. Adjuvant chemotherapy after surgery (usually including anthracyclines and a taxane) is generally given to patients with a high risk of recurrence. HER2-positive breast cancer patients can benefit of HER2-specific monoclonal antibody therapy (trastuzumab, pertuzumab, lapatinib, neratinib) in addition to chemotherapy. Patients with ER- or PgR-positive breast cancer should instead receive endocrine therapy, such as an aromatase inhibitor or tamoxifen[5,6].

While non metastatic disease is potentially curable, metastatic disease is not considered so, and therapy is aimed at extending life span and quality, while minimizing symptoms or side effects.

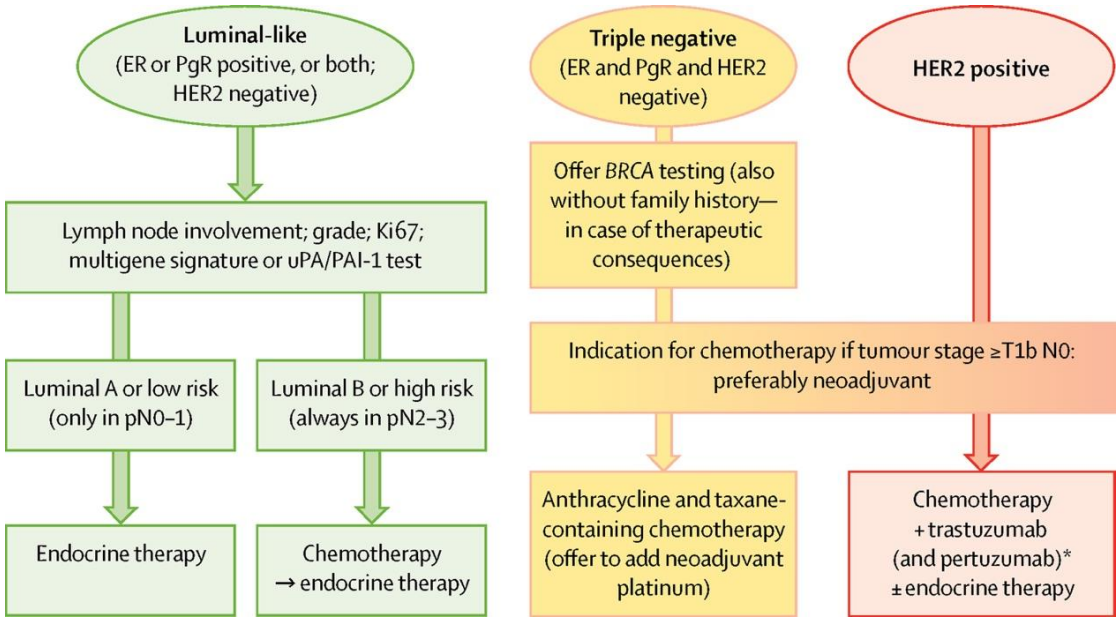

*Figure 1 Principles of systemic therapy in early breast cancer (from Breast cancer. The Lancet 389, 1134–1150 (2017). ER=oestrogen receptor. PgR=progesterone receptor. *Dual HER2 blockade only registered for neoadjuvant setting. Endocrine therapy always indicated if ER or PgR positive, or both.*

2.2. The Tumor Microenvironment

The so-called tumor microenvironment (TME) comprises the complex and variegated environment in which cancer cells grow and interact with surrounding normal cells.

The TME of solid tumors is composed by a number of different cell types: immune cells, like monocytes, tumor associated macrophages (TAMs), dendritic cells (DCs), T and B lymphocytes, neutrophils, myeloid derived suppressor cells (MDSCs), natural

killer cells (NKs) and natural killer T cells; endothelial cells of blood and lymphatic vessels; stromal cells such as cancer associated fibroblasts (CAFs), pericytes, mesenchymal stromal cells and adipocytes. The TME also comprises the extracellular matrix and soluble factors like cytokines, chemokines, growth factors and extracellular vesicles. Stromal cells and fibroblasts secrete growth factors and soluble mediators that deeply influence tumor progression, and act as attractants for other cells to the TME. The TME plays a critical role both in the initial phase of tumor development and throughout disease progression, it can be both tumor-promoting or tumor-suppressive and is also crucial in determining the response to therapies[7,8].

Normal breast ducts are composed of a luminal epithelial cell layer enclosed by myoepithelial cells. Ductal myoepithelial cells contribute to producing the surrounding basement membrane. The breast microenvironment is composed of an extracellular matrix (ECM) and numerous stromal cell types, including endothelial and immune cells, fibroblasts, and adipocytes[9,10].

Breast cancer progression follows well-defined pathological and clinical stages, starting with ductal hyperproliferation that evolves in *in situ* and invasive carcinomas. The most critical step in breast cancer progression consists in the transition from *in situ* to invasive ductal carcinoma. This phase is defined by the disruption of the myoepithelial cell layer and basement membrane. The subsequent spreading of cancer cells to distant organs leads to metastatic disease, the last step of breast cancer[11]. Notably, the TME has been implicated in all of the former phases of tumor progression[12].

Human breast cancer immune infiltrate is typically composed by T lymphocytes (70-80%), B cells (10-20%), macrophages (5-10%), NK cells and antigen presenting dendritic cells[13,14,15]. The possibility of targeting the TME has long being envisaged and different therapeutical approaches have been designed, such as those targeting immune-infiltrating populations such as TAMs, MDSCs or neutrophils or those aimed at enhancing DC and TIL anti-tumor activity; immunotherapies or adoptive T-cell therapy. Moreover, other strategies target the tumor stroma or the tumor vasculature[16,17,18,19].

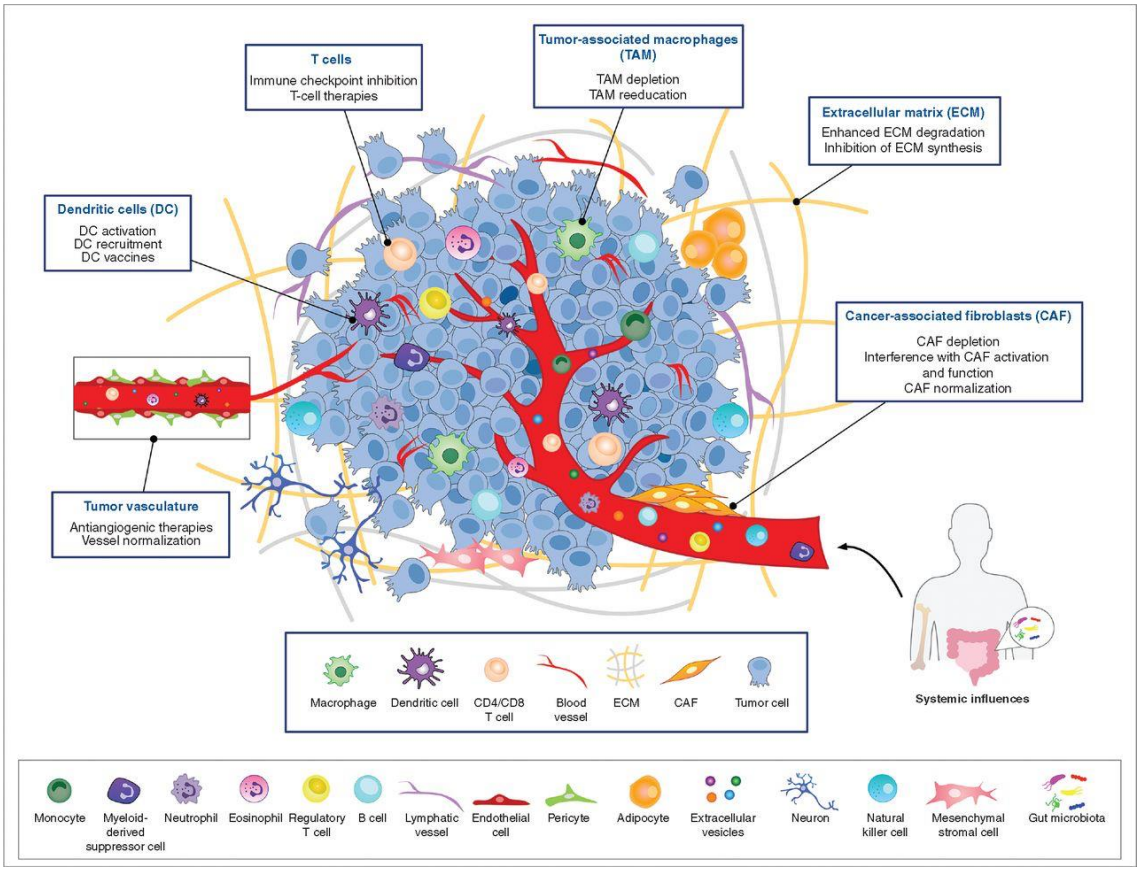

Figure 2 The Tumor Microenvironment. Schematic representation of the TME as potential therapeutic target. Figure from Bejarano L, Jordão MJC, Joyce JA. Therapeutic Targeting of the Tumor Microenvironment. Cancer Discovery 2021

2.2.1. Tumor Associated Macrophages (TAMs)

Macrophages are leukocytes of the mononuclear phagocyte innate immune system and play a critical role in homeostasis and defense against infections. Depending on microenvironmental conditions such as i) released chemokines, cytokines, and other secreted factors from tumor, mesenchymal, and immune cells, ii) anoxia, iii) inflammation or iv) acidosis, monocytic cells in the circulation are recruited to the tumor TME and differentiate as tumor-associated macrophages (TAMs)[20].

TAM infiltration can promote tumor growth and metastasis[21,22], although certain subtypes can also play an anti-tumoral role. Microenvironmental cues can polarize macrophage phenotype. Polarized macrophages can be classified in two main groups: M1 (classically activated macrophages) or M2 (alternatively activated macrophages). Classically activated macrophages are endowed with strong microbicidal properties and sustain strong IL-12-mediated Th1 responses. M1 macrophages can also express nitric oxide synthase (iNOS)[23] and reactive oxygen species (ROS) together with high levels of proinflammatory mediators such as TNF-α or IL-1β. Moreover, M1 TAMs can engulf and kill target cells [24,25,26]. On the contrary, M2 polarized macrophages support Th2-associated effector functions and play a major role in inflammation resolution and express a large number of scavenger receptors and secrete chemokines and cytokines that promote tumor growth, such as IL-6, IL-10, VEGF and matrix metalloprotein (MMP) [27,28,29,30].

However, anti-tumoral M1-polarized and pro-tumoral M2-polarized TAMs represent two extremes of a broad range of functional states. Indeed, during tumor initiation, progression and metastasis TAM phenotype changes dynamically. Of note, conversion of M2 macrophages to M1-like has also been reported in literature[31].

High TAM infiltration is associated with poor patient prognosis[32] and resistance to therapies and depletion of M2 TAMs or their "re-education" as anti-tumor effectors is considered a promising immunotherapeutic strategy [33].

2.2.2. Tumor Infiltrating Lymphocytes (TILs)

The tumor immune infiltrate plays a key role in determining patient's clinical outcome. Different subpopulations of lymphocytes can be recruited to the tumor site and become tumor infiltrating lymphocytes (TILs). The most common lymphocyte subsets, $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ regulatory T cells, fundamentally contribute to tumor eradication. Activated cytotoxic $CD8^+$ T cells are able to secrete large amounts of proinflammatory cytokines such as TNFα (tumor necrosis factor α) and IFNγ (type γ interferon). The second major mechanism is the production and release of cytotoxic

factors, mostly perforin (which forms a pore in the membrane of the target cell) and granzyme (which elicits the apoptosis of target cells)[26,34].

$CD4^+$ T lymphocytes are referred to as T helper lymphocytes (Ths). Ths can be divided into two categories; Th1 and Th2. Th1 cells produce IFNγ, interleukin (IL)-2, and tumor necrosis factor (TNF)-beta, that activate macrophages and promote cell-mediated immunity and phagocyte-dependent responses. Th2 cells instead produce IL-4, IL-5, IL-10, and IL-13, which induce strong antibody production, eosinophil activation, and lead to the inhibition of macrophage functions, mediating phagocyte-independent responses[35]. Activated $CD4^+$ T helper cells can release several cytokines, promote B cell differentiation into plasma cells, and enhance dendritic cells (DCs) ability to trigger $CD8^+$ T cell activation, through stimulation of CD40 on DCs by the expression of CD40 ligand (CD40L)[36 37 34].

$CD4^+$ regulatory T cells (T-regs) constitute a T cell subset playing a critical role in self-tolerance. T-regs are characterized by expression of CD25 and FOXP3 (forkhead box P3 transcription factor) and negatively regulate immune responses. Increased T-regs abundance has been reported in the environment of several malignancies, including breast cancer[38].

A number of clinical studies have evaluated the prognostic and predictive value of tumor-infiltrating lymphocytes (TILs) in breast cancer. For instance, $CD8^+$ cytotoxic T cells infiltration has been strongly associated with patient survival and response to therapy[34,39,40]. Among $CD4^+$ T cells, Th1 cells infiltration has been associated with positive clinical outcomes; on the other hand, Th2 cells have been shown to correlate with anti-tumor response inhibition. The presence of $CD4^+$ regulatory Tregs has been associated with both good and bad prognosis depending on the subtype[39].

During early stages of cancer progression, naïve T cells can be primed in the draining lymph nodes by immunogenic antigens displayed by antigen presenting cells. T cells activation is then followed by their migration to the TME, where they can initiate an effector immune response, eliminating immunogenic tumor cells[41]. Unfortunately, TME-infiltrating $CD8^+$ T cells can assume a state of functional 'exhaustion'[42]. This exhaust phenotype is characterized by reduced responsiveness and upregulation of inhibitory molecules such as PD1, CTLA4, LAG3, TIGIT and TIM3[43].

The malignant transformation of cells greatly depends on the accumulation of DNA damage. The so called neoantigens arise as a consequence of this genetic damage and the immune system is frequently able to mount a response against cells displaying these mutated peptides[44]. Cancer cell neoantigens are synthesized in the cytosol and are presented to $CD8^+$ T cells as peptides fragments bound to MHC class I molecules. MHC I mediated antigen presentation allows $CD8^+$ T lymphocytes to identify and later eliminate cancer cells. Cancer antigens can also be ingested into their endocytic compartments by macrophages, dendritic cells, or B cells and then be presented to $CD4^+$ T cells as peptides bound to MHC II molecules. T cells are able to recognize antigenic peptides via their receptors only if MHC I or MHC II molecules are associated to the antigen on the cell surface[45].

The ability of the host immune system to eradicate and shape the immunogenicity of tumors defines the concept of cancer immunoediting[46]. In fact, it is becoming increasingly clear that the immune system plays a dual role in cancer; not only it suppresses tumor growth by eliminating cancer cells or inhibiting their growth but it also promotes tumor progression either by selecting the fittest cancer cells for surviving in an immunocompetent host or by shaping TME conditions that facilitate tumor outgrowth.

When the immune system fails to completely eradicate cancer cells, tumors can escape immunological surveillance. A crucial role in this escape is played by T cell-inhibiting immune checkpoints such as PD-1 and CTLA-4. T cell activity can be strongly dampened by the binding of programmed death protein 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on T cells with their respective ligands exposed by tumor cells: programmed death ligand 1 (PD-L1) and CD80. Indeed, a tremendous effort in the last years led to the development of immune checkpoint therapies against CTLA4, PD1 and PD-L1 that have shown remarkable success in a subset of patients with intractable cancers[43]. Moreover, altered MHC II expression has been linked to decreased anti-tumor immunity[47]. The interaction of the immune system with tumor cells in breast tumors seems to be breast cancer subtype specific. The TNBC and HER2-positive breast cancer subtypes elicit stronger immune responses and are hypothesized to be more dependent on these immunoediting mechanisms[47].

Indeed, even though breast cancer is not considered a highly immunogenic disease, immunotherapy strategies are being successfully translated in clinical trials[47], especially for TNBC patients[48]. Initial studies evaluating monotherapy immune checkpoint inhibitors in patients with TNBC demonstrated that the activity of these drugs is enhanced when used in the first-line disease setting in patients with PD-L1-positive disease. The IMpassion130 study for instance, established atezolizumab combined with nanoparticle albumin-bound (nab)-paclitaxel as the first-line therapy of choice for patients with advanced TNBC who are positive for PD-L1 on immunohistochemistry[48].

2.2.3. Myeloid Derived Suppressor Cells (MDSCs)

Myeloid derived suppressor cells (MDSCs) derive from myeloid cells that do not differentiate into granulocytes, macrophages, or dendritic cells (DCs). MDSCs in bone marrow and spleen can be triggered by diverse pathological conditions such as infection, autoimmune diseases or cancer[49]. Pro-inflammatory cytokines in the TME induce a chronic inflammation in the tumor, that can perturb the maturation of myeloid cells in favor of MDSCs[50]. These cells then accumulate in the peripheral blood and, in the case of solid tumors, accumulate in the tumor site[49].

In mice, MDSCs are characterized by CD11b and Gr-1 expression, and are further classified into two subsets: granulocytic (polymorphonuclear) MDSCs (G-MDSCs or PMN-MDSCs) and mononuclear MDSCs (M-MDSCs). Murine G-MDSCs are defined as $CD11b^{+}Ly6G^{hi}Ly6C^{lo}$ cells, whereas M-MDSCs are defined as $CD11b^{+}Ly6C^{hi}Ly6G^{-}$cells[49,51].

MDSCs comprise an heterogeneous population of immature immune cells characterized by the ability to suppress T cells and NK cytotoxic functions[52] and direct cell to cell contact is required for such immunosuppressive activity. MDSCs-mediated T cells suppression is achieved via induction of L-arginine shortage via upregulation of Arg1 or iNOS, for which L-arginine is a substrate, leading to inhibition of T cell proliferation. Besides, MDSCs promote the development of the pre-metastatic niche, and contribute to resistance to immunotherapy[53].

Of note, M-MDSCs are currently known as one of the main circulating precursors of TAMs[25].

2.2.4. Tumor angiogenesis

Tumors require nutrients and oxygen supply in order to sustain their growth. The generation of new tumor-associated vasculature exploits the physiological process of angiogenesis, i.e., the sprouting of new vessels from existing ones. This physiologic process in the adult is usually transient. In contrast, during tumor progression, an "angiogenic switch" takes place, causing quiescent vasculature to incessantly sprout new vessels in order to sustain tumor growth[54,55].

This dysregulation of angiogenesis is one of the hallmarks of cancer. Once reached the size of 1–2 mm, the limit of nutrient diffusion, tumors become hypoxic and acidic due to insufficient nutrient supply and metabolic waste clearance by the existing vasculature. Hypoxia can induce the production of angiogenic factors, such as vascular endothelial growth factor (VEGF) within tumors, and cancer cells can release cytokines and growth factors in order to activate normal cells in their microenvironment. At the beginning, angiogenesis provides the tumor with more oxygen and nutrients, nevertheless the ultimate response is poor, and the resulting vascular network is aberrant, with dilated, tortuous, and leaky vessels with irregular blood flow. Normal tissue microvasculature is organized and displays a hierarchical branching order. On the contrary tumor vasculature is characterized by pouches of increased vessel density and others of reduced vessel density[56,57].

Endothelial cells belonging to the tumor vasculature also display poorly organized cell-to-cell contacts and are poorly covered by basement membrane and vascular smooth muscle cells. Pericytes surround and support the vascular endothelium[58]. These cells usually lie within the vessel basement membrane and closely interact with endothelial cells in order to stabilize vessels and to prevent vessel leakage. Pericyte recruitment is critical for the formation of a mature and stable vascular network and occurs in response to a number of molecules including PDGFRβ, sphingosine-1-phosphate-1, Ang-1, and

TGF-β[56]. In tumors, endothelial cells are frequently associated with pericytes that, however, display multiple abnormalities[59].

The overall outcome of the aberrant phenotype of tumor vessels is increased vascular permeability, leading to proteins and fluids to accumulate in the extravascular compartment, and compromising the effective delivery of therapeutic agents[60].

Remarkable effort has been put in the past for the development of anti-angiogenic therapies (AATs) in order to starve tumors of nutrients[61]. The initial clinical trials with the anti-vascular endothelial growth factor (VEGF) antibody bevacizumab failed to improve survival when administered as single therapeutic agent, but could improve the effect of chemotherapy[61]. This apparent paradox is explained by the fact that an hypoxic environment renders cancer cells more aggressive, and leaky vessels indeed favor the metastatic spreading. Moreover, hypoxic and acidic environment reprograms resident immune cells towards a pro-tumorigenic, immunosuppressive phenotype[62]. However, when AATs are properly administered, they can induce a "window of normalization" during which the efficacy of co-administered anticancer drugs is enhanced[61,63]. Vessel normalization results in more efficient oxygen and drug delivery, and increased penetration of drugs improve the outcome of chemotherapy whereas increased oxygen delivery enhance the efficacy of radiation therapy and that of many chemotherapeutic agents[64].

2.3. Semaphorins and their receptors in cancer

Semaphorins comprise a large family of evolutionarily conserved proteins that were originally identified as signaling cues for axonal navigation. Later on, semaphorins were found to be involved in the regulation of diverse biological and pathological processes, in developmental angiogenesis, bone homeostasis and immune response[65]. Accumulating evidence indicates that semaphorin signaling can play a major role also in tumor growth and metastatic progression[66].

The semaphorin family can be subdivided on structural and sequence similarity bases in eight classes. Semaphorin class 1 and 2 comprise invertebrate orthologues, classes 3 to 7 include vertebrate family genes, and class V comprises viral semaphorins. Among

vertebrate semaphorins, class 3 consists of secreted members characterized by a conserved basic charged domain located at the C-terminus. Class 4 to 7 semaphorins are membrane bound proteins[67,68]. All semaphorins share an N'-terminal 500 amino acid long sema domain, arranged in a seven-blade β-propeller shape. The sema domain is coupled to a small cysteine-rich PSI (plexin-semaphorin-integrin) domain. The sema and PSI domains are followed in many semaphorins by immunoglobulin (Ig)-like domains or thrombospondin type 1 repeats[69].

Plexins, the semaphorin receptors, are categorized in four subfamilies on similarity bases. In vertebrates, nine plexins have been identified so far. Plexin-A family comprises 4 members (1 thru 4), Plexin-B family 3 members (1-3), whereas PlexinC1 and PlexinD1 are one of a kind[67]. However, most class 3 semaphorins require binding to co-receptors of the neuropilin family, NRP1 and NRP2, and cannot bind directly to plexins. Alike their ligands, Plexins are characterized by the presence of a Sema domain followed by three consecutive PSI-IPT domains and three more IPT domains. In addition to mediating ligand binding, the extracellular domain can associate with neuropilins and scatter factor receptors[70]. Plexins' intracellular domain is highly conserved, and it has been shown to associate with a number of intracellular signal transducers[70,71].

Plexins lack an intrinsic kinase activity, nevertheless they can trigger the activation of associated receptor tyrosine kinases (RTKs) and non-receptor type tyrosine kinase (nRTKs)[72–75]. Moreover, the plexin cytoplasmic region shows high sequence similarity to a group of Ras-family-specific GTPase-activating proteins (GAPs)[76]. GAPs function by negatively regulating monomeric G proteins activity promoting the hydrolysis of GTP to GDP. Plexin's intracellular domain contains two highly conserved GAP-like subdomains that include tree arginine residues required for the catalytic activity, separated by a linker region[70]. Activation of the intrinsic GAP activity of plexins by semaphorin binding regulates integrin functions, cytoskeletal dynamics, cell adhesion and migration[70,72].

Semaphorin signaling can occur in autocrine, paracrine, or juxtacrine manner. All semaphorins are able to signal through their cognate Plexin receptor via a so-called "forward" signaling pathway. This signal transduction negatively regulates integrin-

mediated adhesion and induces cytoskeletal remodeling. Besides, some transmembrane semaphorins are able to signal through their own cytoplasmic domains, thus acting in "reverse" signaling mode, behaving as receptors rather than ligands for Plexins[77,78].

A huge body of literature supports the idea that semaphorin signaling plays an important role in a variety of cancers; notably, many semaphorins have been implicated in breast tumors and their expression correlates with disease outcome[69]. The secreted SEMA3A, for instance, was found to positively correlate with patient's good prognosis[79]. SEMA3A via NRP-1 attenuates breast tumor progression inducing activation and nuclear translocation of PTEN and FOXO3a[79]. Alike SEMA3A, SEMA3B levels correlate with better prognosis in breast cancer[80]. SEMA3E levels, on the contrary, are associated with distant metastasis[81]. Moreover, SEMA3E signaling via the receptor PlexinD1 promotes carcinoma cell invasiveness and metastatic spreading [82].

Among membrane-bound semaphorins, SEMA4C expression has been found to correlate with poor disease outcome in breast cancer. Targeting of SEMA4C signaling, mediated via PlexinB2, impairs G2/M phase transition and leads to cytokinesis defects and cancer cell senescence[83]. Notably, the reverse signaling of SEMA4C has been found to induce TGF-β/BMP non-conventional signaling, upregulate the expression of ID1/3 transcriptional factors, and reprogram the gene expression profile, with a suppression of epithelial-mesenchymal transition[84].

Lastly, SEMA7A is increased in breast cancer specimens and associates with decreased overall survival[85]. Moreover, SEMA7A has been shown to increase pro-angiogenic TAMs recruitment and induce tumor-associated lymphatic vessels growth[85].

2.4. Semaphorin4D and PlexinB1

A chronic inflammatory state can predispose to cancer development. Moreover, the TME is often infiltrated with inflammatory cells, possibly as a consequence of the aberrant tumor milieu which is typically hypoxic and necrotic.

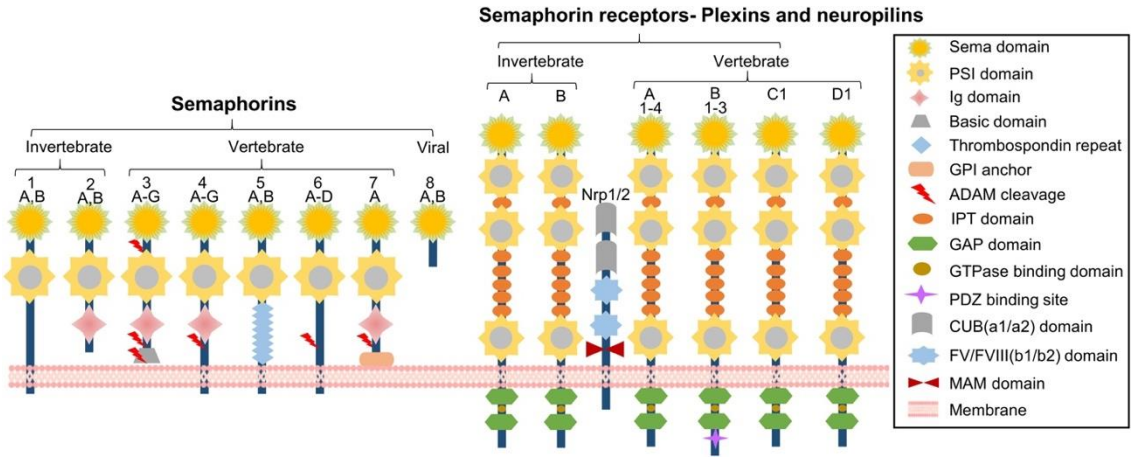

Figure 3 Schematic representation of semaphorins and semaphorin receptors. From Hu S, Zhu L. Semaphorins and Their Receptors: From Axonal Guidance to Atherosclerosis. Front Physiol. 2018

This cancer-associated inflammation is characterized by the recruitment of leukocytes and by the presence of soluble inflammatory mediators such as cytokines and chemokines. This inflammatory process is tightly associated with the angiogenetic and fibrotic process with consequent tissue remodeling[86]. Accumulating evidence indicate that a number of so-called "immune"-semaphorins play a major role in the pathogenesis of immunological diseases and can represent interesting potential therapeutic targets[87]. Semaphorin4D (SEMA4D) has been initially described for its role in the immune system[88]. Known at the beginning as CD100 antigen, it was the first semaphorin family member found to have immunoregulatory activity. SEMA4D shows a conserved seven-blade β-propeller structure and consists of NH2-terminal signal peptide followed by a Sema domain, an Ig domain of the C2 type, a hydrophobic transmembrane region, and a cytoplasmic tail[89]. SEMA4D is synthesized as a transmembrane protein, but membrane type-1 matrix metalloproteinase (MT1-MMP) or the metalloprotease ADAM17 (TACE) mediate cleavage of its extracellular portion[90].

SEMA4D has three known receptors, the highest affinity receptor PLXNB1, the intermediate affinity receptor PlexinB2 and the low affinity receptor CD72[86]. In the immune system, SEMA4D is highly expressed on resting T cells[91]. On the contrary SEMA4D expression is low in resting B cells and antigen-presenting cells but its levels are upregulated upon activation. Through CD72, SEMA4D promotes B cells

aggregation and survival, and enhances antibody production[92]. Study conducted on *Sema4d* deficient mice demonstrated a non-redundant role for SEMA4D-CD72 interactions in the immune system[93]. SEMA4D deficiency, in fact, led to a strong reduction in the number of $CD5^+$ B-1 cells despite other conventional B and T cells developed normally. *In vitro* proliferation and B cells immuno-globulin production were reduced in *Sema4d* deficient mice. Moreover, *in vivo* priming of T cells and humoral response towards a T cell–dependent antigen were defective in *Sema4d* deficient mice[93]. SEMA4D expression on T cells or dendritic cells (DCs) has been shown to contribute to the activation and maturation of DCs. Among its immunological functions, the SEMA4D-CD72 complex induces the production of cytokines by monocytes[94,95].

SEMA4D receptor Plexin-B1 intracellular domain contains two separated Ras GAP-homologous domains, C1 and C2. When the small GTPase Rnd1 associates with Plexin-B1, the resulting Plexin-B1-Rnd1 complex enables a GTPase promoting activity of the plexin for R-RAS. inactivation of R-RAS in response to SEMA4D then reduces cell adhesion and migration, and induces growth cone collapse[96,97]. PLXNB1 shows also strong GAP activity towards the RAP1 GTPase[98].

In the immune system, SEMA4D signaling through PLXNB1 inhibits the migration of monocytic and B-cell lineage cells[99]. Besides, SEMA4D-PLXNB1 interaction was shown to increase $CD5^+$ leukemic but also normal B cells viability and proliferation[100].

SEMA4D binding to the high-affinity receptor PLXNB1 in endothelial and epithelial cells induces angiogenesis and cell migration[101].

SEMA4D-PLXNB1 signaling has also been shown to sustain tumor progression. PLXNB1 can associate with the two RTKs MET and ERBB2 (Fig.4). However, previous work pointed out that the expression of these RTKs in breast cancer cells can cause opposite effects on RhoA via PLXNB1[102]. In particular, activation of PLXNB1–ERBB2 complex activates RhoA through a plexin-associated PDZ-Rho-GEF, promoting directional cell migration. Moreover, in a mouse model of ErbB2 overexpressing breast cancer, PLXNB1 knockdown strongly reduced the occurrence of metastases[74]. In human patients with $ERBB2^+$ tumors, low levels of PLXNB1 expression correlate with good prognosis[74]. On the contrary, coupling of PLXNB1 with MET mediates RhoA inhibition, resulting in migration arrest. Nevertheless, this point

remains controversial since SEMA4D has been shown to activate an invasive growth program by coupling with MET in epithelial cancer cells[103]. SEMA4D binding to PLXNB1 induces the association of PLXNB1 with MET, promoting auto-phosphorylation of the MET receptor leading to tumor cells invasiveness. Besides, directional migration via MET activation has been described also in neurons secreting the neuropeptide gonadotropin hormone–releasing hormone-1 (GnRH-1)[104].

Furthermore, SEMA4D is an acknowledged pro-angiogenic factor *in vitro* and *in vivo* [105,106]. Previous work by Sierra and colleagues, demonstrated that syngeneic breast cancer cells engraftment in *Sema4d* deficient mice resulted in reduced tumor growth and vessel maturation compared to WT animals[107]. Moreover, their work showed that TAM derived SEMA4D is critical for angiogenesis and vessel maturation.

In vitro work by Younis and colleagues showed that Sema4D released by head and neck squamous cell carcinoma (HNSCC) cells can change myeloid cell polarization toward an immune suppressor phenotype ($CD33^+$ $CD11b^+$ HLA-DR2/low MDSCs). These MDSCs could inhibit T-cell proliferation and blunt IFNγ secretion, likely via the production of immunosuppressive factors[108]. Moreover, the down-modulation of SEMA4D in cancer cells inhibited MDSCs-mediated regulation of T cells, promoting the differentiation of effector T cells and decreasing Tregs.

Preclinical studies demonstrated that antibody-mediated SEMA4D blockade in combination with immune checkpoint inhibitors remodels TME by promoting recruitment of effector lymphocytes and APCs, while reducing immunosuppressive cell infiltration. Evans and co-workers showed that antibody-(MAb 67-2) mediated SEMA4D blockade could result in tumor rejection in syngeneic murine carcinoma models of colon and $ERBB2^+$ mammary cancer[109]. SEMA4D levels were higher at the tumor borders and disruption of this gradient via antibody targeting enabled increased infiltration within the tumors of $CD11c^+$ monocytic/APC cells, $CD8^+$ T-cells and increased Teffector/Treg ratio that ultimately led to tumor rejection and stable disease control[109]. Moreover, anti-SEMA4D antibody treatment enhanced the efficacy of anti–CTLA-4, anti–PD-1 immuno-therapies and cyclophosphamide. Later, Clavijo and colleagues demonstrated that treatment with anti-SEMA4D MAb 67-2, in combination with immune checkpoint inhibitors, enhanced tumor rejection in murine syngeneic

models of oral carcinoma and Lewis lung cancer[110]. This effect was obtained through the attenuation of MDSC recruitment to the tumors via reduction of MAPK-dependent chemokine production by cancer cells. Besides, PMN-MDSC suppressive capacity was reduced through inhibition of SEMA4D-driven arginase expression[110].

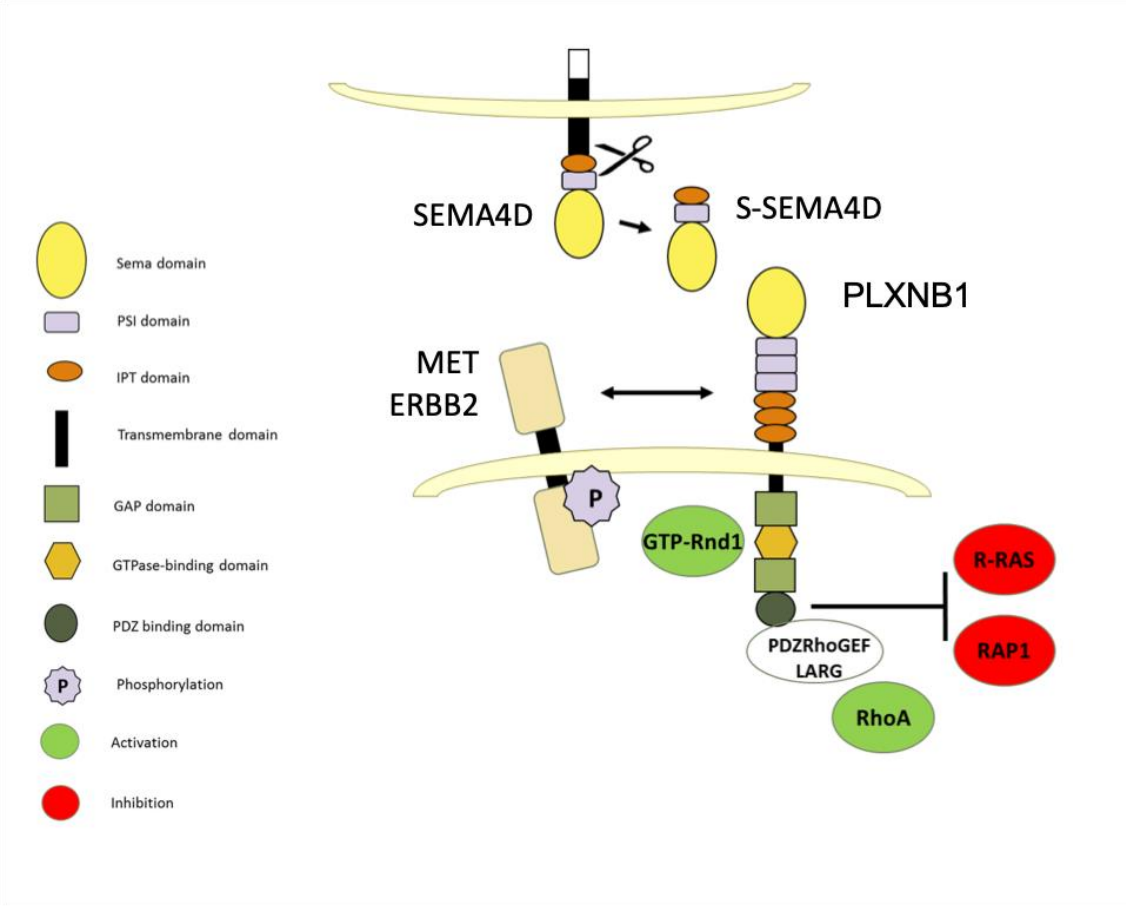

Figure 4 Schematic representation of SEMA4D- PLXNB1 signaling. Membrane-bound SEMA4D or its soluble cleaved form can signal through PLXNB1. PLXNB1 PDZ-binding domain binds to PDZRhoGEF and LARG, resulting in RhoA activation, whereas recruitment of Rnd1 enables intrinsic GAP activity towards R-RAS and RAP1. PLXNB1 can associate and trans-activate MET and ERBB2 RTKs.

On the other hand, an unexpected pro-invasive effect of anti-SEMA4D antibodies was recently reported in a preclinical model of neuroendocrine pancreatic cancer (Rip1-Tag2 mice)[111]. This novel mechanism was shown to be mediated by SEMA4D receptor-independent reverse signaling in tumor associated macrophages. SEMA4D blockade

resulted in increased TAM recruitment to the tumor invasive front and increased their secretion of stromal cell–derived factor 1 (SDF1, also known as CXCL12). SDF1/CXCL12 in turn, promoted invasion and metastasis via the CXCR4 receptor expressed in cancer cells[111].

The implication of these findings for translation in the clinic may be limited to PanNET or similar tumors. However, the presence of a signaling pathway triggered by anti-SEMA4D antibodies in macrophages should be further investigated and future studies should address the question of how to predict tumor specific response to SEMA4D blockade[112]. A humanized version of anti-SEMA4D with the same antigen recognition domain of MAb67-2 has been developed and is named VX15/2503 or pepinemab[113]. VX15/2503 is currently in early phase clinical trials for the treatment of human cancers and Huntington disease[112].

Recently, Bashiruddin and colleagues developed a cyclic peptide with a high affinity for human and mouse PLXNB1, which can inhibit the SEMA4D-PLXNB1 interaction. Chemical dimerization of this peptide resulted in further increased activity and demonstrated a complete rescue of bone loss in mouse model of osteoporosis[114]. In fact, inhibition of SEMA4D-PLXNB1 interaction in osteoblasts has been shown to be a promising target for osteoporosis treatment[65]. SEMA4D expressed by osteoclasts inhibits mineralization by osteoblasts. The feasibility of pharmacologically target PLXNB1 is particularly of interest also in the tumor context and future efforts will evaluate the efficacy and safety in preclinical mouse tumor models.

3. Aims

A growing body of literature supports the idea that semaphorin signaling plays a major role in tumor onset and progression. SEMA4D, in particular, has been intensively studied and its blockade is currently under clinical trial for solid malignancies. SEMA4D signaling in tumors has been shown to affect tumor-induced angiogenesis and, due to its crucial role in the immune system, to be a master regulator of immune cell infiltration in the tumor microenvironment. However, the role of SEMA4D principal receptor PLXNB1 in the tumor microenvironment has been so far poorly characterized. Moreover, a recent work raised some concerns on the safety of targeting SEMA4D in terms of increased metastatic ability in a pancreatic cancer model due to antibody-mediated retrograde SEMA4D signaling in macrophages[111,112]. In this respect, targeting PLXNB1 could represent a novel alternative way to tackle this signaling axis.

This thesis work is aimed at understanding the role of tumor-microenvironmental PLXNB1 in the development of a metastatic TNBC murine model development and its contribution to tumor progression.

We questioned whether targeting PLXNB1 could have a significant impact in modifying stromal and immune cell recruitment to the tumor or on tumor-induced neo-angiogenesis and vessel maturation. Moreover, the final aim of this thesis work is to validate PLXNB1 as a putative molecular target for metastatic breast cancer, alone or in combination with immunomodulatory therapies.

4. Materials and Methods

4.1. Animal model

Six to eight-week-old female WT BALB/c mice were purchased from Charles River. *Plxnb1*$^{-/-}$ mice were previously generated and described[115]. 1x10^{6} 4T1 cells were orthotopically surgically implanted in the 4th mammary fat-pad under anesthesia with isoflurane 2,5%. Tumor growth was monitored by caliper measurement twice a week. Mice were sacrificed after 21 days and the primary tumor weighted. All animal procedures were approved by the Ethics Committee of the University of Torino, and by the Italian Ministry of Health, in compliance with national and international laws (D.Lgs 26/2014 and Directive 2010/63/EU respectively). Lung macro metastasis were counted about staining lungs with indian ink. Anti-CD8 and anti PD-1 antibody were administered intraperitoneally at a dosage of 200µg or 250 µg, respectively.

4.2. Tail vein extravasation assay

In vivo studies were conducted in 6-8 weeks old WT and *Plxnb1*$^{-/-}$ mice. For *in vivo* extravasation assay, 1x10^{6} RFP expressing 4T1 cells were injected into the lateral mouse tail vein. Mice were sacrificed 48 hours after injection and their lungs perfused with PAF 4%. Quantification of metastatic cells in the lungs was done by fluorescence microscopy by analyzing at least 4 microscopic fields per lung, using ImageJ software (NIH) to measure signal intensity.

4.3. Adhesion assay

Endothelial cells were plated at a density of 2,5x10^{5} in 9,6 cm^{2} wells the day before the experiment. Cancer cells were labeled with Vybrant DID cell labeling solution (Life Technologies) according to manufacturer's instructions. Labeled cells were gently detached with 1 mM EDTA and plated on the sub-confluent endothelial cell layer. Cells were let to adhere for 30 minutes at 37°C and 5% CO_2 and then washed 3 times with

PBS. Images were acquired immediately with a DMI4000 B Leica optical microscope at 10X magnification and analyzed using ImageJ software (NIH).

4.4. Gene transfer

Lentiviral-mediated gene transfer in mammalian cells was performed as previously described[116]. Non-replicating viral particles containing the targeted shRNAs (or empty vector noncoding plasmids) were produced in HEK-293T packaging cells by co-transfection with calcium phosphate precipitation method. Target cells were then incubated with the conditioned media derived from transfected 293T cells, in the presence of polybrene 8 μg/ml, for 8–12 hours. PLXNB1 silencing was accomplished with the TRCN0000078913 or TRCN0000078917 shRNA from Sigma-Mission Library.

4.5. Immunofluorescence

Tumor from 4T1 cells were dissected and collected. Tissues were fresh frozen in OCT and 10-μm-thick frozen sections were serially cut using a Leica 2135 cryostat. Dried tumor slices were fixed with Zn fixative for 10 minutes and permeabilized with Tryton 0,1% PBS solution. After saturation with 2% goat serum at room temperature for 1 hour, tumor slices were incubated overnight with optimized primary antibody dilution, washed and incubated with Alexa Fluor secondary antibody for 45 minutes. After staining with dapi, tumor slices were mounted and images acquired with SPEII DM5500 CSQ Leica confocal microscope using Leica LAS AF software. Images were analyzed using ImageJ software (NIH).

For IF stainings the following primary antibodies were used: rat anti-CD68 (1:100, Bio-Rad), goat anti-CD206 (1:100, R&D), rabbit anti-iNOS (1:50, Abcam), rat anti-Meca32 (1:100, BD Pharmigen), Rabbit anti-Ng2 (1:100, Millipore), rat anti-Gr1 (1:100, BD Pharmigen), rat anti-CD4 (1:100, eBioscience), rat anti-CD8 (1:100, eBioscience), rabbit anti-CA IX (1:100, Novus).

4.6. Flow cytometry

Mouse tumors were cut into small pieces, disaggregated with collagenase (1.5 mg/ml) and DNase (100 μg/ml) and filtered through strainers. $1x10^6$ cells were stained with specific antibodies. Flow cytometry was performed using the BD LSR Fortessa 20X and analyzed with FlowJo software. Phenotype analysis was performed with the following antibodies:

BD Horizon™ Fixable Viability Stain 780; BD Horizon™ BV786 Rat Anti-Mouse CD45; BD Horizon™ BB700 Rat Anti-Mouse Ly-6G; BD Horizon™ BV421 Rat Anti-Mouse Ly-6C; BD Pharmingen™ BV480 Rat Anti-CD11b; BD Horizon™; BD Horizon™ PE-CF594 Rat Anti-Mouse F4/80; BD Horizon™ BB700 Armenian Hamster Anti-Mouse CD3e; BD Horizon™ BB515 Rat Anti-Mouse CD8a; BD Horizon™ BV480 Rat Anti-Mouse CD4; BD Pharmingen™ APC Rat Anti-Mouse IFN-γ.

4.7. Real Time quantitative PCR analysis of gene expression

Total RNA from tumor cell lines was isolated using Rneasy Protect Mini kit (Qiagen) according to the manufacturer's instructions. Whole tumor RNA was harvested by embedding in RNAlater Tissue Storage Reagent (Sigma-Aldrich). RNA was extracted with Maxwell® RSC miRNA Tissue Kit (Cat. n°AS4500). cDNA preparation was performed according to standard procedures with High-Capacity cDNA Reverse Transcription Kit (Invitrogen).

PCR was performed by applying the following Taqman probes: CXCL1 Mm04207460_m1; CXCL9 Mm00434946; Actb Mm01205647_g1; GAPDH Mm99999915_g1.

Alternatively, PCR was conducted with SYBR Green Master Mix (Life Technologies), by applying the following primer pairs: CXCL2 FWD 5′-CACCAACCACCAGGCTACA-3′ REV 5′-GCCCTTGAGAGTGGCTATGA-3′; bACT FWD 5′-GATCTGGCACCACACCTTCT-3′ REV 5′-

GAGGCATACAGGGACAGCAC-3′; GAPDH FWD 5′-TCCACTCACGGCAAATTCAA-3′ REV 5′-TACTCAGCACCGGCCTCAC-3′. IFIT1 expression was evaluated with PrimePCR Assay from BioRad Cat#10025636.

5. Results

5.1. PLXNB1 expression in the microenvironment sustains tumor growth and it is critical for metastatic dissemination

In order to study the role of PLXNB1 in the tumor microenvironment we performed *in vivo* orthotopic tumor transplant experiments with the syngeneic murine triple negative breast cancer model 4T1, in WT and PLXNB1 deficient animals (*Plxnb1*$^{-/-}$ mice). 4T1 is a highly metastatic 6-thioguanine resistant cell line that closely mimics stage IV human breast cancer. When 4T1 cells are injected into syngeneic BALB/c mice, they give rise to highly aggressive tumors that spontaneously metastasize to the lung, liver, lymph nodes and brain[117]. For this study, 4T1 cells were orthotopically injected in the mammary fat-pad of female WT and *Plxnb1*$^{-/-}$ mice, as previously described [115].

PLXNB1 constitutive knock-out mice (kindly provided by Prof. Luca Tamagnone) are viable, fertile and do not display major developmental or angiogenesis defects[115]. Genetic ablation of *Plxnb1* did not lead to morphological or functional abnormalities, indicating that this semaphorin receptor is redundant in development, possibly due to compensatory mechanisms from PLXNB2[115].

We observed that the ability of 4T1 breast cancer cells to generate tumor masses was strongly reduced when they were transplanted in the mammary fat-pad of *Plxnb1*$^{-/-}$ mice, compared to WT controls (Fig 5).

Overtime, malignant cells in the primary tumor begin to infiltrate the surrounding parenchyma, and enter into the circulation via a process called blood vessel intravasation. Once in the circulation, these cells are referred to as disseminated tumor cells (DTCs). DTCs can travel to distant organs where they exit the circulation and extravasate into the target tissue, where they colonize the metastatic site by forming small micro-metastatic mass that eventually grows into macroscopic metastasis[118].

We sought to evaluate the impact in the metastatic process of PLXNB1 expression in the TME, and found that, over a 21 days period, tumors growing in *Plxnb1*$^{-/-}$ mice gave

rise to fewer lung macro-metastasis, compared to tumors growing in a WT environment. Interestingly, tumors grown in mutant animals displayed a significantly lower metastatic index, defined as the number of lung metastasis normalized to the primary tumor weight (Fig. 5), indicating that the reduced metastatic burden is not simply dependent on the smaller size of the primary tumor mass.

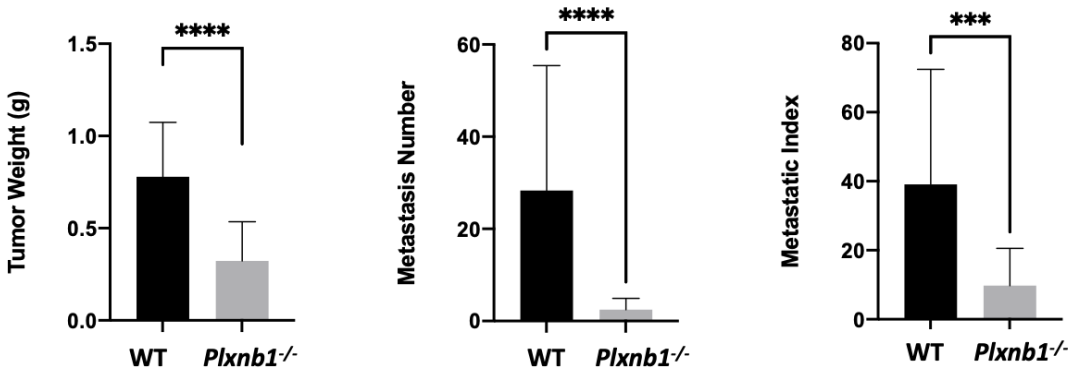

*Figure 5 PLXNB1 expression in the tumor microenvironment sustains tumor growth and it is critical for metastatic dissemination. 1x106 4T1 cells were injected in the mammary fat pad of female WT and Plxnb1$^{-/-}$ mice. Mice were sacrificed at 21 days. Boxplots indicate A) mean tumor weight ± SD. B) mean metastasis number ± SD. C) mean metastatic index (number of lung metastasis per tumor weight) ± SD. Results were analyzed by unpaired Student t test. ***, P<0,001, ****, P<0,0001 (n=23 mice per group).*

5.2. PLXNB1 deficiency does not impact on 4T1 cells extravasation

Given the strong impact of microenvironmental PLXNB1 on 4T1 cells metastatization, we sought to assess if the reduced metastatic ability was a consequence of impaired cancer cell extravasation from blood vessels into the lungs. We thus performed a short-term metastatic dissemination assay of fluorescence-labeled tumor cells. To this end, RFP-labelled 4T1 cells were injected in the lateral tail vein of WT and *Plxnb1*$^{-/-}$ mice. 48 hours later, the mice were sacrificed and the presence of RFP-positive 4T1 micro-

metastatic foci in the lungs was evaluated by fluorescence microscopy (Fig. 6). No significant differences in the RFP positive fluorescent area were observed between WT and mutant mice, indicating that the reduced ability of 4T1 cells to metastasize from primary tumors is not accounted by differences in their ability to extravasate in the lungs.

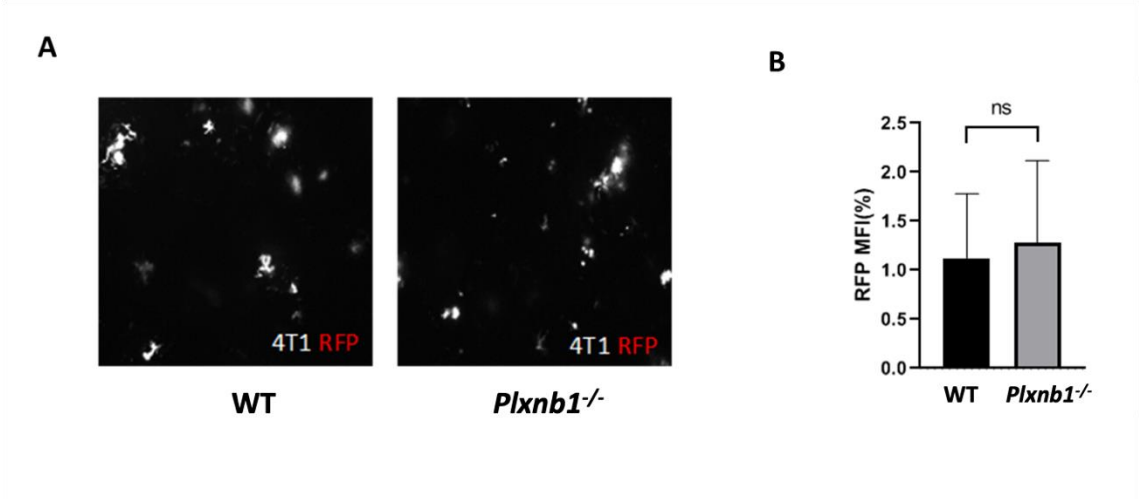

Figure 6 PLXNB1 deficiency does not affect 4T1 cells extravasation ability. 1x10^{6} RFP-labelled 4T1 cells were injected in the tail vein of WT and Plxnb1$^{-/-}$ mice. 48 hours later, mice were sacrificed and their lungs perfused with PAF 4%. A) RFP signal was assessed by fluorescence microscopy. B) Quantification of RFP signal is given as positive area percentage (%) ±SD and normalized to WT. Results were analyzed by unpaired Student t test.

5.3. PLXNB1 deficiency in endothelial cells does not affect cancer cells-endothelial cells adhesion

Previous work demonstrated that SEMA4D participates in monocyte-endothelial cell adhesion via coupling with PLXNB1 and PLXNB2 receptors[119]. 4T1 cells do express a significant amount of SEMA4D (data not shown), and PLXNB1 is highly expressed on endothelial cells[101]. We hence sought to evaluate if PLXNB1 expression by endothelial cells could impact on cancer cell ability to adhere to endothelial cell monolayers. In

order to test this hypothesis, we evaluated 4T1 cancer cell adhesion to mock-transduced and PLXNB1-silenced murine endothelial cells SVEC4-10. We observed that PLXNB1 expression on endothelial cells is not affecting 4T1 cell' ability to adhere to an endothelial cell monolayer (Fig.7), suggesting that other mechanisms are responsible for the reduced metastatic ability of tumors grown in mutant mice or that there might be alternative compensatory signaling pathways, as observed in embryo development. These data are in line with our evidence that environmental PLXNB1 deficiency is not affecting cancer cell extravasation in short term extravasation experiments.

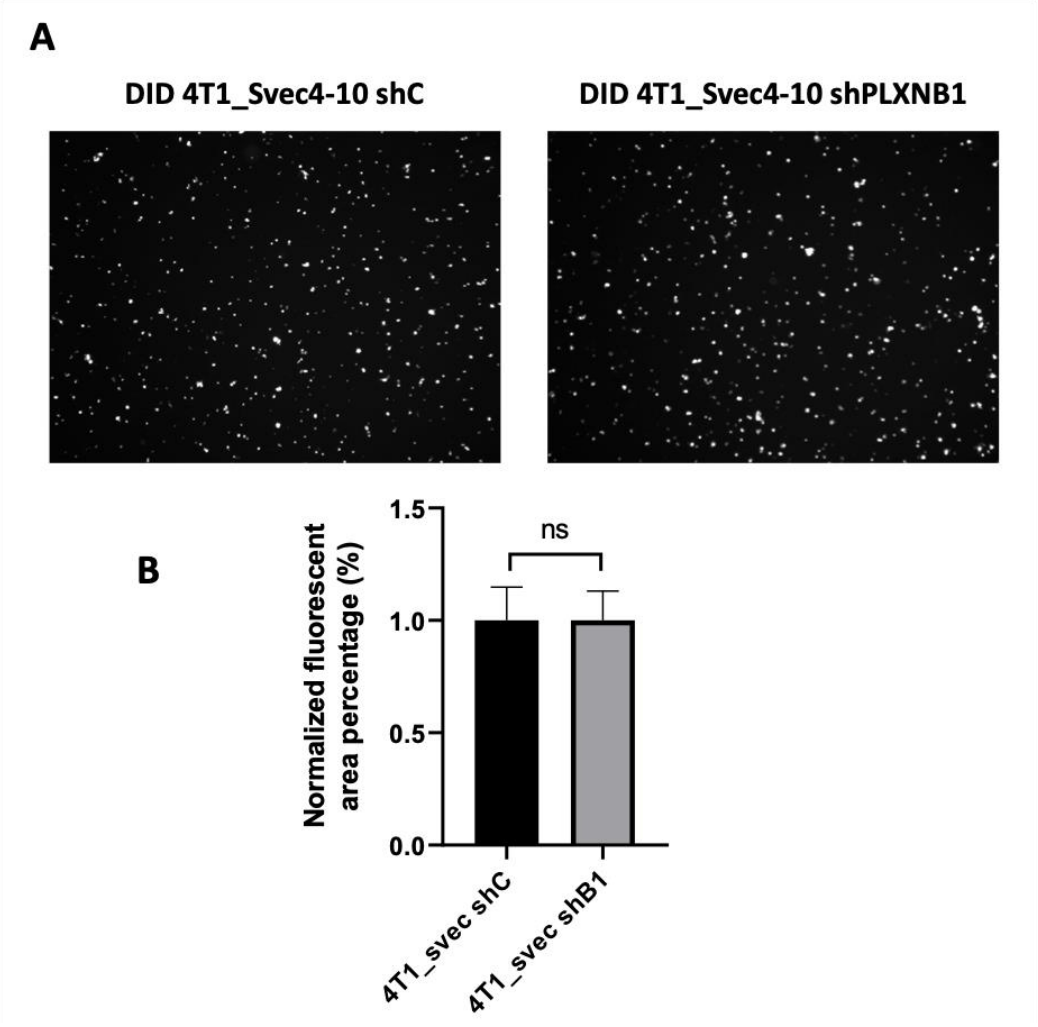

Figure 7 PLXNB1 expressed in endothelial does not affect 4T1 cell-endothelial cell adhesion. Fluorescent labeled 4T1 cells (DID 4T1) were plated onto mock or sh PLXNB1 transfected SVEC4-10 endothelial cells. Cancer cells were let to adhere for 30 minutes, washed and Representative images acquired at 10X magnification. B) Quantification of fluorescent signal is given as positive area percentage (%) ± SD and normalized to mock transfected cells. Results were analyzed by unpaired Student t test.

5.4. PLXNB1 deficiency promotes vessels' normalization

As discussed previously, PLXNB1 ligand SEMA4D is a well-recognized pro-angiogenic factor[105] and tumor induced angiogenesis was found to be impaired in *Sema4d* deficient mice[107]. We hence hypothesized that the reduced primary tumor growth and metastatic dissemination observed in *Plxnb1*$^{-/-}$ mice could be explained in part by impaired tumor angiogenesis.

In order to study vessel growth and maturation within the primary tumors, we performed immunofluorescence (IF) staining of endothelial cells in tumor sections (Fig.8). We did not observe a significant reduction in tumor vessel area of *Plxnb1*$^{-/-}$ vs. WT mice, indicating that tumor-induced angiogenesis was not reduced (Fig.8, B). Accordingly, previous studies conducted in *Plxnb1*$^{-/-}$ mice in the C57BL/6 mouse model showed that PLXNB1 expression did not affect tumor-induced neo-angiogenesis in B16 melanoma transplants[115]. These data point to the fact that SEMA4D pro-angiogenic signaling may be mediated by other receptors on endothelial cells.

Interestingly, upon co-staining tumor sections for endothelial cells and mural cells (NG2 positive pericytes) we observed a higher degree of co-localization of the two cell types in tumors grown in *Plxnb1*$^{-/-}$ mice, compared to the WT counterpart (Fig.8, C). Increased pericyte recruitment to tumor vessels indicates that *Plxnb1*$^{-/-}$ mice tumor vasculature is more mature and displays a more "normalized" phenotype. Tumor vasculature pericyte coverage is a key negative regulator of metastasis[64] and clinical studies suggested that low pericyte coverage correlates with invasive breast cancer and decreased patient survival[120]. Moreover, pericyte ablation resulted in reduced tumor growth in spite of an increased metastatic burden, increased hypoxia, epithelial-to-mesenchymal transition (EMT), and MET receptor activation[120].

Further experiments are required in order to elucidate the molecular mechanisms which underly this observation. Of note, a recent study by Wu et al., showed that SEMA4D levels are increased in a diabetic retinopathy model, leading to endothelial cell and pericyte disfunction. In particular, SEMA4D stimulation increases PLXNB1 association with mDIA1, a protein involved in angiogenesis and vascular permeability. Through mDIA1, Src, VE-cadherin, and Fak phosphorylation was increased, resulting in

weakened endothelial cell adhesion. Moreover, SEMA4D promoted pericytes migration, due to Src kinase-mediated dissociation of the N-cadherin/p120-catenin/β-catenin complex[121]. Future experiments will aim at evaluating if these molecular mechanisms can be of biological relevance also in the tumor-induced angiogenesis context, and may explain the reduced pericyte coverage observed in the PLXNB1 knockout model.

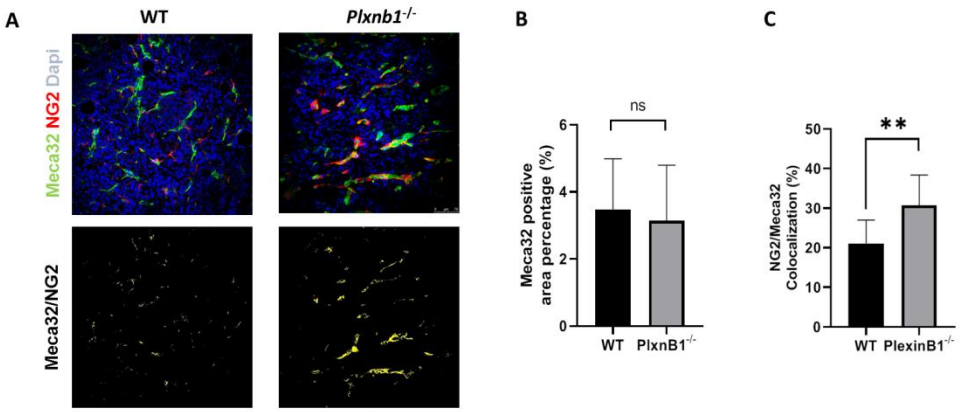

*Figure 8 Tumor-induced angiogenesis is not reduced and tumor endothelial cells of the vessels are better covered by NG2-positive mural cells in PLXNB1 deficient mice. A) Representative images of IF staining of 4T1 tumors from WT (n=9) and Plxnb1$^{-/-}$ (n=9) tumors for endothelial cells (Meca32) in green and pericytes (NG2) in red. Meca32/NG2 colocalization signal is shown in yellow. Images are representative of 5 fields per mouse. B) Vessel area is measured by Meca32 positive area percentage ±SD. C) Pericyte coverage was evaluated using Imagej "colocalization" plugin and normalized to total vessel area. Results were analyzed by unpaired Student t test *, P<0,05.*

We then wondered if this increase in vessel coverage observed in *Plxnb1*$^{-/-}$ mice was functionally relevant in terms of vascular normalization. Notably, increased pericyte recruitment seems to relieve intra-tumoral hypoxia. Indeed, IF of tumor sections, showed a significant reduction in the levels of carbonic anhydrase IX (CAIX) in tumors grown in a PLXNB1 deficient microenvironment compared to WT animals (Fig.9); this is a well-known hypoxia-inducible enzyme that has been previously associated with acidosis, invasiveness, and drug resistance[122].

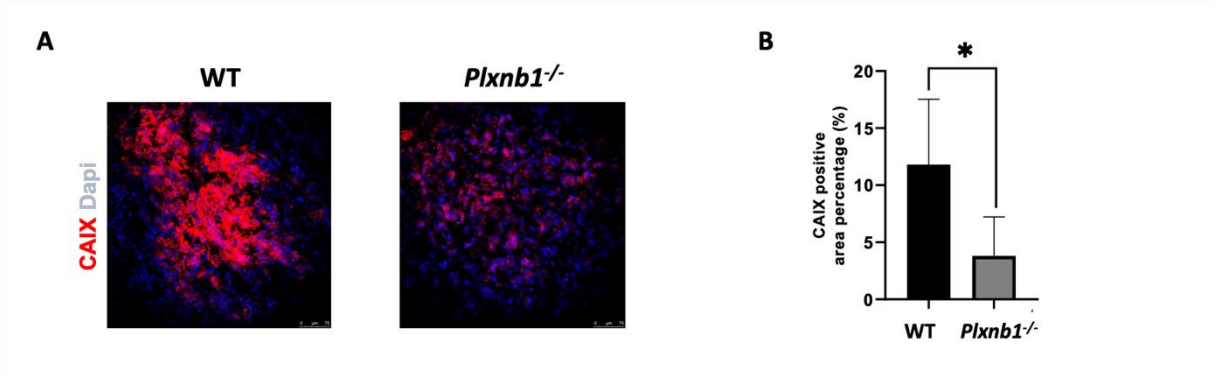

*Figure 9 Reduced levels of CAIX expression in tumors grown in a Plxnb1$^{-/-}$ deficient microenvironment. A) CAIX immunofluorescence staining of 4T1 tumors from WT (n=5) and Plxnb1$^{-/-}$ (n=5) mice. Images are representative of 5 fields per mouse. B) CAIX immunofluorescence staining quantification measured as positive area percentage (%) ± SD. Results were analyzed by unpaired Student t test. *, P<0,05*

5.5. TAM infiltration is increased in tumors grown in *Plxinb1*$^{-/-}$ mice

Antibody-mediated blockade SEMA4D can enhance activated monocytes and lymphocytes recruitment to the tumor site, thus skewing the balance of stromal cells and cytokines in the TME towards an anti-tumor, pro-inflammatory, environment[109,110]. Hence, we investigated stromal cell recruitment in the tumor microenvironment of *Plxinb*1$^{-/-}$ and WT mice, by IF staining of the infiltrating immune cell populations in tumor sections. Macrophages frequently represent the most abundant component of the leukocyte infiltrate and are able to deeply influence tumor development and metastasis. We observed an overall increase of infiltrating CD68^{+} macrophages in the microenvironment of tumors grown in a PLXNB1 deficient environment (Fig. 10 A, B) compared to those grown in WT mice. The IF results were also validated via flow cytometry analysis of whole tumor digests (Fig.10, C).

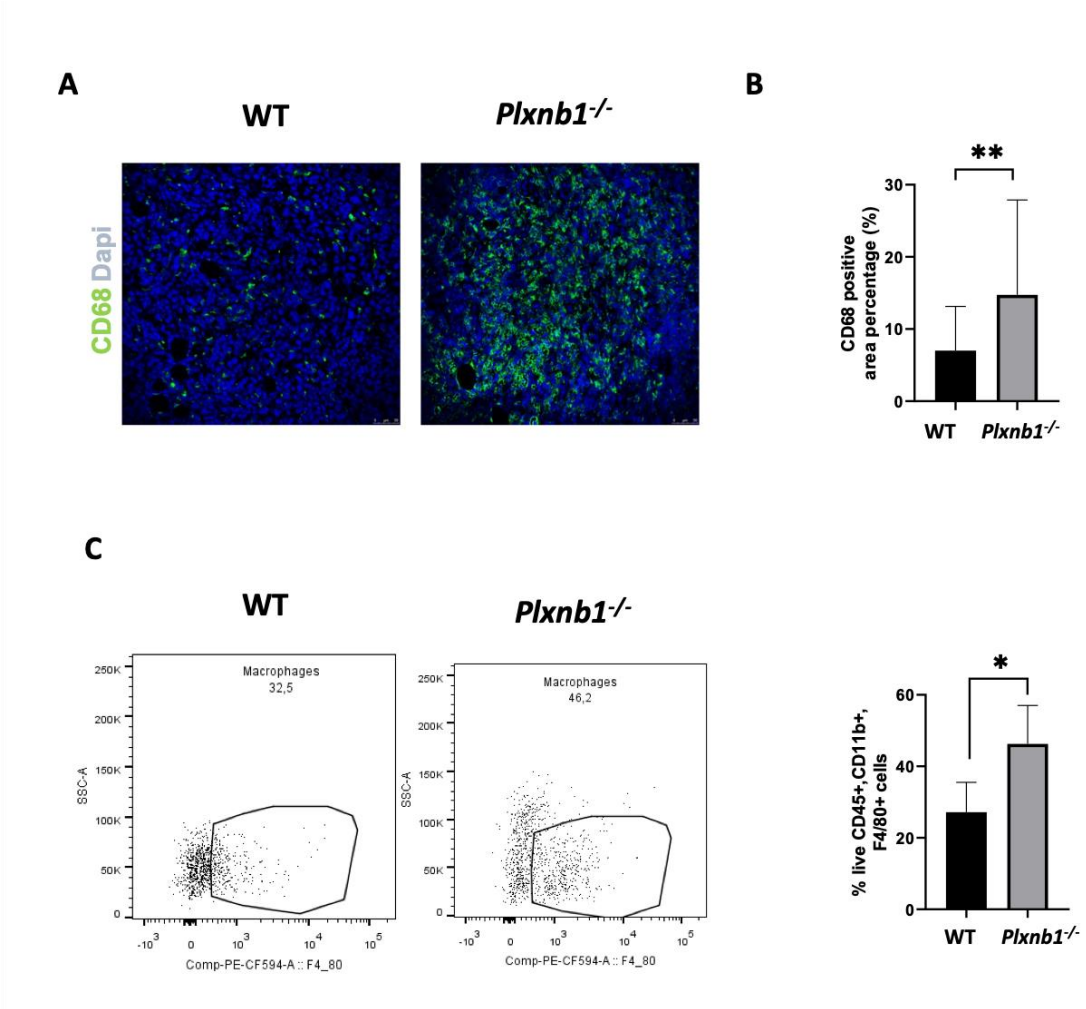

*Figure 10 PLXNB1 depletion induces higher number of infiltrating macrophages in 4T1 tumors. A) CD68 immunofluorescence staining of 4T1 tumors from WT (n=30) and Plxnb1$^{-/-}$ (n=27) mice. Images are representative of 5 fields per mouse. B) CD68 immunofluorescence staining quantification measured as positive area percentage (%). ± SD Results were analyzed by unpaired Student t test **, P<0,01. C) Whole tumor digests analyzed via flow cytometry for CD45; CD11b and F4/80 positive cells. Results were analyzed by unpaired Student t test*, P<0,05*

5.6. Switch of macrophage polarization markers towards M1 phenotype in tumors grown in *Plxnb1*$^{-/-}$ mice

High density of TAM was significantly associated with late clinical staging in patients with breast cancer[123] and TAMs are increasingly considered as a potential therapeutic

target in breast cancer[124]. However, TAMs can exist in different polarization states in response to microenvironmental cues. We investigated the pro-tumoral or anti-tumoral nature of this infiltrating macrophages by IF to assess the co-localization of the macrophage marker CD68 with classical polarization markers, such as the M1-like marker iNOS (inducible nitric oxide synthase) and the M2-like marker mannose receptor MRC1 (also known as CD206) (Fig. 11, A). We found a decrease of M2-like (pro-tumoral) macrophages and a concomitant increase of the M1-like (anti-tumoral) population (Fig. 11, B) in *Plxnb1*$^{-/-}$ mice, as indicated by co-staining of CD68 with the MRC1 and iNOS markers respectively.

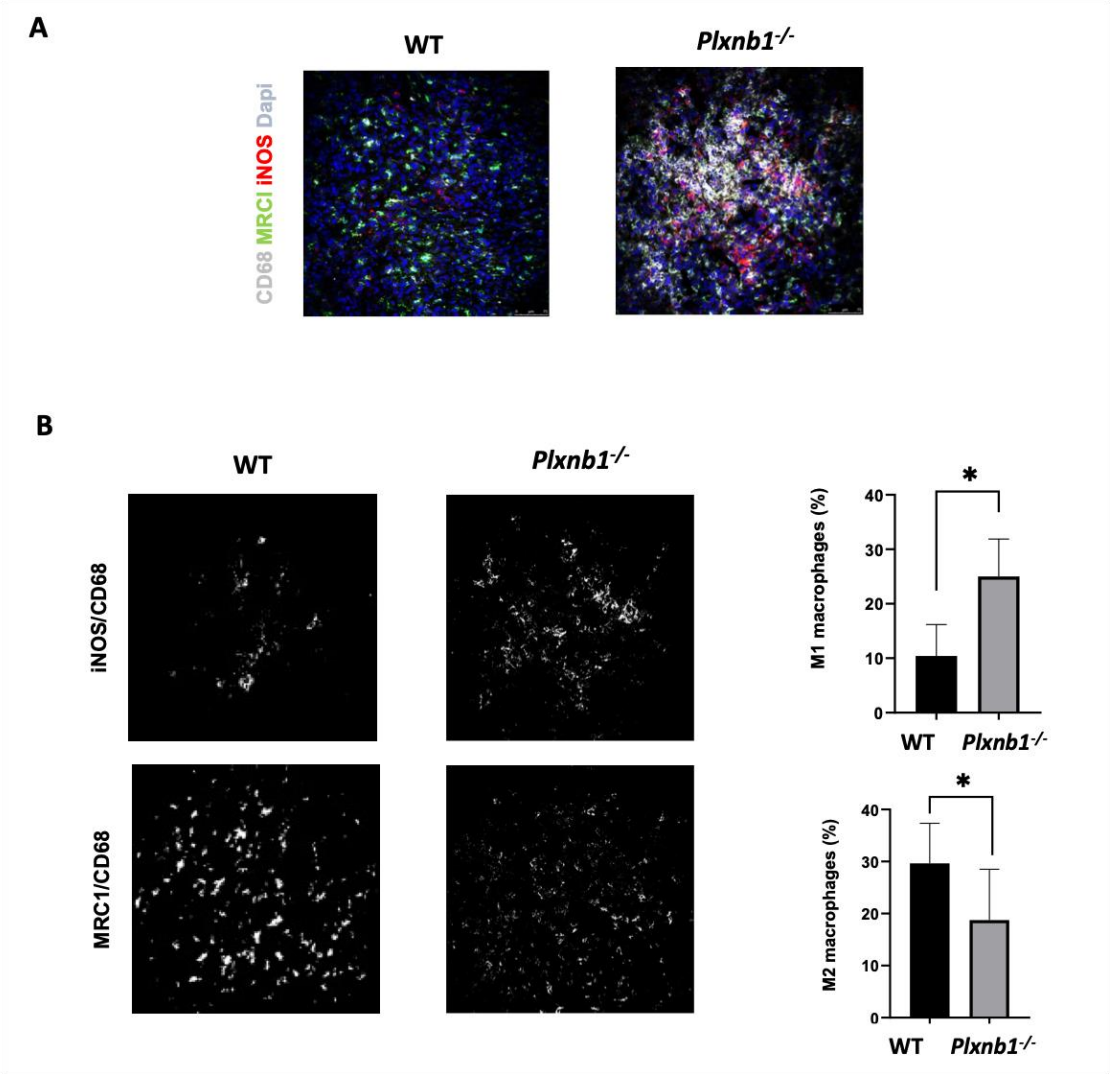

*Figure 11 PLXNB1 deficient mice have reduced percentage of M2 polarized macrophages within the TME and display an increased recruitment of macrophages belonging to the M1 pro-inflammatory type. A) CD68 (far-red), MRC1 (green) and iNOS (red) immunofluorescence staining of 4T1 tumors from WT (n=at least 4) and Plxnb1$^{-/-}$ (n=at least 4) tumors. Images are representative of 5 fields per mouse. B) Quantification of M2 and M1 polarized macrophages. M2 and M1 polarization was evaluated respectively as the percentage of green (MRC1) or red (iNOS) fluorescence signal overlapping the far-red (CD68) channel using Imagej "colocalization" plugin and normalized to total macrophage area. Results were analyzed by unpaired Student t test. *, P<0,05.*

5.7. CD11c^{+} APCs are increased in tumors grown in *Plxnb1*$^{-/-}$ mice

Previous studies demonstrated an inhibitory effect of SEMA4D on monocytic cell migration[99]. SEMA4D has been found to negatively regulate the migration of human monocytes and immature DCs via PlexinC1 and B1, respectively[125]. The same study observed a significant increase in the levels of IL-10 secretion, together with a reduction of the pro-inflammatory cytokines IL-6, IL-8 and TNF-a in response to SEMA4D. Since PLXNB1 is not expressed in macrophages[119], to better investigate the myeloid subtype mostly recruited in 4T1 tumors grown in *Plxnb1*$^{-/-}$ mice, we started to evaluate the amount of infiltrating CD11c^{+} cells, a DC marker, at the tumor site.

DCs are professional APCs and present extracellular antigens to CD4^{+} T cells on MHC-II molecules. These cells can also present extracellular antigens on MHC-I molecules to CD8^{+} lymphocytes, in a process known as cross-presentation[126]. Interestingly, we observed an increase in the number of CD11c^{+} APCs in the tumor stroma of mutant mice (Fig.11) Even though CD11c^{+} DCs were abundant in the TME of *Plxnb1*$^{-/-}$ mice compared to WT, we cannot formally exclude that other subtypes could be present and formally relevant in an environment lacking PLXNB1. Further experiments are needed to better characterize the diverse myeloid cell subtypes recruited in the absence of PLXNB1.

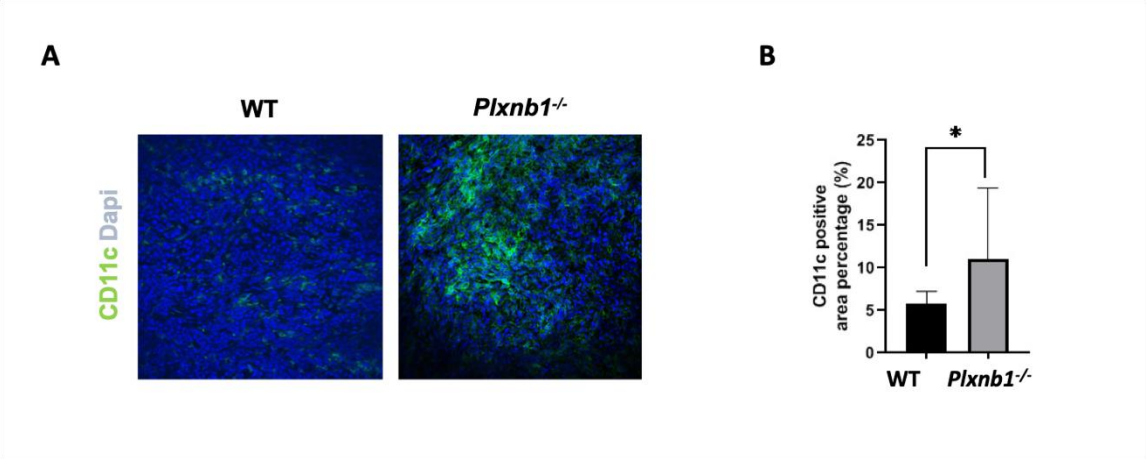

*Figure 12 Increased number of infiltrating CD11c^{+} APCs in tumors grown in Plxnb1$^{-/-}$ mice. A) CD11c immunofluorescence staining of 4T1 tumors from WT (n=12) and Plxnb1$^{-/-}$ (n=12) tumors. Images are representative of 5 fields per mouse. B) CD11c immunofluorescence staining quantification, measured as positive area percentage (%). ± SD Results were analyzed by unpaired Student t test *, P<0,05.*

5.8. Reduced MDSCs infiltration in the tumor stroma of *Plxnb1*$^{-/-}$ mice

Next, we further started investigating the role of immunosuppressive myeloid cells, myeloid derived suppressor cells (MDSC). MDSCs are a heterogeneous population of immature myeloid cells that are able to induce T cell dysfunction[127].

MDSCs express CD11b and Gr-1 markers and can be classified into two main subsets: granulocytic (polymorphonuclear) MDSCs (G-MDSCs or PMN-MDSCs) or mononuclear MDSCs (M-MDSCs). Murine G-MDSCs are defined as CD11b^{+}Ly6G^{hi}Ly6C^{lo} cells, whereas M-MDSCs are defined as CD11b^{+}Ly6C^{hi}Ly6G^{-}cells[49,51]. Interestingly, as assessed by immunofluorescence staining of tumor sections, we observed a reduced recruitment of Gr1^{+} myeloid cells in tumors grown in mutant mice compared to WT controls (Fig.13 A, B) and thus sought to better characterize this population by means of flow cytometry analysis of whole tumor digests. We found a reduced recruitment of a putative immunosuppressive, CD45^{+} CD11b^{+} F4/80^{-} Ly6G^{high} and Ly6C^{int} myeloid cell population known as

polymophonuclear-myeloid derived suppressor cells (PMN-MDSCs) in tumors grown in Plxnb1$^{-/-}$ mice (Fig.13 C, D).

Of note, we cannot exclude that part of this population is comprised by mature neutrophils on a morphology or surface marker base[128]. Future experiment will be aimed at assessing *ex vivo* their immunosuppressive ability. Myeloid derived suppressor cell and tumor associated neutrophil (TAN) recruitment to the TME is thought be mediated mainly by tumor and stromal cell release of the CXCR2 ligands CXCL1, CXCL2 and CXCL5[129,130]. On the basis of reduced MDSCs recruitment observed in tumors grown in *Plxnb1*$^{-/-}$ mice, we sought to investigate the expression levels of these chemokines within the TME of WT and knock-out mice. We found that mRNA expression of CXCL1 was strongly reduced in *Plxnb1*$^{-/-}$ mice compared to WT controls (Fig. 14), possibly explaining the reduced recruitment of the immunosuppressive population within the tumors. In addition, we observed a trend towards a reduction of CXCL2 mRNA.

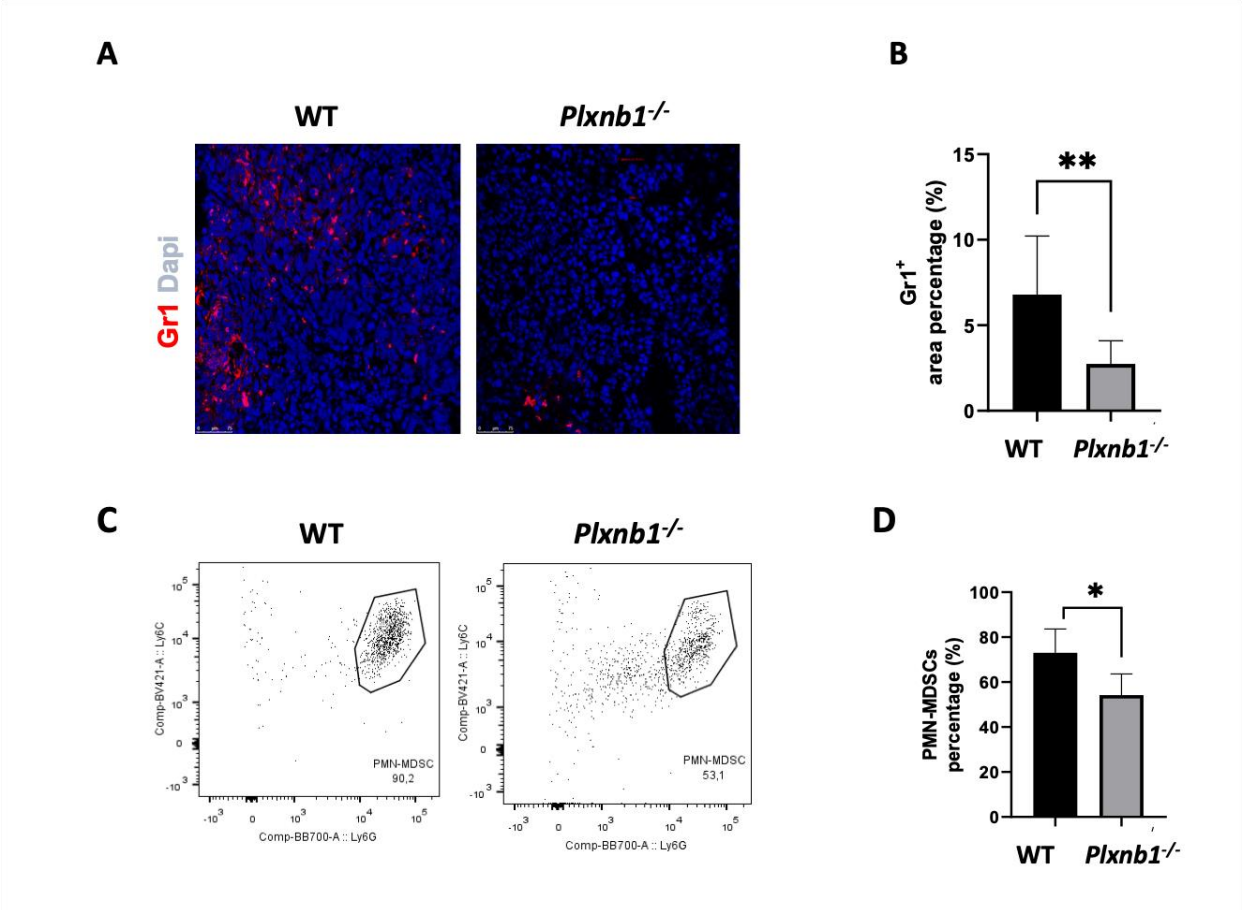

*Figure 13 Reduced number of infiltrating $Gr1^+$ myeloid cells and PMN-MDSCs in tumors grown in $Plxnb1^{-/-}$ mice. A) Gr1 immunofluorescence staining of 4T1 tumors from WT (n=9) and $Plxnb^{-/-}$ (n=8) mice. Images are representative of 5 fields per mouse. B) Gr1 immunofluorescence staining quantification measured as positive area percentage (%) ± SD. Results were analyzed by unpaired Student t test. **, P<0,01 C) Representative plots of whole tumor digests analyzed via flow cytometry for $Ly6G^{high}$ and $Ly6C^{int}$ myeloid cells infiltration (PMD-MDSC). This population was gated as $CD45^+$, $CD11b^+$, $F4/80^-$. D) Bars indicate average percentage of $Ly6G^{high}$ and $Ly6C^{int}$ myeloid cells infiltrating WT and $Plxnb1^{-/-}$mice ±SD. Results were analyzed by Mann-Whitney test. *, P<0,05 (n=4 mice per group).*

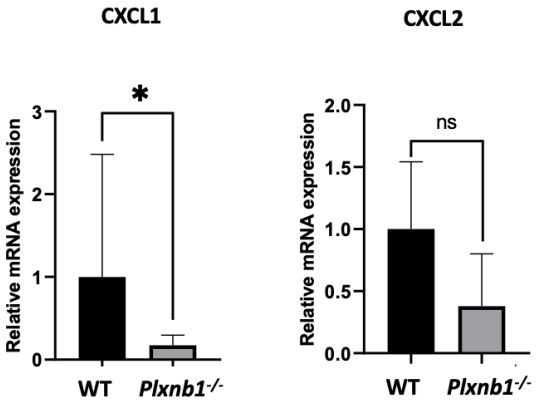

Figure 14 m-CXCL1 and m-CXCL2 expression levels are reduced in the TME of $Plxnb1^{-/-}$ mice. Graph bars represent relative mRNA expression normalized to the housekeeper gene.

5.9. TIL recruitment is increased in *Plxnb1*$^{-/-}$ mice

Myeloid cells represent the most prominent component of immune cell infiltrate within the TME. However, the presence, activation, and co-stimulation of immune system lymphoid components are required to develop a successful antitumor immune response[37]. Therefore, we investigated differential recruitment of TILs in the TME of

WT and mutant mice. Notably we observed an increased recruitment of T lymphocytes in the TME of tumors grown in *Plxnb1*$^{-/-}$ mice. Particularly important for their tumor suppressing ability, cytotoxic CD8^{+} T cells were increased within *Plxnb1*$^{-/-}$ stroma compared to WT controls, as assessed both in IF staining of tumor slices and flow cytometry analysis of whole tumor digests (Fig.15 A, C). Moreover, we also observed an increase in the number of infiltrating CD4^{+} T lymphocytes (Fig. 15 B, C) in the TME of mutant mice compared to WT controls. As previously discussed, T helper cells support cytotoxic T lymphocyte functions due to their ability to promote CD8^{+} T cell priming, resulting in the release of cytokine (such as IL-12, IL-15, and IFNγ) upregulation of costimulatory ligands, recruitment of B and naive CD8^{+} T cells and increased antigen presentation[37]. However, other CD4^{+} subsets such as Tregs have a strong immunosuppressive function, and future experiments will be aimed at characterizing the relative contribution of different CD4 T cell subtypes.

Further experiments will be aimed at understanding the molecular mechanisms regulating increased T cells infiltration to the tumor site. For instance, one possible explanation may involve PLXNB1 intrinsic GAP activity towards R-RAS[76]. Indeed, previous work showed that R-RAS signaling plays a critical role in T cells proliferation, migration, and activation[131]. Interestingly, PLXNB1 has been found expressed by activated human T lymphocytes[100] and DCs[125]. Future experiments will also be addressed at investigating R-RAS signaling in T cell mediated immune response in PLXNB1 deficient tumors.

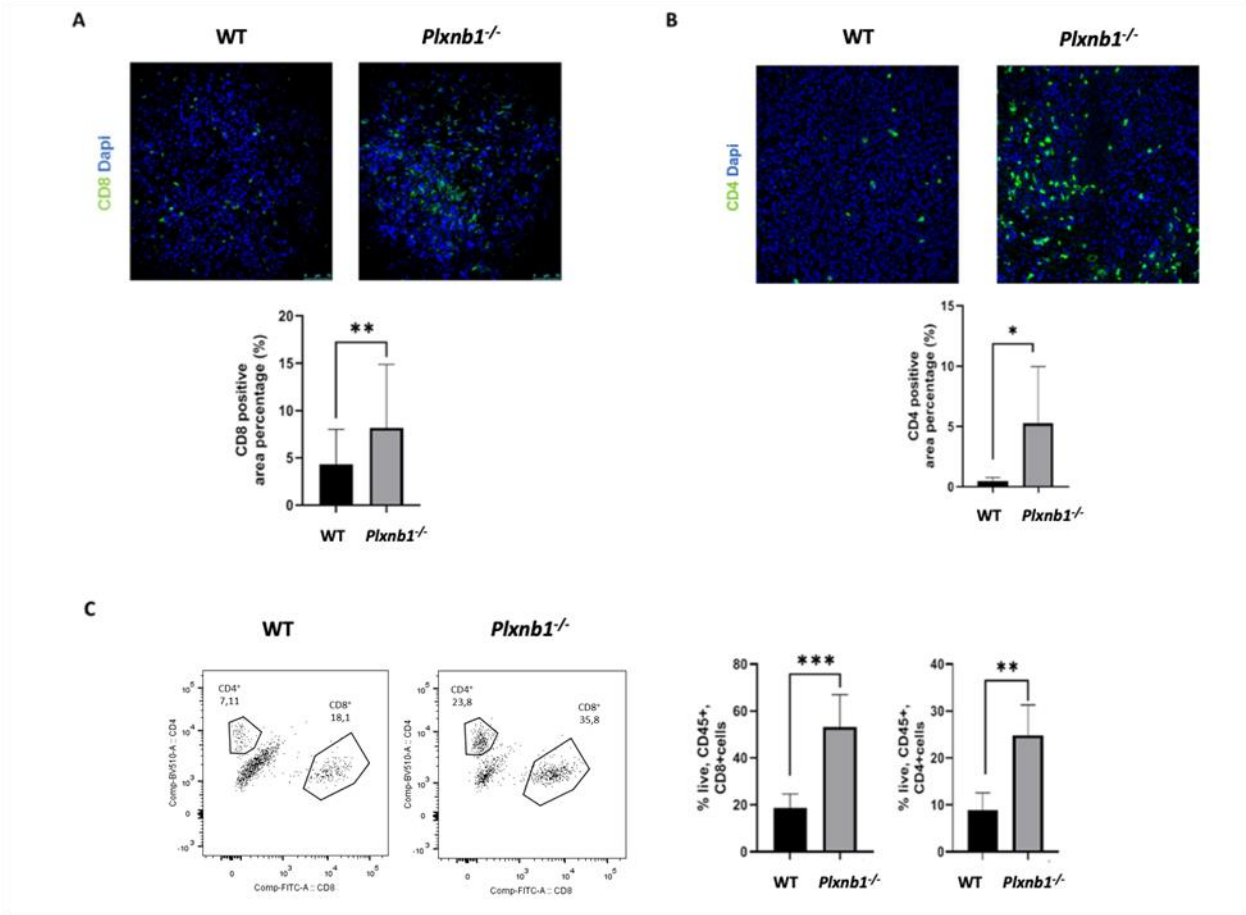

*Figure 15 Increased tumor-associated T cells in Plxnb1$^{-/-}$ mice. A) Top: CD8 immunofluorescence staining of 4T1 tumors from WT (n=30) and Plxnb1$^{-/-}$ (n=27) mice. Images are representative of 5 fields per mouse. Bottom: CD8 immunofluorescence staining quantification measured as MFI ±Sl. B) Top: CD4 immunofluorescence staining of 4T1 tumors from WT (n=6) and Plxnb1$^{-/-}$ (n=4) mice. Images are representative of 5 fields per mouse. Bottom: CD4 immunofluorescence staining quantification measured as MFI ±SD and normalized to control. C) Representative dot plots of whole tumor digests analyzed with flow cytometry for CD8 and CD4^{+} T cells and quantification. (n=at least 4). Results were analyzed by unpaired Student t test. *, P<0,05; **, P<0,01.*

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5.10. $CD8^+$ T cells recruitment is responsible for the reduced tumor growth and metastatic burden in PleixnB1 knockout mice

Given the strong impact of environmental PLXNB1 deficiency in increasing CD8 T cell recruitment we sought to investigate the selective functional role of CD8-mediated cytotoxic anti-tumor activity in *Plxnb1*$^{-/-}$ mice. To this aim we treated tumors growing in *Plxnb1*$^{-/-}$ mice and their control counterparts with anti-CD8 blocking antibodies (Fig.16, A) and found that the decrease in tumor growth observed in *Plxnb1*$^{-/-}$ mice was abrogated (Fig.16, B). Likewise, the number of lung metastases was significantly increased upon CD8 blockade (Fig.16, C). The reduction in the number of metastases between untreated WT and *Plxnb1*$^{-/-}$ mice is consistent with previous experiments; nevertheless, the difference is not significant, most probably due to the fact that we had some toxicity issues and, for this reason, we limited the experiment to 15 days. Taken together, these data suggested that $CD8^+$ T cells recruitment into tumors grown in *Plxnb1*$^{-/-}$ mice is, at least in part, responsible for the suppression of tumor growth and metastatic burden.

5.11. *Plxnb1*$^{-/-}$ TME is enriched in IFNγ and upregulates IFNγ inducible genes.

It has been shown previously that 4T1 cells ability to grow and metastasize is increased when transplanted into IFNγ$^{-/-}$ mice[132]. Moreover, increase in the expression of IFNγ-inducible molecules such as B7-H1, I-A^d, and ICAM-1 is observed when these cells are transplanted in syngeneic Balb/c mice, indicating the presence of biologically active IFNγ in the TME. Since cytotoxic $CD8^+$ T cells are among the most likely IFNγ producers, and their number is significantly increased in the TME of *Plxnb1*$^{-/-}$ mice, we looked at IFNγ production in the TME of our tumor models. By means of IF staining and flow cytometry techniques, we found that the stroma of *Plxnb1*$^{-/-}$ mice was strongly enriched in IFNγ which is, at least in part, released by $CD8^+$ T cells (Fig.17).

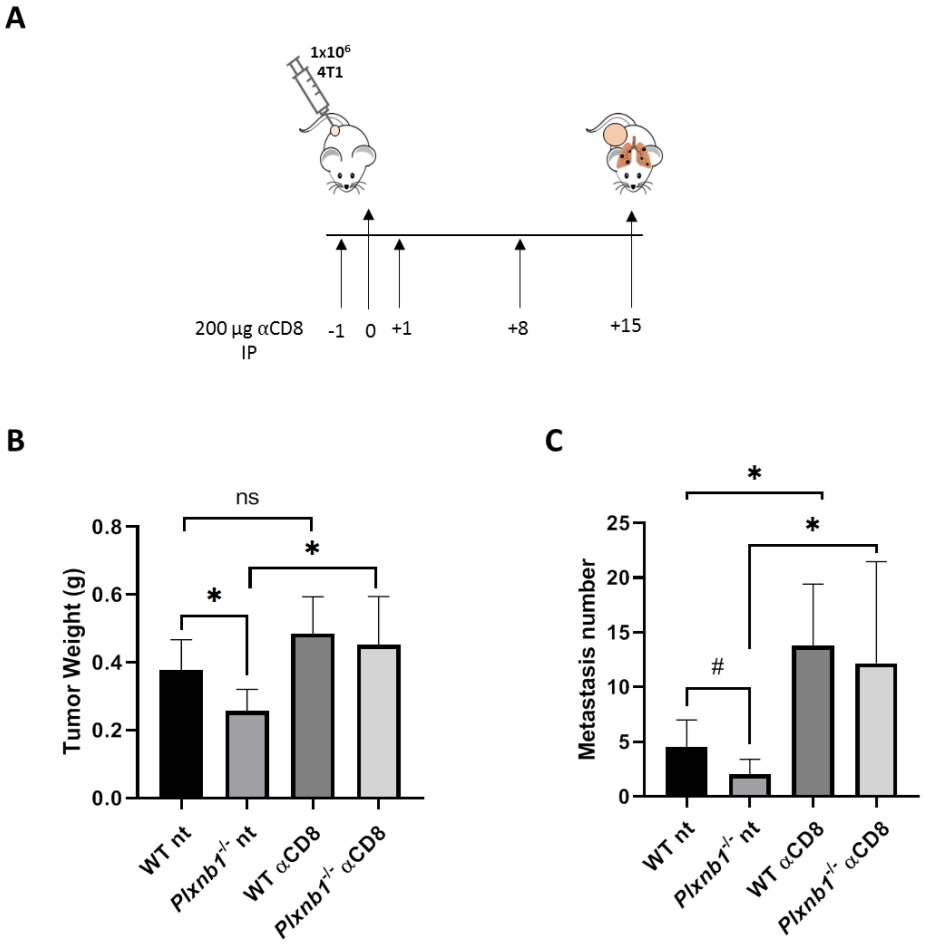

*Figure 16 CD8^{+} T cell recruitment to tumors grown in Plxnb1$^{-/-}$ mice contributes to reduce tumor growth and metastatic burden. A) Schematic representation of experimental design. 1x10^{6} 4T1 cells were injected in the mammary fat pad of female WT and Plxnb1$^{-/-}$mice at day 1. Anti-CD8 antibody treatment (200 ug IP) or saline solution as negative control (nt) was given at day -1, 0, 1 and 8. Mice were sacrificed at day 15. B) Bar graphs indicate average tumor weight ± SD. C) Bar graphs indicate average lung metastasis number ±SD. Results were analyzed by unpaired Student t test. *, P<0,05 #; P=0.07(n=6 mice per group).*

Consequently, we investigated IFNγ-induced responses in 4T1 cells *in vitro*. In fact, 4T1 cells express IFNγR1, the ligand binding subunit of the IFNγ receptor complex[133], and 4T1 cells treatment with recombinant IFNγ is able to promote the expression of IFNγ inducible molecules such as IFIT1 and CXCL9 (Fig.18, A)

IFIT1 (Interferon-induced protein with tetratricopeptide repeats 1) belong to the IFIT protein family[134]. These proteins are devoid of any known enzymatic activity but could inhibit virus replication by binding and functionally regulating cellular and viral proteins as well as RNAs. Moreover IFIT1 have been extensively used as a readable outputs for studying the transcriptional regulation of interferon-stimulated genes[134].

Chemokine (C-X-C motif) ligand 9 (CXCL9) is a chemokine whose expression is driven by IFNγ and correlates with immune cell migration and activation[135]. CXCL9, together with CXCL10 and CXCL11 are mainly secreted by monocytes, endothelial cells, fibroblasts, and cancer cells in response to IFN-γ and constitute selective ligands for the CXCR3 receptor[135,136]. CXCL9 is also known as a monokine induced by gamma interferon (MIG) and its expression is induced only by gamma-type interferon. Its major function is to mediate lymphocyte infiltration and suppression tumor growth[137]. Remarkably, expression of CXCL9 expression is more induced *in vivo* in tumors grown in PLXNB1 deficient environment, compared to WT controls (Fig.18, B), thus suggesting a biological role for IFNγ in *Plxnb1*$^{-/-}$ mice TME.

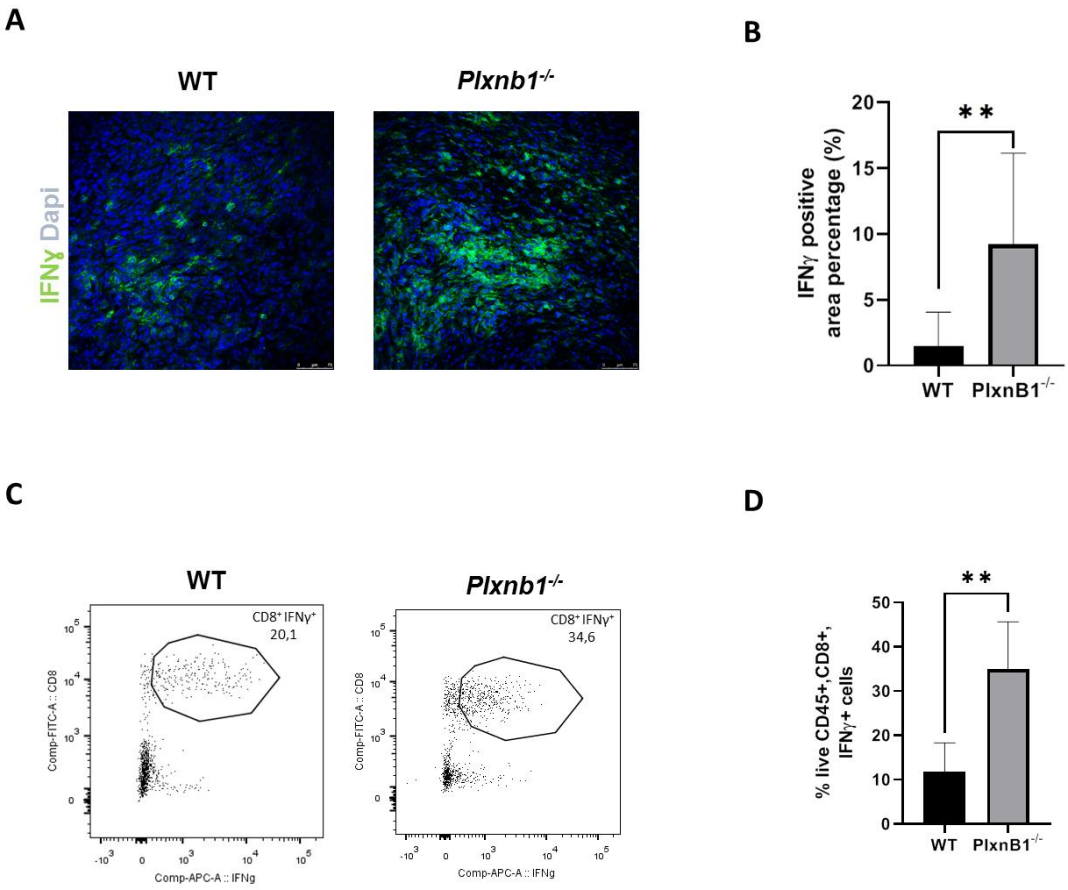

*Figure 17 Increased IFNγ in the TME of Plxnb1$^{-/-}$ mice. A) IFNγ immunofluorescence staining of 4T1 tumors from WT (n=9) and Plxnb1$^{-/-}$ (n=9) mice. Images are representative of 5 fields per mouse. B) IFNγ immunofluorescence staining quantification measured as MFI ±SD and normalized to control. C) Representative dot plots of whole tumor digests analyzed with flow cytometry for CD8^{+} T cells and IFNγ and D) Flow cytometry quantification (n=at least 4) Results were analyzed by unpaired Student t test. **, P<0,01.*

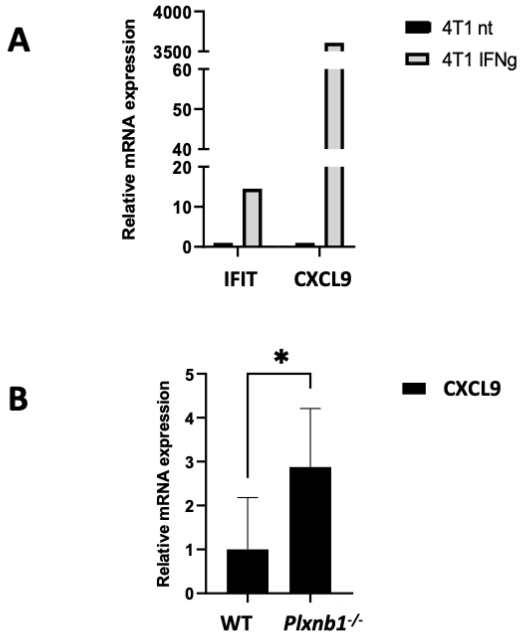

Figure 18 A) IFNγ-induced transcriptional changes in 4T1 cells. 4T1 cells have been stimulated with 200 ng IFNγ for 48 hours. Graph bar represent relative mRNA expression of IFIT1 and CXCL9 of IFNγ stimulated cells compared to controls normalized to the housekeeper gene. B) Relative expression of CXCL9 in total RNA extract from tumors from WT and PLXNB1 deficient mice (n=6). Graph bars represent relative mRNA expression normalized to WT mice.

5.12. PLXNB1 deficiency enhances anti-PD-1 immunotherapy efficacy

Increased levels of infiltrating TILs correlate with good prognosis in TNBC patients, even in the absence of adjuvant therapy, suggesting a prognostic function[14]. Even though breast cancer is not generally considered a highly infiltrated tumor, the TNBC subtype displays a high degree of immune cell infiltration and could potentially benefit from immune-checkpoint therapy. Nevertheless, monotherapy with anti-PD-1/PD-L1 only achieved mild responses [138].

Given the strong increase in infiltrating TILs observed upon PLXNB1 knockout in the TME, we sought to investigate the outcome of combining PLXNB1 targeting with the administration of anti-PD1 antibody. We found that while PD-1 blockade in 4T1 tumors engrafted in WT mice barely reduced primary tumor size, in *Plxnb1*$^{-/-}$ mice PD-1 treatment almost achieved almost complete tumor rejection (Fig.20). Indeed, at the 21 days endpoint, only 3 out of 6 mice in the treated cohort had measurable tumors. Moreover, the number of lung metastases was strongly reduced in *Plxnb1*$^{-/-}$ mice compared to controls, consistent with previous data, and PD-1 treatment could further significantly reduce metastasis number only in the mutant cohort. These results show that not only PLXNB1 targeting is a promising therapeutic approach for TNBC, but it also enhances responses to known immunomodulatory therapies.

Future trials will be aimed at confirming these results and evaluating the efficacy of combined therapeutic approaches using drugs targeting ICIs, such as PD-L1, whose expression is detectable in 4T1 cells both *in vitro* and *in vivo* (data not shown), or CTLA-4.

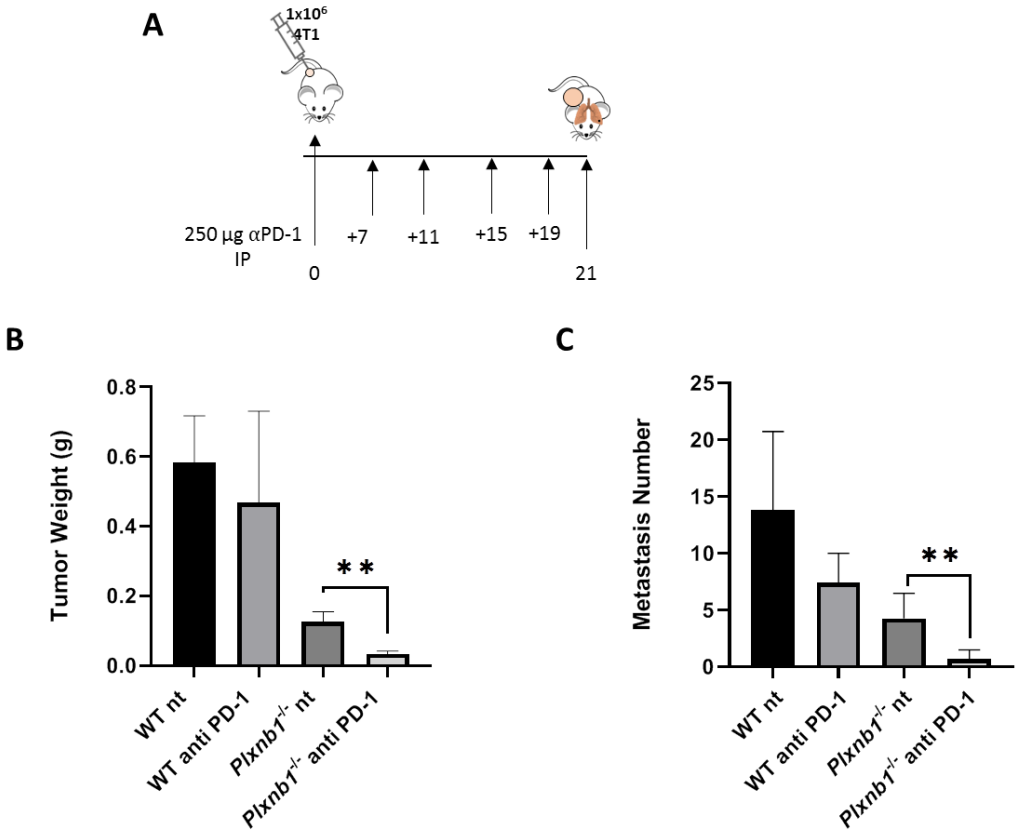

*Figure 19 A) Schematic representation of experimental design. 1x10^6 4T1 cells were injected in the mammary fat pad of female WT and Plxnb1$^{-/-}$mice at day 0. Anti-PD-1 antibody treatment (250 ug IP) or saline solution as negative control (nt) were given every 4 days starting from day 7. Mice were sacrificed at day 21. B) Bar graphs indicate tumor weight at explant ±SD C) Bar graphs indicate average lung metastasis number ± SD. Results were analyzed by unpaired Student t test. **, P<0,01 (n=6 mice per group).*

6. Discussion

The importance of semaphorin signaling in cancer onset and development is becoming increasingly clear. The immune semaphorin SEMA4D in particular has been intensively studied in this context, and its antibody-mediated blockade is currently being translated into the clinic[113]. Tumor growth depends on dynamic interactions within the tumor ecosystem and between the malignant and stromal cells, including lymphocytes and macrophages, which influence tumor growth and invasion. SEMA4D and its receptor PLXNB1 are widely expressed in murine and human tumors, and their expression has been found to correlate with invasive disease[139]. Previous work demonstrated that syngeneic breast cancer cells engrafted in SEMA4D knock-out mice undergo impaired tumor growth and vessels maturation[107]. Moreover, it was shown that antibody-mediated blockade of SEMA4D can enhance the recruitment of activated monocytes and lymphocytes into the tumor, modifying the balance of stromal cells and cytokines in the TME toward a pro-inflammatory environment [109,110]. However, the role of the SEMA4D main receptor PLXNB1 in the regulation of the tumor microenvironment has so far been poorly characterized so far.

In order to study the role of PLXNB1 in the TME we performed in vivo orthotopic tumor transplants of the BALB/c syngeneic highly metastatic 4T1 breast cancer cells in WT mice and *Plxnb1*$^{-/-}$ mice. We found that the ability of these cancer cells to grow in the mammary fat pad of *Plxnb1*$^{-/-}$ mice was strongly reduced, compared to controls; moreover, their ability to metastasize was severely impaired. This latter aspect was particularly intriguing, considering that the reduced metastatic ability was not merely a consequence of the reduced primary tumor size, as indicated by the reduction of the metastatic index in mutant mice. In order to better investigate this issue, we checked if the reduced metastatic ability seen in *Plxnb1*$^{-/-}$ mice was a consequence of impaired cancer cell extravasation. Short term extravasation assay of labeled cancer cells showed no significant differences, indicating that the reduced ability of 4T1 cells to metastasize from primary tumors is not accounted by differences in their capacity to extravasate in the lungs. PLXNB1 expression on endothelial cells surface, has been previously implicated in monocyte-endothelial cell adhesion via interaction with SEMA4D[119].

However, we found that PLXNB1 expression on endothelial cells was not accountable for 4T1 cells ability to adhere to an endothelial cell monolayer, indicating that early endothelial-cancer cell adhesion is primarily supported by other mechanisms likely to involve additional well-known adhesion and signaling molecules, e.g. selectins, integrins, cadherins, CD44 and immunoglobulin superfamily receptors[140].

Even though SEMA4D signaling has been extensively studied for its impact on the TME composition, PLXNB1 contribution in the mouse tumor TME did not gain as much attention as its ligand. Most of the known SEMA4D immune functions are mediated by its low affinity ligand CD72 which is widely expressed in the immune system[141]. Nevertheless, PLXNB1 has been demonstrated to play a role in immature dendritic cell migration[99,125]. Moreover, PLXNB1 expression on bone marrow stromal cells, follicular dendritic cells, and activated T lymphocytes has been shown to sustain B cells proliferation and survival[100].

PLXNB1 expression is also found in endothelial cells, and SEMA4D ligation induces PLXNB1 to form a complex with the MET RTK[133]

eliciting MET oligomerization and activation, and leading to an angiogenic response[105]. In vivo studies from Sierra and colleagues demonstrated that tumor angiogenesis is reduced in tumors growing in a SEMA4D-deficient environment[107]. However, in the PLXNB1 knockout model we observed that tumor-induced angiogenesis was not affected. This is consistent with previous published studies and may be accounted by the presence of alternative SEMA4D receptors (e.g. PLXNB2)[115].

Interestingly, we observed an increase in vessel pericyte coverage upon PLXNB1-loss, suggestive of normalization of the tumor vasculature. In addition, increased pericyte coverage was paralleled by a reduction in intra-tumoral hypoxia as indicated by reduced levels of CAIX. Actually, SEMA4D-PLXNB1 signaling has already been directly linked to vascular permeability alterations. In a rat model of stroke, soluble SEMA4D increased blood brain barrier permeability and endothelial cells tight junctions disruption, and the effect was abrogated by PLXNB1 silencing in pericytes [142]. Moreover, SEMA4D levels were increased also in a diabetic retinopathy model, where SEMA4D-PLXNB1 signaling induced endothelial cells and pericytes disfunction leading to vascular leakage[121].

Additional indirect evidences strengthen the link between PLXNB1 and tumor vessel normalization. As mentioned above, the intracellular domain of PLXNB1 carries intrinsic GAP activity towards R-RAS. Indeed, R-RAS knockout mice show greater angiogenic responses after arterial injury or tumor challenging[143]. Moreover, R-RAS positively regulates maturation and functional integrity of endothelial cells[144]. Future experiments will aim at deciphering the precise molecular mechanisms responsible for increased pericyte coverage and evaluating from a functional perspective PLXNB1 impact on tumor vessels leakage and perfusion.

We next sought to characterize differential immune infiltrate within tumors growing in *Plxnb1*$^{-/-}$ mice. In the first place, we observed a striking increase in the total number of CD68^{+} cells. The co-staining of CD68 with macrophage polarization markers the iNOS demonstrated an increase in the M1-polarized macrophages in mutant mice TME. This macrophage polarization switch was nicely paralleled by concomitant decrease in the percentage of M2 TAMs as indicated by co-staining of CD68 and MRC1. Macrophages are one of the most represented immune cell population in the TME and strongly impact on tumor growth and metastatic process. SEMA4D has been shown to inhibit monocyte migration[99]. However, macrophages express PLXNB2 but not PLXNB1, possibly excluding a direct impact of PLXNB1 deficiency in macrophage recruitment. Besides, PLXNB1-mediated signaling negatively influences the migration of immature DCs[125]. However, CD68 is also expressed by other mononuclear phagocyte lineages such as microglia, osteoclasts, and, more important in the tumor context by myeloid dendritic cells[145]. Notably, we observed an increase in CD11c^{+} cells recruitment in the TME of PLXNB1 deficient animals, suggesting an increased infiltration of professional APCs within mutant mice stroma.

Among other immune cells populating the TME, we observed a reduction in Gr1^{+} myeloid cells. This marker is expressed on the surface of immune-suppressive myeloid cells known as myeloid derived suppressor cell (MDSCs). MDSCs are a tumor-promoting population given their ability to promote T cell dysfunction mainly via inhibition of T cell proliferation by inducing L-arginine shortage[51]. Deeper characterization of surface markers showed that PMN-MDSCs were particularly reduced in the TME of mutant mice compared to control counterparts. Interestingly, recruitment of the same population was found to be reduced in a mouse oral carcinoma

model upon antibody mediated inhibition of SEMA4D[110]. This reduced MDSCs recruitment was explained by MAPK-dependent decreased expression of the CXCR2 ligands, CXCL1, 2 and 5 upon SEMA4D blockade in cancer cells. Of note, we observed a significant down-modulation of CXCL1 and CXCL2 levels in the TME of *Plxnb1*$^{-/-}$ mice. Even though in our model SEMA4D-PLXNB1 signaling in cancer cells was not affected, we cannot rule out that the proposed molecular mechanism is relevant in other CXCR2 ligands expressing cells. CXCL1 in particular has been shown to be expressed by TAMs and endothelial cells[146,147] and its expression in BC positively correlates with migration and invasion[148].

A crucial step in the mounting of anti-tumor immune response is the recruitment and activation of adaptive immune system cells. Characterization of the lymphoid immune infiltrate showed a strong increase in the amount of infiltrating $CD8^+$ cytotoxic and $CD4^+$ T cells in *Plxnb1*$^{-/-}$ mice TME. Again, these findings are in line with the observation of Evans and colleagues, studying the tumor infiltrate following SEMA4D blockade[109]. The precise molecular mechanism for increased T cells recruitment awaits elucidation but a number of mechanisms can be proposed. On one side, increased TIL recruitment could be an indirect effect of increased pro-inflammatory TAM or DC recruitment. Alternatively, PLXNB1 deficiency could be directly involved in regulating T cells homing. We can speculate that PLXNB1 deficiency results in increased R-RAS signaling due to PLXNB1 intrinsic GAP activity[76]. Previous work showed that R-RAS signaling plays a critical role in T cells proliferation, migration, and activation[131]. Lymph nodes are the place where the innate immune responses encounters acquired immunity and are crucial for mounting an immune response[149]. T cell trafficking in the high endothelial venules (HEVs) of lymph nodes is a fine-tuned regulated process involving dynamic interactions between T and endothelial cells. Previous work demonstrated that R-RAS deficient mice have smaller lymph nodes and immature HEVs, and these characteristics correlate with functional defects in T cells proliferation, migration, and activation[131]. Besides, Yan and colleagues showed a lower surface expression of L-selectin on $CD4^+$ and $CD8^+$ T cells from R-RAS knockout mice, together with proliferative and trafficking defects and reduced ability of R-RAS deficient T cells to bind to ICAM1 upon CCL21 stimulation. PLXNB1 has been shown to be expressed by activated human T lymphocytes[100] and DCs[125] and PLXNB1

deficiency could possibly result in increased R-RAS signaling in these cell types and explain increased T cell mediated immune response in PLXNB1 deficient tumors.

From a functional perspective, we wondered if the increase in $CD8^+$ T cells could be sufficient to explain PLXNB1 mutant mice phenotype. Upon CD8 blocking antibody treatment we found that previously observed difference in growth and metastasis between tumors grown in *Plxnb1*$^{-/-}$ and WT mice was lost, rescuing the growth and metastatic ability in WT animals. However, we observed a strong basal effect of CD8 targeting in empowering 4T1 tumor metastatic ability also in tumors grown in WT mice, suggesting that cytotoxic CD8 T cells are fundamental in maintaining disease control in the 4T1 model.

In line with the observed anti-tumor CD8 cytotoxic activity, we found that the TME of *Plxnb1*$^{-/-}$ mice is strongly enriched with IFNγ. IFNγ-producing immune cells play a critical role in the elimination of tumor cells, either by directly affecting proliferation and survival of cancer cells or increasing antigen presentation, thus generating a favorable feedback loop that ultimately sustains tumor eradication[150]. However, cancer cells can activate alternative signaling pathways to elude direct IFNγ-mediated toxicity and induce the expression of anti-inflammatory mediators in order to escape immune surveillance. 4T1 cells are responsive to IFNγ[132,151] and upregulate known IFNγ inducible genes such as IFIT1 and CXCL9. Lack of CXCL9 expression in tumors has been correlated with increased tumorigenesis, and this chemokine expression promotes lymphocyte recruitment and anti-tumor immunity[135]. Notably tumors growing in mutant mice display increased levels of this chemokine, possibly suggesting the presence of biologically active IFNγ in the TME.

The ability of tumors to evade immune surveillance is one of the hallmarks of cancer. Cancer cells display on their membrane a number of inhibitory signaling proteins that can lead to immune cell dysfunction and apoptosis. Among these molecules, PD1 and its ligand PD-L1 have been intensively studied and their antibody-mediated blockade is currently exploited in the clinic for the treatment of a variety of cancers[152]. PD-1 is expressed on T-cells, B-cells, dendritic cells and natural killer T-cells, and the binding to its PD-L1 ligand suppresses anti-cancer immunity. Therefore, anti-PD-L1 and anti-PD-1 antibodies have been used for the treatment of cancer, with notable results[153].

Finally, we found that PD-1 blocking antibody treatment in *Plxnb1*$^{-/-}$ mice prompted a strong anti-tumor response that led to disease control and, in some mice, even to tumor rejection. Moreover, anti PD-1 treatment reduced the average lung metastasis number both in WT and in *Plxnb1*$^{-/-}$ animals but in a significant manner only in the mutant cohort

Solid tumor TME is frequently defined as "cold" or "hot", based on the amount of T lymphocyte infiltration and pro-inflammatory cytokine production. Hot tumors, characterized by higher levels of pro-inflammatory cytokines and T cell infiltrate, display a better response to immunotherapy such as IC targeting the PD-L1/PD-1 axis[154,155]. Hence, turning a cold tumor into a hot one has been the focus of many recent efforts in the field. Our results suggest that targeting PLXNB1 in breast tumor TME can enhance the recruitment of T cells, turning the TME into a warmer one and increasing the outcome of anti PD-1 immunotherapy. Moreover, these findings are promising from a translational perspective and open the gates for new combination therapy with other immunomodulatory agents and standard-of-care therapies.

In conclusion, this thesis work demonstrated that PLXNB1 expression in mouse mammary tumor microenvironment strongly impacts on primary tumor growth and metastatic dissemination. The reduced primary tumor growth and metastatic dissemination is primarily due to increased infiltration in the TME of immune-suppressive populations such cytotoxic $CD8^+$ and $CD4^+$ T helper lymphocytes, and $CD11c^+$ APCs. At the same time reduced recruitment of MDSCs and a concomitant switch in the polarization status of TAMs towards a pro-inflammatory phenotype was observed. Moreover, PLXNB1 deficiency in the TME led to increased vessel normalization as indicated by increased pericyte coverage and intra-tumoral reduction of hypoxia. As result of these profound changes in TME, PLXNB1 deficiency in the TME enhanced the efficacy of anti-PD-1 immunotherapy. These results show that PLXNB1 is a promising therapeutic target for metastatic breast cancer and, in a more translational perspective, experiments with PLXNB1-blocking peptides are currently planned. Moreover, due to the strong impact of PLXNB1 deficiency in triggering anti-tumor immune response and enhancing the efficacy of anti-PD-1 immunotherapy, we envisage the combination of PLXNB1 blockade with other immunomodulatory therapies.

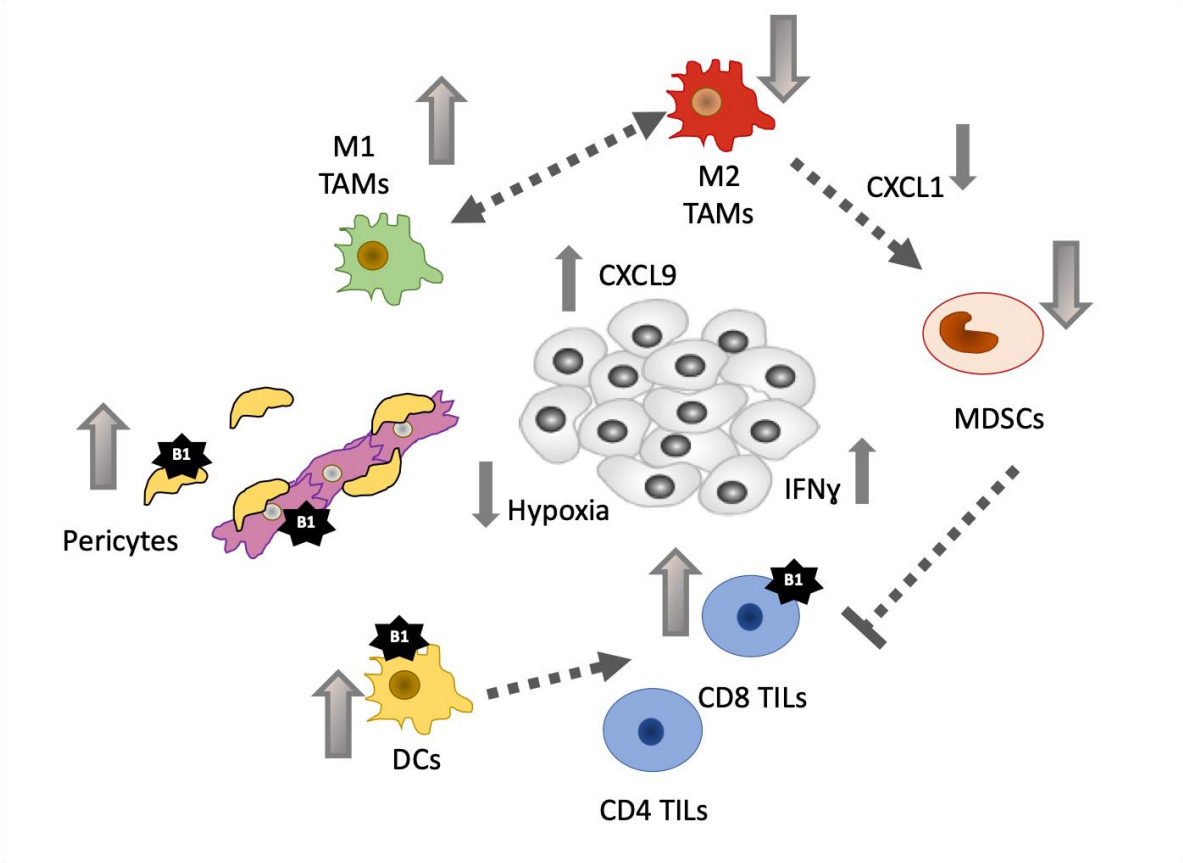

Figure 20 Graphical abstract. PLXNB1 deficiency strongly influences 4T1 syngeneic tumors TME shifting the balance towards a pro-inflammatory milieu. Increased pericytes recruitment results in vascular normalization leads to reduced intra-tumor hypoxia possibly impacting on metastasis. Moreover, dramatic changes occur in immune cell recruitment. PLXNB1 deficiency strongly increases macrophages and CD11c positive APC recruitment to the tumor and promotes macrophage polarization shift from pro-tumoral M2 to anti-tumoral M1 phenotype. Besides, TIL recruitment is strongly upregulated together with IFNγ and CXCL9 induction. On the contrary MDSC recruitment is reduced, possibly via reduction of CXCL1 levels.

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