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Year:       "[Enter print year]"

Title:      Role of the microenvironment and lymphatics in cancer development
            "[Enter subtitle]"

Author:     Eli Sihn Samdal Steinskog

Print:      University of Bergen
**Scientific environment**

The present study was carried out in The Circulation Research Group at the Department of Biomedicine, University of Bergen during the years 2012-2016. Professor Helge Wiig M.D, Ph.D. was main supervisor, Professor Olav Tenstad M.D, Ph.D and Hans Petter Eikesdal M.D, Ph.D were co-supervisors for the duration of the Ph.D work. Professor Rolf Bjerkvig Ph.D was co-supervisor from 2012-2015 and Marek Wagner Ph.D was co-supervisor from 2015-2016.

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKT</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>ATM</td>
<td>Adipose tissue macrophages</td>
</tr>
<tr>
<td>BNML</td>
<td>Brown Norwegian acute myelocytic leukemia</td>
</tr>
<tr>
<td>CAF</td>
<td>Cancer associated fibroblast</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic T-cell-associated protein</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicles</td>
</tr>
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</table>
FAB  French-American-British
FBS  Fetal Bovine Serum
FGF  Fibroblast growth factor
FoxP3  Forkhead box P3
GAGs  Glycosaminoglycans
GM-CSF  Granulocyte-macrophage colony stimulating factor
IFN-γ  Interferon-γ
IHC  Immunohistochemistry
IL  Interleukin
LC-MS/MS  Liquid chromatography-mass spectrometry
LPS  Lipopolysaccharide
LYVE-1  Lymphatic endothelium-specific hyaluronan receptor-1
M-CSF  Macrophage colony stimulating factor
MCP  Monocyte chemoattractant protein
MDSC  Myeloid-derived suppressor cell
MHC  Major histocompatibility complex
M-MDSC  Monocyte myeloid-derived suppressor cell
MMP  Matrix metalloproteinases
NOS  Nitric Oxide Synthase
NRP-2  Natriuretic peptid receptor B
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>PD</td>
<td>Programmed cell death protein</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
</tr>
<tr>
<td>PIGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>PMN-MDSC</td>
<td>Polymorphonuclear myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, normal T cell expressed and secreted</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem cell factor</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell derived factor</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumor-node-metastasis</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
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Abstract

Although cancer is one of the oldest diseases described in human history, it remains one of the most common causes of death worldwide. Cancer represents an assembly of diseases, all characterized by abnormal cell growth, invasion of neighboring tissue and metastatic capacity. These acquired abilities of malignant cells develop as a multistep process of genetic mutations. Further, tumor initiation and progression is promoted through interactions between cancer cells and their surrounding cells (stroma), an interplay often referred to as "tumor-stroma interactions".

This interplay has been the framework of all the research papers presented in this thesis; as our overall aim has been to study interactions between the tumor microenvironment and the lymphatic compartment, in solid cancers and leukemia. Additionally, we have addressed macrophages found in tumor surrounding stroma, i.e. adipose tissue, and their potential role in tumorigenesis.

High lymph vessel density is associated with increased frequency of lymph node metastases, and more negative prognosis, in several solid cancers. At the same time, lymphatic vessels are the main route of immunological response, and therefore the consequences of targeting lymphatic vessels should be carefully examined. Lymph is generated through filtration over the blood capillary wall and transports nutrients and waste products back into circulation. As the fluid interacts closely with the resident cells before entering lymphatic capillaries, the lymph naturally contains locally secreted substances, and potentially tumor antigens, from its origin. This offers an opportunity to study the secretome of a particular region or organ, which again can provide unique insight to local alterations during disease development, e.g. in cancer.

We have assessed how halted lymphatic capacity affects the tumor microenvironment, in three different mouse models. We report a reduced immunological reaction as a result of impaired lymphangiogenesis, with lower immune cell infiltrate and a weakened inflammatory response in the tumor microenvironment. Additionally, in murine breast cancer and sarcoma, we found accelerated tumor growth, while in murine melanoma, we observed less metastatic
propensity and enhanced therapeutic effect of adoptive T cell transfer. Of notice, the effect on tumor growth varied between different murine models of impaired lymphangiogenesis, and in particular, an altered macrophage profile was observed in our breast cancer model, when compared to mice with normal lymphatic vessels.

We also addressed the opposite setting; whether leukemic cell (AML) infiltrates in the spleen affected its microenvironment, and if these changes were reflected in draining lymph. We found significant alterations in both intracellular signaling patterns and proteomic profile in leukemic spleen lymph, at an early stage of AML development. Thus, we provide proof-of-concept evidence that interstitial fluid can be used to detect local changes in a microenvironment during cancer development.

The last decades have revealed an extensive heterogeneity and plasticity in the tumor microenvironment. The complexity of cancer represents a huge challenge in our search for improved therapy. Thus, it is important to increase our knowledge of the interactions within the cancer microenvironment; to target essential steps of malignant transformation, and to enhance our ability to adjust cancer treatment in a personalized manner.
List of publications

This thesis is a summary of the following papers, which is referred to by their roman numerals in the text:


**Impaired lymphatic function accelerates cancer growth.**


**Lymphatic vessels regulate immune microenvironments in human and murine melanoma.**
*J Clin Invest.* 2016 Sep 1;126(9):3389-402. doi: 10.1172/JCI79434.

III. Steinskog ES, Finne K, Enger M, Helgeland L, Iversen PO, McCormack E, Wiig H & Tenstad O

**Isolation of lymph shows activation of STAT3 and CREB pathways in the spleen during AML development in a rat model**
*Manuscript*

IV. Wagner M, Steinskog ESS, Wiig H.

**Adipose tissue macrophages: the inflammatory link between obesity and cancer?** (review)

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1. Introduction

1.1 Cancer development and progression

1.1.1 Epidemiology and characteristics of solid cancers

Cancer is the common term of an assembly of diseases, all characterized by their abnormal cell growth, ability to invade neighboring tissue and disseminate to other sites of the body (DeVita & Rosenberg, 2012; Hanahan & Weinberg, 2011). It is one of the oldest diseases described in human history, and the underlying mechanisms that maintain cancer are closely intertwined with the mechanisms that keep us alive, which make them difficult to target in an effective manner (DeVita & Rosenberg, 2012; Sudhakar, 2009). The World Health Organization (WHO) reported 8.2 million cancer related deaths and 14 million new cases of cancer in 2012, a number that is expected to rise with 70% the next two decades (Cancer, 2014). Cancer subsequently remains one of the leading causes of death worldwide, and new therapeutic strategies are therefore critically warranted.

**Hallmarks of cancer**

In 2000, Douglas Hanahan and Robert A. Weinberg published a review article in the journal *Cell* that defined later research in the field (Hanahan & Weinberg, 2000). They summarized decades of research and proposed 6 “hallmarks of cancer”; traits which define the neoplastic cell. The article quickly became a framework for cancer researchers and physicians to navigate the complexity of tumor biology. In 2011, they updated their review in *Cell*, “Hallmarks of cancer: the next generation”, providing updates and new perspectives on tumor biology from the last decade (Hanahan & Weinberg, 2011). Following the publication of the first article, research has confirmed the importance of the original hallmarks (Hanahan & Weinberg, 2011), and the characteristics of neoplastic cells can still be summarized by their ability to:

1) Sustain proliferative signaling
2) Evade growth suppressors
3) Resist cell death
4) Enable replicative immortality
5) Induce angiogenesis
6) Activate invasion and metastasis

These acquired capabilities do not only discern cancer cells from normal cells, they lay the foundation for the unrestricted and aggressive cell growth that characterizes cancer development. In addition, Hanahan and Weinberg proposed two new hallmarks that emerge as prominent characteristics of neoplastic cells; their ability to *deregulate cellular energetics* and *avoid immune destruction*, the latter being the target of immune therapy, which is the “rising star” of new therapeutic approaches in the field of cancer therapy (Mahoney, Rennert, & Freeman, 2015). They also suggested two enabling characteristics underlying these hallmarks; *genomic instability/mutations* and *tumor-promoting inflammation*.

**Figure 1.** Hallmarks of cancer, emerging hallmarks and enabling characteristics.

*Modified from (Hanahan and Weinberg, 2011, Cell)*
The metastatic process

While the primary tumor rarely threatens the patient’s life, cancer mortality is most commonly due to increasing metastatic deposits in critical inner organs. The course of metastatic spread is a process of several steps, all controlled by mechanisms not yet fully understood (Talmadge & Fidler, 2010; Valastyan & Weinberg, 2011). The initial step is the ability of cancer cells to invade neighboring tissue, followed by the detachment of small cell clusters that leave the primary tumor site. These cells proceed to intravasate blood or lymphatic vessels where they travel through the natural vasculature, before extravasating in a different site of the body. Surrounded by a new microenvironment, they form micrometastases, and reshape their milieu to promote further growth in the last step, named colonization (Steeg, 2016). Understandably, many molecular pathways are involved and altered for these events to take place, and they all represent potential targets of therapy, either as metastatic prevention or treatment of existing metastatic disease (Steeg, 2016).

1.1.2 Epidemiology and characteristics of leukemia

Leukemia is a malignant condition that originates from the hematopoietic compartment, and is characterized by massive infiltration of immature, white blood cells in the bone marrow and blood stream. Leukemia has traditionally been divided into four main categories based on morphology and cell line origin: 1) acute myeloid leukemia, 2) acute lymphoid leukemia, 3) chronic myeloid leukemia and 4) chronic lymphoid leukemia. In acute leukemia, the malignant cells are predominantly blasts (i.e. immature cells), whereas in chronic leukemia the malignant cells are more differentiated. The terms myeloid and lymphoid describe the cell lineage from which the malignant cells originate (Craig, McClelland, & Watson, 2010). Leukemia is an extremely heterogeneous condition, and the last decades have dramatically increased our knowledge about the different genetic and epigenetic changes that underlie this disease (Forthun, Hinrichs, Dowling, Bruserud, & Selheim, 2016). Today, the traditional four categories are only starting points for further sub-classification and mutational analysis, before a final diagnosis and treatment decisions are made.
According to The World Cancer Report from 2014, the incidence of leukemia worldwide was 5.6/100 000 for men and 3.9/100 000 for women. This makes leukemia a rather rare malignant condition, when compared to lung cancer with an incidence of 34.2/100 000 and 13.6/100 000 or colorectal cancer with an incidence of 20.6/100 000 and 14.3/100 000 for men and women, respectively. Still, the level of research performed on hematological malignancies is extensive, and in many cases more advanced than on solid tumors, probably in some part due to the fact that the malignant cells are so easily accessible (Chizuka et al., 2006). Accordingly, many in vitro and in vivo models have been developed to study leukemia (Kennedy & Barabe, 2008).

**Acute myeloid leukemia development, classification and treatment.**

Acute myeloid leukemia (AML) develops from cells which are arrested at an early stage of myeloid differentiation. This arrest can happen at different stages of cell maturation, and in different myeloid cell subpopulations, which explain in part the extensive cell heterogeneity seen in AML patients (Forthun et al., 2016). The arrest in differentiation is caused by genetic mutations and, as with solid cancer, additional genetic alterations are essential for AML development, e.g. increased survival, enhanced proliferation and telometric maintenance (Warner, Wang, Hope, Jin, & Dick, 2004). AML has traditionally been classified according to the French-American-British (FAB) criteria (M₀-M₇), using morphology and cytochemical methods to describe eight separate differentiation stages of leukemic blasts (Bennett et al., 1976, 1985). In 2008, WHO presented a new diagnostic system with seven categories, based both on genetic alterations in leukemic blasts and clinical and prognostic considerations (Vardiman et al., 2009). Although our knowledge on subtypes and molecular and cytogenetic changes in AML is substantial, this has not yet been translated into major therapeutic improvements, and the standard AML therapy is based on the same principles as it has been for the last decades (Dohner, Weisdorf, & Bloomfield, 2015). However, it is likely that we will see a shift in guidelines and move toward more individualized treatment protocols in the years ahead (Dohner et al., 2015).
1.2 Tumor microenvironment

1.2.1 Definition of the tumor microenvironment

The tumor microenvironment consists of the cells, structural components, interstitial fluid and vessels embedded in the tumor, or immediately surrounding the malignant cells, and can constitute more than 50% of the primary tumor tissue (Hanahan & Weinberg, 2011; Swartz & Lund, 2012; Wiig, Keskin, & Kalluri, 2010). Although the interaction between the malignant cells and their stroma is of critical importance to tumor development and progression, much is still unknown about the molecular mechanisms driving these processes or the consequences of targeting them (Balkwill, Capasso, & Hagemann, 2012). And indeed, tumor-stroma interactions have been the framework of all the papers which are presented in this thesis; i.e. the effect of an altered microenvironment on tumor development (paper I and II), whether stromal components (macrophages) support carcinogenesis (paper IV) and how malignant (leukemic) cells affect the function of a lymphoid organ (spleen) (paper III).

Although the tumor stroma comprises many of the same constituents as normal stroma, the structure and cellular density are quite dissimilar. In the following sections, the different compartments of the tumor stroma, and their role in tumor progression, will be addressed.

1.2.2 Cellular components of the tumor microenvironment

**Immune cells**

Multiple immune cell subtypes (i.e. macrophages, lymphocytes, myeloid-derived suppressor cells, dendritic cells) have been identified within the tumor stroma, with both pro- and anti-tumorigenic properties (Hanahan & Coussens, 2012; Ivanova & Orekhov, 2015).

**Lymphocytes** are present in the tumor microenvironment in different subpopulations, and their effects on tumor growth and progression are diverse. The accumulation of
cytotoxic CD8\(^+\) memory cells, CD4\(^+\) T helper 1 cells (Th1) and γδ T lymphocytes is in general associated with good prognosis, through the cytotoxic activity of these cell types and their secretion of pro-inflammation markers (Fridman, Pages, Sautes-Fridman, & Galon, 2012; Hannani et al., 2012). In contrast, high numbers of CD4\(^+\) T helper 2 cells (Th2), Th17 cells or T regulatory cells (Tregs) in the tumor microenvironment are associated with poor prognosis in many types of cancer, due to their anti-inflammatory or immunosuppressive abilities (Chaudhary & Elkord, 2016; Fridman et al., 2012). Lymphocyte subpopulations exert huge plasticity depending on their surrounding stimulus, which could explain why some studies show favorable patient outcome in the presence of “immunosuppressive” lymphocytes (Balkwill et al., 2012; Ivanova & Orekhov, 2015), whereas the above studies show the opposite.

**Myeloid-derived suppressor cells (MDSC)** are cells of myeloid origin that, as their name indicate, have immunosuppressive capability. They exist in two subtypes, monocyte-MDSC (M-MDSC) and polymorphonuclear-MDSC (PMN-MDSC) that share morphological and phenotypical characteristics with monocytes or neutrophils, respectively (Marvel & Gabrilovich, 2015). MDSC execute immunosuppression through inhibition of T-cells responses (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; Lu et al., 2011), recruitment of Tregs (Lindau, Gielen, Kroesen, Wesseling, & Adema, 2013) and by differentiating into TAMs (Narita, Wakita, Ohkur, Chamoto, & Nishimura, 2009). Additionally, they support cancer growth through stimulation of tumor angiogenesis (Tartour et al., 2011), contribution to pre-metastatic niches (Yan et al., 2010) and promotion of epithelial-to-mesenchymal transition (EMT), which facilitate dissemination of epithelial malignancies (Cui et al., 2013).

**Macrophages** are mononuclear cells derived from the myeloid lineage that are characterized by unique heterogeneity and plasticity, and they serve a variety of different functions in physiological and pathological conditions (Sica, Erreni, Allavena, & Porta, 2015). Macrophages found in the tumor microenvironment originate either from blood monocytes or from tissue-resident macrophages, and although there is accumulating evidence that they both take part in instigating a tumor-promoting local environment, there is still much to elucidate regarding the
interplay between these leukocyte subpopulations (Ostuni, Kratochvill, Murray, & Natoli, 2015; Wagner, Samdal Steinskog, & Wiig, 2015). The function and phenotype of macrophages are determined by the signals from the microenvironment that surrounds them, which trigger transcriptional programs that will skew the macrophages into different polarization states (Ostuni et al., 2015). The extremes of macrophage polarization have traditionally been classified into M1 ("classically activated") macrophages, induced by lipopolysaccharide (LPS), interferon-γ (IFNγ), tumor necrosis factor α (TNFα) or granulocyte-macrophages colony stimulating factor (GM-CSF), and M2 ("alternatively activated") macrophages, induced by interleukin-4 (IL-4), interleukin-13 (IL-13), interleukin-10 (IL-10) or transforming growth factor-β (TGFβ). M1 macrophages are known to be "pro-inflammatory" by secreting inflammatory cytokines and trigger Th1 responses, and they can also have an anti-tumor effect through their cytotoxic activity. M2 macrophages execute "anti-inflammatory" properties, induce tissue healing, stimulate angiogenesis and trigger Th2 responses (Sica et al., 2015).

Tumor-associated macrophages (TAMs) are in general thought to express a M2-like phenotype and exhibit tumor-promoting features; e.g. through secretion of mediators that stimulate tumor cell growth, promoting angiogenesis, releasing enzymes and proteases that degrade the extracellular matrix and suppressing the adaptive immune response by halting T-cell responses or recruiting T-regulatory cells (Tregs) (Kitamura, Qian, & Pollard, 2015; Ostuni et al., 2015). However, the last decade has challenged the traditional approach to macrophage nomenclature (Murray et al., 2014), and due to the heterogeneity of macrophage activation, different subpopulations have even been found within the same tumor microenvironment (Movahedi et al., 2010). Importantly, the plasticity that makes macrophages difficult to characterize, is also the feature that possibly can be exploited in the clinical setting, by attempting to repolarize them into an anti-tumor phenotype. Their abundance and well-described functions within the tumor microenvironment, clearly indicate that macrophages could be a potentially important therapeutic target in cancer treatment (Ostuni et al., 2015; X. Tang, Mo, Wang, Wei, & Xiao, 2013).
**Dendritic cells** are another immune cell type of myeloid origin which is known to influence the tumor microenvironment. In the normal setting, dendritic cells stimulate the immune system through antigen-presentation and subsequent activation of T-cell mediated immunity (M. Tang, Diao, & Cattral, 2016). However, in the tumor microenvironment, differentiation of dendritic cells is disrupted by locally secreted factors (i.e. vascular endothelial growth factor-A (VEGF-A) or signal transducer and activator of transcription 3 (STAT3)-signaling followed by an impairment of their immunogenic capability (Conejo-Garcia, Rutkowski, & Cubillos-Ruiz, 2016). The local tumor milieu can therefore suppress the antigen-presenting ability of dendritic cells (Herber et al., 2010). Further, dendritic cells can inhibit T-cell responses (Scarlett et al., 2012) and stimulate Treg accumulation (Klebanoff et al., 2013).

**Cancer associated fibroblasts (CAFs)**

Fibroblasts are another non-neoplastic cell type which executes important cancer promoting functions in the tumor microenvironment, and multiple fibroblast subtypes have been identified in the cancer stroma (H. P. Eikesdal, Kalluri R., 2011; Sugimoto, Mundel, Kieran, & Kalluri, 2006). Cancer-associated fibroblasts (CAFs) originate from surrounding tissue or bone marrow, and are recruited and activated by factors secreted from the developing tumor, e.g. fibrin, TGF-β and platelet derived growth factor (PDGF) (Hanahan & Coussens, 2012; Mezawa & Orimo, 2016; Wiig, Tenstad, Iversen, Kalluri, & Bjerkvig, 2010). Once activated, they commonly express alpha-smooth muscle actin (α-SMA) and secrete numerous cytokines and growth factors that lay a foundation for several of the hallmarks of cancer such as proliferation (Cirri & Chiarugi, 2011; Kalluri & Zeisberg, 2006), angiogenesis (Crawford et al., 2009; Rasanen & Vaheri, 2010), tissue invasion (Zhuang et al., 2015) and metastasis (Del Pozo Martin et al., 2015; X. H. Zhang et al., 2013). Recent studies have even implicated CAFs in the development of drug resistance to both conventional chemotherapy and immune therapy (Feig et al., 2013; Lotti et al., 2013). However, there are also indications that CAFs may execute tumor-suppressing abilities (Augsten, 2014; Mezawa & Orimo, 2016; Ozdemir et al., 2014). It needs to be further elucidated whether this is due to plasticity of CAFs, or due to the innate tumor-
suppressing ability of non-activated fibroblasts, in the tumor microenvironment (Mezawa & Orimo, 2016).

**Cancer stem cells**

The involvement of stem cells in cancer initiation and sustentation was proposed already a century ago (Maehle, 2011). The foundation of our knowledge on cancer stem cells (CSCs) was built through decades of leukemic research (Bonnet & Dick, 1997; J. C. Wang & Dick, 2005), and the presence of cancer cells with stem cell-like properties has been verified during the last decade in many solid tumors (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003; Visvader & Lindeman, 2008). However, the existence of CSCs is still debated (Nguyen, Vanner, Dirks, & Eaves, 2012); are they stem cells which acquired mutations and became CSCs or are they cancer cells which have become dedifferentiated and acquired stem cell-like features? Cancer cells with stem-like properties exist only in small numbers in the tumor microenvironment, and are characterized by their ability to create new tumors after inoculation into recipient mice and the capacity for self-renewal (Hanahan & Weinberg, 2011; Koren & Fuchs, 2016). Studies report their involvement in the metastatic process (Malanchi et al., 2012) as well as in EMT (Ishiwata, 2016). They also promote therapeutic resistance to chemotherapy and radiation therapy, through mechanisms like increased expression of drug efflux transporters (McIntosh, Balch, & Tiwari, 2016), upregulated aldehyde dehydrogenase activity (Marcato, Dean, Giacomantonio, & Lee, 2011), enhanced expression of anti-apoptotic proteins (Madjd et al., 2009), quiescence (Dembinski & Krauss, 2009) or augmented DNA-repair mechanisms (Bao et al., 2006). Altogether, these characteristics have led to the suggestion that cells with stem-like properties participate in cancer initiation, maintenance, metastasis and even cancer relapse (Koren & Fuchs, 2016).

**Adipocytes**

Historically, adipocytes were regarded merely as an energy repository, but research during the last few decades have deciphered their involvement in many different types of human disease (Kumar, Nayak, & Kumar, 2015). These cells vary their
secretion of adipokines (cytokines secreted from adipocytes), depending on their site and surrounding circumstances (Coelho, Oliveira, & Fernandes, 2013). Several studies have confirmed the cancer promoting potential of adipocytes, both in vitro and in vivo, through secretion of cytokines, growth factors and increased lipolysis when exposed to different tumor cell lines (Bussard, Mutkus, Stumpf, Gomez-Manzano, & Marini, 2016).

Importantly, adipose tissue which is surrounding or embedded in the tumor microenvironment, does not only consist of adipocytes, but also contain a stromal-vascular fraction (SVF), the latter being an assembly of pre-adipocytes, stem cells, fibroblasts and immune cells i.e. lymphocytes and macrophages (Han, Sun, Hwang, & Kim, 2015). These cell populations are all part of the tumor stroma, with known influence on cancer cell growth and survival.

It is well-established that obesity, which is defined as an excess of adipose tissue, is associated with cancer development (Gilbert & Slingerland, 2013; J. Park, Morley, Kim, Clegg, & Scherer, 2014). In our review (paper IV), we point to the role of adipose tissue macrophages in this process, as instigators of angiogenesis and promoters of chronic inflammation. Of notice, chronic inflammation creates a mutagenic environment and stimulates cancer progression (Pesic & Greten, 2016). It has also been shown that cells from adipose tissue, both surrounding the tumor and in other locations of the body, might be recruited to the tumor microenvironment to support cancer progression (Wagner et al., 2012; Y. Zhang et al., 2009).

1.2.3 Extracellular matrix

The structural compartment of the interstitium (the extracellular matrix) is built up of different types of collagen fibers, glycosaminoglycans (GAGs) and elastin, and the composition differs in various tissues (Wiig & Swartz, 2012). In the tumor extracellular matrix, this structural framework has some distinct characteristics when compared to healthy tissue:
Collagen

A developing solid malignancy is clinically often described as a “stiffened” mass, due to the expansion of cells, increased interstitial fluid pressure (addressed later) but also stiffening of the extracellular matrix itself (Butcher, Alliston, & Weaver, 2009). Although the amount of collagen has been shown to vary amongst different tumor types, the stiffening of the matrix can be due to increased collagen deposition and reorganization e.g. linearization and upregulated crosslinks between collagen fibers (Levental et al., 2009; Wiig & Swartz, 2012). The changes in collagen structure to a stiffer organization, have been shown to facilitate growth-factor-dependent cell migration and subsequently a more invasive tumor phenotype (Egeblad, Rasch, & Weaver, 2010). Although tumor cells migrate easier along these aligned collagen structures, the increased density of collagen may also hinder tumor cell movement. An acquired ability to induce collagen proteolysis has therefore been associated with accelerated cell invasion in different tumor cell lines (Wolf et al., 2007). In addition, cleavage of collagen has been linked to angiogenesis, associating various cancer hallmarks to collagen metabolism in malignant tumors (Seandel, Noack-Kunnmann, Zhu, Aimes, & Quigley, 2001).

Glycosaminoglycans (GAGs)

GAGs are negatively charged polysaccharide chains that not only provide a structural support to the tissue, but also add a gel-like composition to the interstitium (Wiig & Swartz, 2012). They exist in four main classes, heparan sulfate, chondroitin sulfate, keratin sulfate and hyaluronan, and are covalently bound to a protein backbone, which together is referred to as a proteoglycan. Especially hyaluronan and chondroitin sulfate have been found upregulated in the tumor extracellular matrix (Svensson et al., 2011; Toole, 2002).

Hyaluronan is capable of modulating the tumor microenvironment through both direct and indirect mechanisms (Chanmee, Ontong, & Itano, 2016). Hyaluronan accumulation has been linked to TAM recruitment and activation in preclinical studies, and lately also in human breast cancer (Tiainen et al., 2015). The binding of
myofibroblasts to CD44 (a hyaluronan surface receptor), has been shown to induce a more invasive phenotype, and increased levels of hyaluronan has therefore been linked to induction of EMT (Y. Li et al., 2011; Zoltan-Jones, Huang, Ghatak, & Toole, 2003).

**Chondroitin sulfate proteoglycans** have been associated with tumor cell migration and increased invasiveness, both in vitro and in vivo (Fthenou et al., 2009; Silver & Silver, 2014). In addition, upregulation of chondroitin sulfate proteoglycan in mammary tumors accelerated hematogenous metastases in breast cancer, through facilitated adhesion of malignant cells to platelets and endothelial cells (Cooney et al., 2011).

**Elastin**

Elastin fibers provide elasticity to different tissues in the body (e.g. skin, arteries, lungs), but the relative amount of these fibers in most tissues is small (Wiig & Swartz, 2012). Although elastin fibers normally are quite resistant to proteolysis, matrix metalloproteases (MMPs) have been known to degrade elastin during cancer development, leaving elastin peptides that again modulate cell behavior (Toupance et al., 2012). The resulting elastin peptides have been shown to enhance tumor cell growth and invasiveness in e.g. a melanoma mouse model (Devy et al., 2010) and a lung cancer cell line (Toupance et al., 2012).

**1.2.4 Interstitial fluid**

The fluid phase of the interstitium (the interstitial fluid/IF) is surrounding the parenchymal and stromal cells of a tissue, and serves as an important transport-medium for nutrients and waste products between the circulation and the tissue in which it is located. Interstitial fluid also contains locally produced substances, e.g. cytokines and proteins from the parenchyma. The concentration gradients of these signaling molecules between interstitial fluid and plasma may provide functional information that is of particular interest as we try to increase our knowledge of tumor-stroma interactions (Baronzio et al., 2012; Wiig, Tenstad, et al., 2010).
**Tumor Interstitial fluid (TIF) production**

Interstitial fluid is generated through filtration over the capillary wall, determined by forces summarized in the Starling equation, i.e. hydrostatic pressure, colloid osmotic pressure, permeability, surface area and the reflection coefficient (Wiig & Swartz, 2012). However, the tumor microenvironment influences several of these factors (Fukumura & Jain, 2007). The tumor vasculature is known to exhibit increased permeability, leading to elevated protein transport over the capillary wall and a higher colloid osmotic pressure in the interstitium (Hofmann et al., 2009). The modified driving forces of interstitial fluid generation do not only affect the volume of filtrated TIF, but also the composition of the fluid.

**TIF isolation**

To study the fluid phase of the tumor microenvironment, it is essential to use techniques that will isolate as representative interstitial fluid as possible, i.e. reduce cellular disruption, bleeding and inflammation (Jain, Martin, & Stylianopoulos, 2014). A summary of available techniques for isolating TIF is found in Table 1 (p.29). In the present work, we used the tissue centrifugation method (paper I) that previously has been found to generate reproducible results in solid tumor analysis (Haslene-Hox et al., 2011; Salnikov et al., 2006; Wiig, Aukland, & Tenstad, 2003). We also used lymph cannulation for isolating interstitial fluid in spleens that were infiltrated by leukemic (AML) blasts (paper III).

**TIF composition**

Some of the characteristics of the tumor microenvironment, and the alterations in TIF formation, are reflected in the composition of the fluid. TIF has been shown to have a reduced pH level (high H⁺, CO₂, lactic acid and low O₂, glucose), and acidity has been found to correlate with tumor mass (Jain, Shah, & Finney, 1984). This is secondary to low perfusion and high metabolic rate within the tumor, elevating the extracellular lactate level (X. Zhang, Lin, & Gillies, 2010). In addition to hypoperfusion, it is believed that tumor cells tend to use the anaerobic glycolytic pathway, known as the “Warburg effect” to generate lactate, also when adequate O₂
levels are present (Kato et al., 2013). The consequences of the resulting acidic environment are many; they inhibit T-cell function (Fischer et al., 2007), promote chronic inflammation by stimulating macrophages to produce IL-17 (Yabu et al., 2011), stimulate angiogenesis (Vegran, Boidot, Michiels, Sonveaux, & Feron, 2011) promote metastasis (Cheng et al., 2016) and even enhance resistance to chemotherapy and radiotherapy (Doherty & Cleveland, 2013; Sattler et al., 2010).

Although not confirmed yet, it is likely that TIF contains extracellular vesicles (EVs), also referred to as exosomes, microvesicles or apoptotic bodies. These are cell derived vesicles secreted by tumor and stromal cells and used for intercellular communication in both health and disease (Wagner & Wiig, 2015). Tumor-derived EVs have been shown to modulate cancer cell behavior and enhance cancer progression, and are potential therapeutic targets in cancer treatment (Antonyak & Cerione, 2014; J. E. Park et al., 2010).

Proteins are being secreted from tumor cells, as part of the tumor “secretome” i.e. molecules and proteins shed from living cells, and these tumor specific proteins have the potential of being biomarkers of the emerging tumor (Makridakis & Vlahou, 2010). Secreted proteins are found in higher concentration in TIF than in plasma, and this compartment can therefore provide unique information of tumor and stromal cell behavior during cancer pathogenesis (Wagner & Wiig, 2015). The possibility of isolating TIF, and current access to advanced proteomic technology, laid the foundation for our study of the spleen microenvironment during the progression of leukemia (paper III).

**TIF pressure**

Several independent factors contribute to an elevated interstitial pressure in tumors, e.g. irregular blood vessel structures and increased permeability, resulting in accelerated protein transport over the vascular wall and high colloid osmotic pressure in the interstitium (Jain et al., 2014). Absent or dysfunctional intratumoral lymphatics, solid pressure from growing neoplastic tissue and a stiffened ECM, are additional factors that raise TIF pressure (Ariffin, Forde, Jahangeer, Soden, &
Hinchion, 2014). The clinical consequences of the elevated TIF are many; hypoperfusion resulting in hypoxia which may induce a more aggressive tumor phenotype (Sitkovsky, Kjaergaard, Lukashev, & Ohta, 2008). Moreover, hypoxia reduces the efficacy of radiation therapy (Wilson & Hay, 2011) and uneven distribution of chemotherapy is often a consequence of both heterogeneous intratumoral perfusion and altered pressure gradients across the vessel wall (Ariffin et al., 2014; Khawar, Kim, & Kuh, 2015). Targeting the elevated TIF has therefore been suggested as an additional strategy to enhance cancer treatment efficiency (Ariffin et al., 2014; Jain et al., 2014), primarily to reduce the hypoxia within the tumor microenvironment. Whether this strategy alone will improve drug uptake and can be used clinically, or if other vascular factors are equally important in the delivery of drugs to a heterogeneous microenvironment, needs to be elucidated further (Wiig & Swartz, 2012).

<table>
<thead>
<tr>
<th>Method</th>
<th>How performed</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass capillaries</td>
<td>Insertion by blunt dissection in vivo</td>
<td>In vivo native fluid</td>
<td>Bleeding, inflammation, cellular disruption</td>
<td>High level of intracellular enzymes in isolated fluid</td>
</tr>
<tr>
<td>Implantable chambers</td>
<td>Chronically implanted</td>
<td>In vivo native fluid, continuous and repeated sampling</td>
<td>Inflammation in early phases, scar formation</td>
<td>Requires chronic restraining of animal</td>
</tr>
<tr>
<td>Implanted wicks</td>
<td>Implanted acutely or chronically</td>
<td>In vivo, native fluid</td>
<td>Bleeding, inflammation, cellular disruption</td>
<td>Chronic implantation more representative than acute</td>
</tr>
<tr>
<td>Microdialysis</td>
<td>Insertion of semipermeable membrane</td>
<td>In vivo continuous and repeated sampling</td>
<td>Inflammation, incomplete recovery, dilute fluid</td>
<td>Recovery especially low for macromolecules</td>
</tr>
<tr>
<td>Capillary ultrafiltration</td>
<td>Negative pressure applied to semipermeable membrane</td>
<td>In vivo continuous and repeated sampling</td>
<td>Inflammation, incomplete recovery</td>
<td>Recovery especially low for macromolecules</td>
</tr>
<tr>
<td>Tissue elution</td>
<td>Elution of excised tissue</td>
<td>Technically easy</td>
<td>Ex vivo single samples, dilute fluid</td>
<td>Contamination by intracellular proteins likely</td>
</tr>
</tbody>
</table>

**Table 1.** Methods for TIF isolation. Modified from (Wiig, Tenstad et al. 2010, Fibrogenesis & Tissue Repair)
1.3 Tumor blood vessels

The importance of angiogenesis in tumor development and progression has long been recognized, and substantial research on angiogenesis inhibitors has been conducted, both in the preclinical and clinical setting (Hanahan & Weinberg, 2011; Welti, Loges, Dimmeler, & Carmeliet, 2013). This has increased our knowledge on tumor angiogenesis but also revealed complex questions that still need to be elucidated, e.g. how to adapt anti-angiogenesis therapy to the diverging vascular biology found in different cancers and how to defeat therapy resistance (H. P. Eikesdal & Kalluri, 2009; Vasudev & Reynolds, 2014; Welti et al., 2013).

1.3.1 Tumor angiogenesis

Physiological angiogenesis and vascularization have been extensively studied and these processes are well characterized (Chung & Ferrara, 2011). Tumor angiogenesis share many features with steady-state non-malignant angiogenesis, but due to deregulated expression of proliferating signals, normal regulatory mechanisms are halted (Chung & Ferrara, 2011). The tumor requires neo-vascularization as it exceeds 1-2 mm in diameter (Folkman, 1971), or perhaps even at the size of 100 µm (C. Y. Li et al., 2000). Subsequently, a plethora of angiogenic factors, e.g. vascular endothelial growth factor A-D (VEGFA-D), fibroblast growth factor (FGF) and placental growth factor (PlGF), are secreted by tumor and stromal cells as a response to hypoxia and elevated metabolic demands to initiate angiogenesis (Ferrara & Adamis, 2016; Hanahan & Weinberg, 2011; Welti et al., 2013). This heterogeneity of proangiogenic stimuli in tumors is one of several reasons why targeting angiogenesis has proven difficult, as many of the developed drugs mainly have focused on VEGF/VEGFR inhibitors (H. P. Eikesdal & Kalluri, 2009).

The most studied model of tumor vascularization is the vessel sprouting model, where new vessels are formed from pre-existing capillaries. Tip cells migrate toward angiogenic signals, with subsequent stalk cell proliferation that form vascular lumens, before invasive sprouts finally fuse into new blood vessels (Ferrara, 2010; Welti et al., 2013). Tumors are also capable of generating different routes of vascularization,
e.g. vascular co-option (Donnem et al., 2013) and vascular mimicry (Qiao et al., 2015), that add further complexity to targeting angiogenesis in cancer.

1.3.2 Tumor vessel morphology

The disproportion between pro- and anti-angiogenic signals seen in tumor angiogenesis leads to morphologically altered blood vessels, often described as dilated, highly branched and hyperpermeable (Goel et al., 2011). The blood vessels inhabit morphological abnormalities at all levels of the vascular tree, generating a chaotic vascular architecture (Dudley, 2012). At the histological level, tumor endothelial cells do not form organized monolayers as in normal blood vessels (Hashizume et al., 2000), the basement lamina varies in thickness and content of collagen IV (Kalluri, 2003) and the cell junctions of both endothelial cells and pericytes are loosely connected to each other (Baluk, Hashizume, & McDonald, 2005). The leakiness of the tumor blood vessels does not only affect blood supply to the tumor, but also contributes to elevated interstitial pressure, as addressed in the previous section. The increased interstitial pressure also contributes to compression of intratumoral vessels, adding to the variable diameter of tumor blood vessels and inadequate tumor blood supply (Hida, Maishi, Sakurai, Hida, & Harashima, 2016). The altered structure and pathological properties of these vessels may cause both chronic and acute hypoxia due to the unstable perfusion, i.e. open vessels are not necessarily perfused, some vessels collapse temporarily and multi-directional blood flow may occur (Nagy, Chang, Dvorak, & Dvorak, 2009).

1.3.3 Tumor endothelial cells

Tumor endothelial cells (TECs) are heterogeneous both within the tumor and between different tumor types (Dudley, 2012; Hida et al., 2016). TECs are irregular in shape and size and often exhibit cytoplasmic projections extending across the vessel lumen. These filopodia-like sprouts are believed to originate from oxygen seeking tip cells in hypoxic areas of the tumor (Mazzone et al., 2009). There are varieties of defects in the endothelial layer; gaps (1.5 μm) extending through the vessel wall, intercellular gaps between adjacent TECs and additionally, transcellular fenestrae (0.5 μm) are all
abnormalities known to appear in tumor blood vessels (Dudley, 2012). Further, these vascular anomalies do not only cause hemorrhage and plasma leakage, but also provide openings for tumor cells to intravasate into the circulation (Dudley, 2012).

Another noteworthy feature is the heterogeneity between TECs in different tumor types. TECs from tumors with different metastatic profiles have been shown to express dissimilar characteristics regarding proliferation, motility, sensitivity to VEGF and invasiveness (Ohga et al., 2012). This indicates that an individualized targeted strategy might be required for anti-angiogenesis treatment in cancer, based on the characteristics and growth factors involved in each particular case. Finally, reprogramming tumor metabolism has the last decade been identified as an enabling hallmark of cancer (Hanahan & Weinberg, 2011), and preclinical studies indicate that TEC metabolism not only is a regulator of tumor angiogenesis, but also that targeting the metabolic shift in TECs can be exploited to impair pathological vessel sprouting (Zecchin, Borgers, & Carmeliet, 2015). Thus, there is accumulating evidence that TECs are actively influencing crucial steps of tumor development.

1.3.4 Pericytes

Pericytes are elongated cells localized in the vessel wall of capillaries and venules throughout the body. They share basement membrane with endothelial cells and provide structural stability, regenerative capacity and physiological support to endothelial cells and the microvasculature (Ribatti, Nico, & Crivellato, 2011). In the tumor vasculature, pericytes differ from their normal counterparts in both structure and function (Morikawa et al., 2002) and the degree of pericyte coverage varies between tumor types (Raza, Franklin, & Dudek, 2010). The consequence of targeting pericytes in tumor vessels has been somewhat difficult to interpret. Increased pericyte coverage has been associated with a more aggressive tumor phenotype and represents an obstacle for adequate anti-tumor immune recognition and proper drug delivery (Cao et al., 2013; Meng et al., 2015). Simultaneously, impaired pericyte coverage is correlated with increased metastasis, invasiveness and poorer overall survival (Cooke et al., 2012; Yonenaga et al., 2005). The interplay between pericytes and tumor
angiogenesis makes these cells interesting therapeutically, but since their depletion also have been associated with adverse outcome, several aspects of targeting these cells still need to be elucidated (Ribeiro & Okamoto, 2015).

1.4 Tumor lymphatic vessels

Lymphatic vessels have multiple functions in body homeostasis, e.g. transport of extravasated fluid and proteins back to the circulation, fat absorption and immune surveillance, i.e. bringing antigens from peripheral tissue to the draining lymph node, and subsequent transport of effector immune cells into the circulation (Alitalo, 2011). In tumors, malignant cells often metastasize through lymphatic vessels, and increased lymphatic vessel density has been found to be a negative prognostic marker in different types of cancer (Gao et al., 2006; Pastushenko et al., 2014; Sundov et al., 2013). Additionally, the lymphatic system contributes to the development of immune tolerance in tumors (Swartz & Lund, 2012), further underlining the importance of this compartment in tumor development and progression.

1.4.1 Tumor lymphangiogenesis

Lymphangiogenesis is the generation of new lymphatic vessels from pre-existing ones, a process that share features with angiogenesis, but the underlying molecular mechanisms are somewhat different. Lymphangiogenesis is induced by several factors, but the most prominent ones are vascular endothelial growth factor-C and D (VEGF-C/D). They bind to vascular endothelial growth factor receptor-3 (VEGFR-3) on lymphatic endothelial cells (LEC) and activate intracellular signaling pathways, e.g. extracellular signal-regulated kinase 1 and 2 (ERK1/2) and protein-kinase B (AKT), resulting in proliferation, sprouting, migration and tube-formation of LECs (Stacker et al., 2014). For effective sprouting of lymphatic capillaries, binding of the VEGF-C co-receptor neuropilin-2 (NRP-2) and the ephrin tyrosine kinase (Ephrin B2) are also required (Alitalo, 2011; Karpanen et al., 2006).
In the tumor microenvironment, both malignant cells and stromal cells (macrophages and CAFs) overexpress VEGF-C and D, and subsequently promote intra- and peritumoral lymphangiogenesis, lymph node lymphangiogenesis and enlargement of collecting lymphatic vessels (Alitalo, 2011; Dieterich & Detmar, 2016). This overexpression has been related to hypoxia and elevated levels of pro-inflammatory mediators in the tumor microenvironment (Dieterich & Detmar, 2016). Lymphangiogenesis is observed in both acute as well as chronic inflammation, where it facilitates resolution of tissue edema and accumulation of inflammatory cells and modulate the immune response (Wiig & Swartz, 2012). Inflammation is a common feature of malignant tumors, and lymphangiogenesis related to elevated inflammatory mediators has been correlated to increased lymph node metastasis in several cancer types (Liu et al., 2010; Lyons et al., 2014; Morita et al., 2012). Anti-inflammatory drugs have therefore been proposed as a mechanism to inhibit inflammatory induced tumor lymphangiogenesis (Dieterich & Detmar, 2016).

**Fig 2.** VEGF receptors in lymphangiogenesis and angiogenesis. *Modified from (Tammela et al. 2005, Cardiovascular research)*
1.4.2 Lymphatic vessels in the tumor microenvironment

**Lymphatic capillaries**

Lymphatic capillaries are thin-walled vessels that consist of one layer of lymphatic endothelium interconnected with button-like junctions and a discontinuous basement membrane (Wiig & Swartz, 2012). There are no pericytes in the vessel wall, and together these histological features enable passage of fluid and cells. These small caliber vessels are anchored with filaments to the surrounding ECM which support them mechanically (Alitalo, 2011).

LECs have been shown to secrete chemokines that modulates cellular activities favoring cancer progression; e.g. the chemokine (C-C motif) ligand 21 (CCL21) that promote cell migration towards lymphatic capillaries of C-C chemokine receptor 7 (CCR7) expressing cells (tumor cells, immune cells), or epithelial growth factor (EGF) and stromal-derived factor-1 (SDF-1/CXCL21) that promotes tumor cell proliferation and invasiveness (Kim et al., 2010; Lee, Pandey, & Popel, 2014; Shields, Kourtis, Tomei, Roberts, & Swartz, 2010). LECs have also been shown to express major histocompatibility complex (MHC) I and II and modulate T-cell responses, assigning them an active role in adaptive immunity (Card, Yu, & Swartz, 2014).

Elevated interstitial pressure is believed to cause intratumoral lymphatic vessels to collapse, and these vessels are primary believed to be non-functional (Dieterich & Detmar, 2016). In contrast, peritumoral lymphatic vessels are often seen dilated and tortuous, but this might vary between different tumor types (Dieterich & Detmar, 2016). The elevated pressure also causes an increased interstitial flow toward surrounding normal tissue in the tumor margin, promoting an elevated lymph flow from the tumor microenvironment (Swartz & Lund, 2012).

**Pre-collecting/collecting vessels**

Lymphatic capillaries drain into pre-collecting vessels, that again drain into larger collecting vessels with an increasing vessel wall diameter, now supported with a peri-
endothelial smooth muscle layer and a basement membrane (Wiig & Swartz, 2012). Larger lymph vessels are no longer permeable for fluid absorption, and active intrinsic pumping in combination with external (muscular) pressure and lymphatic valves, drives lymph flow towards lymph nodes and centrally to the blood circulation (Dieterich & Detmar, 2016). Although long considered only passive channels of lymph flow, collecting vessels are also remodeled in the presence of tumors (Stacker et al., 2014). Collecting vessels have recently been proposed to have an active role in metastatic spread through the lymphatic route, as tumor secreted VEGF-C and D has been shown to promote enlargement of collecting lymphatic vessels, and subsequent facilitation of metastatic propensity (Gogineni et al., 2013; Karnezis et al., 2012; Stacker et al., 2014).

1.4.3 Lymphatic system

In addition to the lymphatic vessels, the lymph nodes and lymphoid organs (i.e. the spleen, thymus, tonsils and adenoids) constitute the lymphatic system in the body. In the context of cancer, the lymphoid structures of particular interest are lymph nodes and spleen, and they will be described briefly in this section.

Lymph nodes

Lymph nodes are small, bean-shaped lymphoid organs located along the pathways of lymphatic vessels. This is where the innate and adaptive immune system has their first real encounter with new antigens and where systemic immune reactions towards antigens are initiated (von Andrian & Mempel, 2003). The lymphatic vessels are a common route of metastatic spread, and lymph node metastasis is such a common phenomenon that it is an integrated part of how solid cancers are staged in the TNM classification; i.e. nodal (N) status (Alitalo, 2011). Several mechanisms have been proposed for the development of lymph node metastasis; infiltrative growth by the tumor in lymphatic vessels, chemokine secretion (CCL21/CXCL21) that stimulate intravasation of tumor cells into lymphatic vasculature and increased interstitial fluid flow where tumor cells passively move into the lymphatic system (Nathanson, Shah, & Rosso, 2015; Swartz & Lund, 2012).
Recent studies also implicate lymph nodes as important sites of immune modulation, as LECs in draining lymph nodes have been found to suppress both CD8 T cells (Lund et al., 2012) and CD4 T cells functions (Dubrot et al., 2014). The well-established association between tumor lymphangiogenesis, lymphatic metastasis and worse overall survival for several types of cancer, has pointed towards lymphatic vessels as an attractive therapeutic target in cancer (Alitalo, 2011). Moreover, targeting the immune modulation within the lymphatic system therapeutically, for instance by blocking the interaction between antigen presenting cells (APCs) and T-lymphocytes with CTLA-4 antibodies, improves survival in patients with metastatic melanoma (Hodi et al., 2010). This indicates that additional anti-tumor effects can be achieved beyond targeting the intravasation of tumor cells in peritumoral lymphatics per se (Dieterich & Detmar, 2016).

**Spleen**

The spleen is the largest lymphoid organ in the body and has a variety of functions; e.g. fluid volume regulation, removal of damaged erythrocytes, recycling of iron, involvement in immune reactions (Kaufman & Deng, 1993; Mebius & Kraal, 2005), modulation of systemic inflammation (Semaeva et al., 2010) and contribution to extramedullary hematopoiesis (Kiel & Morrison, 2008). In leukemic conditions, the spleen and liver are known to enlarge substantially as a result of leukemic cell infiltration (Lowenberg, Downing, & Burnett, 1999). It is likely that this infiltration elicits interactions between the malignant cells and the spleen parenchyma, as seen in the microenvironment of solid tumors, especially considering the presence of abundant immune cells and the active role in inflammation previously described in the spleen.

### 1.5 Immune therapy

Based on the profound influence of impaired lymphangiogenesis on the immunological response that was observed in paper I-II, some key points in tumor
immune response and potential targets of therapeutic immune modulation, will be addressed hereunder.

Immune therapy in cancer treatment is an emerging field, and the last decade has shown that by utilizing the host immune system in a targeted manner, it is possible to improve overall survival in various cancers subtypes (Palucka & Coussens, 2016). Immune cell infiltrates are found in all tumor stroma, but to a different extent. And although they are commonly “edited” to support the tumor microenvironment (Cavallo, De Giovanni, Nanni, Forni, & Lollini, 2011), recent studies have shown that the presence of abundant tumor lymphocyte aggregates are associated with improved response to immune check-point blockade (Topalian, Drake, & Pardoll, 2015). Thus, the presence of immune cells in the tumor microenvironment, and the consequences of targeting them, need to be assessed carefully.

1.5.1 The myeloid compartment

As discussed in section 1.2.2, myeloid immune cells (e.g. macrophages, dendritic cells, MDSC) have been associated with many tumor promoting abilities and the cells display substantial plasticity depending on environmental factors (Palucka & Coussens, 2016). Therapeutic approaches linked to this compartment have primarily focused on macrophages and evolve around two main strategies;

1) Deplete macrophages at the tumor site, by inhibiting recruitment or suppress survival (X. Tang et al., 2013).
2) Modulate macrophage behavior by enhancing tumoricidal activity or block tumor-promoting ability (X. Tang et al., 2013).

Several pre-clinical studies have provided promising results and clinical trials are ongoing, either as monotherapy or in combination with other therapeutic agents (Palucka & Coussens, 2016; X. Tang et al., 2013).

1.5.2 The lymphoid compartment

However, the greatest success in cancer immunotherapy so far is based on T-cell targeted approaches. Again, there are different strategies;
1) Blocking the inhibitory co-receptors cytotoxic-T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) on T-cells to enhance their natural cytotoxic abilities (Palucka & Coussens, 2016). Antibodies against these proteins have already been approved in the treatment of melanoma, non-small cell lung cancer and kidney cancer (Topalian, Taube, Anders, & Pardoll, 2016).

2) In contrast to checkpoint blockade, that instigates T cell responses in general, adoptive T cell transfer aims toward more individualized responses. This treatment is based on harvesting T cells from patients, expanding and stimulating them in vitro before transferring them back into circulation, a strategy that might improve survival in certain patients (Perica, Varela, Oelke, & Schneck, 2015). Adoptive T cell transfer is especially effective if the patient undergoes depletion of other immune cells by chemo- and radiation therapy before the modified T cells are re-injected (Perica et al., 2015).

3) The last branch in T cell targeted therapy is cancer vaccines aimed towards tumor-specific antigens. Vaccines have so far been most successful in premalignant conditions, i.e. related to human papilloma virus (HPV) infection (Basu, Banerjee, Singh, Bhattacharya, & Biswas, 2013), and have not proven as effective in established malignancies. This is probably due to difficulties in selecting the proper antigen, and the already established immune evasion in the tumor microenvironment (van der Burg, Arens, Ossendorp, van Hall, & Melief, 2016).
2. Aims of study

The synergy between neoplastic cells and their surroundings (stroma) is of crucial importance for tumor growth and progression. The overall aim of this project was to study interactions between the lymphatic compartment and tumor microenvironment, in solid tumors and leukemia. We therefore examined the role of the tumor microenvironment in three different contexts thus resulting in the following specific aims:

1. To study how an impaired ability to induce lymphangiogenesis affects primary tumor growth, tumor interstitial fluid pressure, tumor vasculature, lymphatic drainage and tumor immune response in a murine breast cancer and sarcoma model (paper I).
2. To study the influence of lymphatic vessels on tumor-associated inflammation and immune response in a melanoma mouse model where the mice lack dermal lymphatic vessels (paper II).
3. To study changes in the spleen microenvironment as reflected in draining lymph during development of acute myeloid leukemia in the BNML rat model (paper III).
4. To give an overview of existing literature linking excess adipose tissue (obesity), within and surrounding the tumor stroma, to carcinogenesis (paper IV, review).
3. Methods

This section lists the main methods used in paper I-III. A more detailed description and protocols can be found in Materials and Methods in the respective papers. Methodological considerations will also be addressed in the Discussion.

3.1 Animal models

The use of animal models has been essential for the work presented in this thesis.

Paper I:

To study tumor development in a microenvironment where lymphangiogenesis is halted, we used the Chy mouse model, where the mice have a heterozygous mutation in the gene coding for VEGFR3. This genetic alteration, results in reduced number of lymphatics in the dermis but not in the visceral organs. Chy mice can phenotypically be identified by transient chylous ascites shortly after birth and persistent lymphedema in the extremities (Karkkainen et al., 2001). We used female Chy mice kept on a C3H strain background and wt littermates (8-16 weeks) for breast cancer injection in the mammary fat pad, while mice of both sexes (8-16 weeks) where used to implant sarcoma in the back skin.

Paper II:

We used K14-VEGFR3-Ig mice to study lymphatic influence on tumor-associated inflammation and tumor immune response (Makinen et al., 2001). These mice lack dermal lymphatics due to a fusion protein of the extracellular domain of VEGFR-3 and IgG, that binds and inactivates VEGF-C and VEGF-D, and subsequently inhibits lymphangiogenesis. They can, as for Chy mice, be phenotypically identified by edema in their extremities. The K14-VEGFR3-Ig mice were kept on a C57Bl/6 background, and K14-VEGFR3-Ig mice and their wt littermates (10-12 weeks) of both sexes were used for tumor implantation. In this project we also studied tumor immunity in an environment where immunological response have been shown to be
impaired and therefore implanted breast cancers subcutaneously on the ears of Chy mice and their wt littermates (8-10 weeks).

**Paper III:**

To study the spleen microenvironment during leukemic development, we used the BNML rat model of acute myeloid leukemia (Iversen, Leukemia 2002). Female Brown Norwegian rats were injected in the tail vein with BNML spleen cells, 10-18 days prior to experiments, to induce AML.

### 3.2 Cell lines

Two murine syngeneic cancer cell lines; breast cancer (C3HBA) and sarcoma (KHT-1) were used for orthotopic tumor implantation in Chy mice and wt littermates in **paper I**. In **paper II**, B16F10 and B16F10.OVA murine melanoma cells, were injected subcutaneously in K14-VEGFR3-Ig mice and wt littermates. Additionally, C3HBA breast cancer cells were implanted as ear tumors in Chy mice and wt littermates in **paper II**. All cell lines were maintained in culture and propagated at 37°C in 5% CO₂, in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma), supplemented with non-essential amino acids, 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml of streptomycin and 400 µM L-glutamine (all products: Lonza). In **paper III**, BNML spleen cells were injected i.v. into the tail vein of Brown Norwegian rats to induce AML. The BNML cells were kept in liquid nitrogen in 50% FBS, 40% DMEM, 10% DMSO until 1 hour prior to i.v. injection.

### 3.3 Immunohistochemistry

Immunohistochemistry is a semi-quantitative method that we used to identify vessels, extracellular matrix components and immune cells in PFA-fixed tissue in paraffin sections in **paper I** and **II**. To visualize blood and lymphatic vessels, we stained with antibodies for CD31/PECAM1 and LYVE-1, respectively. Immune cells were identified and quantified with antibodies against leukocytes (CD45), antigen-presenting cells (MHC II), macrophages (F4/80, CD11b, CD163), T-cells (CD3), T-
reg (FoxP3) and B-cells (CD20). Collagen fibers were visualized using Picosirius Red staining, and cancer-associated fibroblasts were quantified with an antibody against α-SMA.

3.4 Isolation of interstitial fluid

**Centrifugation method**

In **paper I**, we isolated interstitial fluid from murine mammary tumors and sarcomas, utilizing the centrifugation method developed in our group (Wiig et al., 2003). Tumor tissue was placed on nylon mesh (pore size 15-22 µm), followed by centrifugation in an Eppendorf tube at 106 g for 10 minutes. The fluid extracted from the tissue was sampled and frozen at -20 °C for later analysis.

**Lymph vessel cannulation**

Initial lymph is considered representative of interstitial fluid, from an organ during steady state conditions, when sampled directly from the organ (Wiig & Swartz, 2012). To study the microenvironment of the spleen during leukemic development in **paper III**, we harvested lymph from a vessel as close to the organ as possible. In all the BN rats, we identified several small efferent lymphatic vessels that converged in a small splenic lymph node located in a cluster of 2-3 lymph nodes, where one of these nodes always was most prominent. When examined in the proximal direction, it became evident that the vessel draining to the prominent lymph node, came directly from the liver. Since the lymph node cluster most proximal to the spleen also received lymph from the liver, we ligated the afferent lymph vessel from the liver, before carefully dissecting the efferent splenic lymph vessel for cannulation.
3.5 Quantification of lymphatic transport

*Transport of lymphatic fluid*

In *paper I* we measured lymphatic drainage of fluid by measuring the washout of Alexa 680-conjugated albumin from the peritumoral area with optical imaging (Karlsen, McCormack, Mujic, Tenstad, & Wiig, 2012).

In *paper II*, transport of fluid by the lymphatics was measured by quantification of FITC dextran in draining lymph nodes, subsequent to intratumoral injection (Bollinger, Jager, Sgier, & Seglias, 1981).

*Transport of solid antigens*

In *paper II* we measured cellular transport of solid antigens to the draining lymph node by injecting FITC⁺ latex beads that, due to their size, do not enter lymphatic capillaries and need to be actively transported by dendritic cells to the lymph node (Randolph, Inaba, Robbiani, Steinman, & Muller, 1999).

3.6 Western blot

This semi-quantitative method of protein detection was used in *paper I* for identification of VEGF family ligands (VEGF, VEGF-C, VEGF-D, PlGF), inflammation-related cytokines (IL-1β, IL-10, GM-CSF, M-CSF, TGF-β, IL-6) and STAT3 phosphorylation (STAT3, pSTAT3(Ser707), pSTAT3(Tyr705)), all from tumor lysates. In *paper II*, western blot analysis was used to analyse the level of inflammatory mediators (GM-CSF, MCP-1, IFN-γ, TNF-α, NOS2, IL-1β, IL-10, VEGF, M-CSF, TGF-β) in tumor lysates.

In *paper II*, we also used a cytokine antibody array kit with 22 different antibodies (G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40/p70, IL-12p70, IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, SCF, sTNF R1, TNF-α, thrombopoietin, VEGF) to detect and compare the cytokine and chemokine levels in tumor lysates.
3.7 ELISA

The enzyme-linked immunosorbent assay (ELISA) is a quantitative method used in paper I to analyse the level of inflammatory mediators (IL-1β, IL-10, GM-CSF, M-CSF, TGF-β, IL-6) in tumor interstitial fluid.

3.8 Flow cytometry analysis

Flow cytometry is a laser/impedence-based technology for cell counting and cell sorting that we used to quantify immune cells in paper II. Tumors and lymph nodes were incubated with collagenase D and filtered through a 70 µm cell strainer. The cell suspension was incubated with antibodies to characterize immune cell populations/subpopulations (CD45, CD3, CD4, CD8, F4/80, CD25, FoxP3, CD11c, MHC II, gp38, CD31, IFN-γ, CD11b) from the homogenized tissue.

In paper III, lymph and plasma samples were stained for phospho-flow cytometry with: p-STAT3, p-STAT5, p-CREB and p-p38. Samples were run on a BD LSR Fortessa with four lasers (488nm, 561nm, 635nm and 407nm) and Diva software version 8.0. BD Cytometry Setup and Tracking beads were used for standardization of application setup.

3.9 High performance liquid chromatography

This technique utilizes different physical characteristics of molecules (i.e. protein, carbohydrates or nucleic acids), to separate them. A liquid mobile phase is run through a column (solid phase) and the components of the liquid phase is separated due to the nature of the column. We used liquid chromatography in paper III to perform immunodepletion, i.e. the removal of the seven most abundant plasma proteins, to access lower abundant proteins in our lymph and plasma samples (Smith et al., 2011). In addition, we also used size-exclusion chromatography, which
separates molecules based on their molecular size, to separate the proteins from our samples in different fractions.

3.10 Mass spectrometry

After liquid chromatography had been performed, our samples were analysed with mass spectrometry in paper III (Aebersold & Mann, 2003; Mesri, 2014). The proteins in the sample were digested with trypsin into peptides and run by liquid chromatography-mass spectrometry (LC-MS/MS) on an iontrap. The basic principle is to generate ions from the injected peptides and characterize them qualitatively and quantitatively based on their mass-to-charge ratio and abundance. The specific information generated can identify the amino acid sequence of the different peptides, and from that, protein identification is possible.
4. Results

**Paper I: Impaired lymphatic function accelerates cancer growth**

In this paper, we report accelerated tumor growth using the C3HBA breast cancer and KHT-1 sarcoma syngeneic tumor cell lines, in a mouse model (Chy) with impaired lymphangiogenesis in the dermis, due to heterozygous inactivation of VEGFR3. We observed a reduced lymphatic washout from the peritumoral area, attenuated innate immune response reflected in fewer peritumoral macrophages and lower levels of macrophage-associated inflammatory cytokines in the breast cancer model (C3HBA). Furthermore, we found an increased M2/M1 ratio in the tumor microenvironment. IL-6 was found upregulated in both tumor lysates and tumor interstitial fluid, and treatment with an anti-IL-6 receptor antibody (tocilizumab) inhibited C3HBA tumor growth in both Chy and wt mice. Tumor interstitial fluid pressure and blood vessel density were not found significantly altered between Chy and wt mice in the breast cancer model. In addition to primary tumor growth, we selectively assessed interstitial fluid pressure and intratumoral IL-6 levels in sarcomas (KHT-1) from Chy mice, and both were found no different from the control group.

**Paper II: Lymphatic vessels regulate immune microenvironments in human and murine melanoma**

We assessed the influence of lymphatic drainage on tumor inflammation, using a mouse model that lack dermal lymphatic vessels (K14-VEGFR3-Ig mice). We report a significant downregulation of tumor immune response in the transgenic mouse model, reflected in reduced immune cell infiltration and inflammatory cytokine levels, coinciding with an impaired dendritic cell migration to the draining lymph nodes. Primary tumor growth was not affected in this model. Furthermore, the metastatic dissemination was reduced in K14-VEGFR3-Ig mice, and dermal prophylactic vaccination did not control tumor growth in the absence of peritumoral lymphatic vessels. However, adoptive transfer of cytotoxic T-cells more efficiently
controlled tumor growth in the transgenic model due to the lack of local immune suppression. Gene expression analysis of human melanoma samples showed a strong correlation between markers of lymphatic vessels and immune infiltration in the tumors.

**Paper III: Isolation of lymph shows activation of STAT3 and CREB pathways in the spleen during AML development in a rat model**

Based on previous reports that interstitial fluid mirrors local changes in the microenvironment of the spleen, we asked whether the presence of infiltrating leukemic blasts during AML development influence interstitial fluid in a way that could be detected in efferent spleen lymph. After cannulation of spleen lymphatic vessels, the lymph was analyzed with flow cytometric and proteomic methods. We report increased STAT3 and CREB signaling in leukemic spleen lymphocytes and a significant upregulation of proteins related to these pathways in leukemic spleen lymph compared with control lymph. Furthermore, proteins related to leukemic development were upregulated in AML spleen lymph, and the local production of SPARC-like 1 protein, a biomarker of AML patient outcome, was enhanced even at an early time-point of disease development.

**Paper IV: Adipose tissue macrophages: the inflammatory link between obesity and cancer? (review)**

There is accumulating evidence of a mechanical link between obesity and cancer development. Moreover, tumor-associated macrophages are known to promote tumor progression, but there is still much that needs to be elucidated regarding the role of resident adipose tissue macrophages in these processes. In paper IV, we summarize some of the literature on the topic and highlight issues that need to be further addressed.
5. Discussion

The overall aim of this project was to study interactions between the lymphatic compartment and tumor microenvironment, in solid tumors and leukemia. Additionally, in paper IV we discuss the role of adipose tissue surrounding solid tumors, and the potential cancer promoting ability associated with resident tissue macrophages found within the adipose tissue.

In paper I and II, we demonstrate that the impairment of lymphangiogenesis results in a reduced immune response in the tumor microenvironment, while in paper III, the presence of leukemic cells in the spleen alters the composition of efferent lymph. Altogether, these studies show that lymphatics and the microenvironment they originate from are uniquely intertwined, and influence each other during cancer development.

5.1 Methodological considerations

5.1.1 Animal models (paper I-III)

Animal models have been essential for all the research papers (I-III) presented in this thesis. Animal models are, unquestionably, one of our most valuable tools for studying cancer biology in general, but also to target or study specific mechanisms in malignant transformation (Lunardi, Nardella, Clohessy, & Pandolfi, 2014). Through our collaboration with the Kari Alitalo research group in Finland, we gained access to two genetically modified mouse models with impaired lymphangiogenesis capacity (Karkkainen et al., 2001; Makinen et al., 2001), that provided us with the opportunity to study lymphatic influence on the tumor microenvironment in paper I-II. In paper III, collaboration with the McCormack group in Bergen provided us with the BNML leukemia model (Martens, Van Bekkum, & Hagenbeek, 1990), a rat model of acute myeloid leukemia, which we used in our study of the spleen microenvironment. All three animal models were well characterized in previous publications.
Animal models provide us with research opportunities, e.g. genetic modifications, standardized study populations, tumor growth under controlled and repeatable conditions, relatively low costs and short breeding time, conditions that for obvious reasons are inapplicable in human studies. However, there are numerous limitations with animal models that are important to keep in mind. Differences in genetic, immunological and cellular mechanisms between animals and humans are reported as possible reasons why only 8% of drugs originating from animal research, make it successfully through clinical phase I studies (Mak, Evaniew, & Ghert, 2014). Although specific processes within disease development can be replicated in animals, it is currently still impossible to mimic the whole range of physiological alterations that occur in human disease (Mak et al., 2014), and the results of animal experiments should therefore be considered hypothesis-generating and explorative.

5.1.2 Flow cytometric analysis (paper II-III)

Flow cytometry is a laser/impedence-based technology for cell sorting that we used to quantify immune cells in paper II and intracellular phospho-signalling in paper III. The technique is able to provide both qualitative and quantitative information of single-cell suspensions, and there is a broad availability of antibodies that makes characterization of multiple cell populations possible, even from small tissues or samples (Brown & Wittwer, 2000; Orr, Kennedy, & Hasty, 2013). Limitations of the technique that are important to keep in mind, however, are subjective data interpretation and poor standardization across institutions (X. M. Wang, 2014). In addition, the multistep-protocol to disaggregate cells from solid tissue requires trained operators to prevent losing or damaging of cells. Thus, flow cytometric analysis is much easier to standardize and perform in leukemias than in solid cancers.

5.1.3 Proteomics (paper III)

In paper III, we used liquid chromatography-mass spectrometry (LC-MS/MS) to identify and quantify proteins in efferent spleen lymph. This technique requires only small volumes of fluid to identify a large set of proteins, which gives unique possibilities to detect changes in the microenvironment in both healthy and diseased
tissues (Haslene-Hox et al., 2013). A noteworthy limitation with this technique is that repeated analyses of the same sample not necessarily reproduce the same part of the proteome, partly due to the stochastic selection of precursor-ions (Tabb et al., 2010). Another limitation in our AML study was the limited number of animals included.

5.1.4 Immunohistochemistry (paper I-II)

Immunohistochemistry was chosen to identify lymphatic vessels and immune cells in paper I, and immune and stromal cells in paper II. The advantages of immunohistochemistry are the low cost of the technique, preserved morphology of the tissue, preservation of proteins and availability of numerous antibodies to detect them (Matos, Trufelli, de Matos, & da Silva Pinhal, 2010). The use of different fluorescence markers or chromogenic substrates makes staining for two or more antigens possible at the same time, thus enabling the identification and localization of several epitopes simultaneously on the same slide. Although extensively used in research, it is important to remember that the technique has multiple possibilities of reaction bias, i.e. differences in tissue fixation, tissue processing, antigen retrieval and detection which may profoundly influence the results. As recently addressed in Nature, the reproducibility of antibodies also represents a great challenge in research that is important to consider when using this method (Baker, 2015). In addition, there could also be interpretation bias, i.e. in selection of antibodies, selection of quantification method and in interpretation of results (Matos et al., 2010).

5.1.5 TIF isolation

We isolated tumor interstitial fluid with the centrifugation technique (Wiig et al., 2003) in paper I and its surrogate, efferent lymph, in paper III. The centrifugation technique has been developed and validated in our research group to preserve the composition of native interstitial fluid. Weaknesses with the technique are the need for ex vivo analysis, which requires standardized conditions, and it also has to be performed from single samples (Wiig, Tenstad, et al., 2010). The cannulation of lymphatic vessels from an organ in steady-state, provide samples of true interstitial fluid. The procedure is performed in vivo and there is minimal inflammatory reaction
in relation to it. However, the technique requires trained operators, it is not applicable for regional sampling in small animals, and it can be difficult to determine the exact origin of the lymph (Wiig & Swartz, 2012).

5.2 The involvement of lymphatics on tumor immune response (I + II)

High lymph vessel density is associated with increased frequency of lymph node metastases and poorer prognosis in several different solid cancers (Alitalo, 2011). Anti-lymphangiogenic treatment has therefore been proposed as a potential approach in cancer therapy, and in the last few years, several agents targeting lymphangiogenesis have been developed and entered clinical trials (Dieterich & Detmar, 2016). Apart from the inhibition of lymphangiogenesis, preclinical studies have also demonstrated anti-angiogenetic properties of blocking VEGFR-3, further advocating a possible anti-tumor effect of this therapeutic approach (Tammela et al., 2008).

Our mouse models in paper I and II had, through two different mechanisms, impaired VEGF-C/VEGFR3 signaling and therefore mimicked pharmacological blockade of this pathway in a cancer setting. Of notice, we reported decreased metastatic dissemination, reflected in less lung metastases in the transgenic mice (paper II), in concordance with one of the proposed benefits of targeting this pathway (Stacker et al., 2014). However, in both paper I and II, we also conclude that targeting VEGF-C/VEGFR3 significantly diminish the immunological tumor response. In paper I, breast cancer and sarcoma growth was accelerated, while in paper II, a melanoma model, the tumor growth was unaffected by the impaired immune response.

In light of the reduced number of lung metastases in our transgenic model, our data suggest a more metastatic tumor phenotype in the presence of lymphatic vessels. Substantial evidence link tumor associated immune cells to the metastatic propensity of cancer cells, and this observation could therefore result from a reduced presence of immune cells in the tumor microenvironment (Kitamura et al., 2015).
Interestingly, in paper II we found that anti-OVA T-cell transfer enhanced OVA-expressing melanoma regression in the transgenic model. This observation is in line with previous observations that adoptive transfer of tumor-targeted lymphocytes has enhanced the anti-tumor effect following immune cell depletion (Perica et al., 2015). It has been suggested that this is because of elimination of immunosuppressive cells from the tumor site, and that transferred cells have more access to homeostatic cytokines (Wrzesinski et al., 2010). Adding anti-lymphangiogenic agents could therefore possibly enhance the effect of adoptive T-cell transfer in humans. Conversely, treatment with checkpoint inhibitors improved treatment response in the presence of lymphocyte aggregates surrounding the tumor (Palucka & Coussens, 2016). Further assessment of the interaction between these therapeutic approaches is therefore warranted.

Since pharmacological blockade of VEGFR-3 has been reported to alter blood vessel morphology and have an antiangiogenic effect in the tumor microenvironment, we wanted to assess whether this appeared in the current tumor models. However, no significant differences were established regarding blood vessel density neither in paper I nor II. There were indications in both models that the blood vessels were altered through impaired VEGF-C/VEGFR3 signaling; in paper I, there was a tendency toward enlarged blood vessels and less hypoxia in Chy mice tumors assessed by IHC (data not shown), but we were not able to show significant differences in tumor blood perfusion using lectin perfusion. In paper II, there was a decrease in the percentage area stained with CD31+, but whether this was the result of VEGFR3 inhibition or less inflammation in the transgenic model, cannot be determined without further assessment.

In summary, we report a reduced tumor immune response, lower frequency of lung metastases and enhanced effect of adoptive T-cell transfer, as a consequence of VEGF-C/VEGFR3 blockade. Additionally, in paper I, accelerated primary tumor growth was also observed in mice with impaired lymphangiogenesis. Thus, the effects of targeting lymphangiogenesis are, not surprisingly, heterogeneous in
different tumor models, and depends on the outcome we study (primary tumor growth versus extent of distant metastases).

5.3 Macrophages and cancer development (I + IV)

There is substantial evidence linking TAMs to several tumor-promoting features, both within the tumor microenvironment and during metastatic development (Biswas, Allavena, & Mantovani, 2013). As addressed in previous sections (2.2.1 and 5.1), the various roles of tumor-associated macrophages and their natural plasticity, make them a potential target in cancer therapy.

In paper I, we report a reduced number of macrophages both in the peritumoral space and within the C3HBA tumors in our Chy model, and also a shift in M2/M1 ratio. In the same tumors, we also found reduced levels of inflammatory cytokines known to be secreted by macrophages, and we concluded that this cell population seemed to be affected by the impaired VEGFR3 signaling. The enhanced tumor growth, together with these changes in the macrophage population, led us to the conclusion that M1-like macrophages either protect against tumor growth in an early phase of tumor development in wt mice, or that the elevated M2/M1 ratio promotes tumor growth in our model. This shows an unexpected and undesirable effect of blocking VEGFR3 signaling in the early stages of murine breast cancer development.

Increased tumor growth and progression have been linked with M2-like macrophages (Ostuni et al., 2015; Sica et al., 2015). In light of these observations, our finding of accelerated tumor growth in the presence of elevated M2/M1 ratio is not surprising, nor is the observation that macrophages may have anti-tumor effects in an early tumor phase (M1). We found elevated levels of IL-6 in both whole tumor lysates and in the tumor interstitial fluid in C3HBA breast cancer in Chy mice, which we suggest could have contributed to the M2 polarization in the tumor microenvironment, in line with previous observations (Heusinkveld et al., 2011; Mauer et al., 2014). However,
the underlying mechanisms or cells responsible for the elevated IL-6 levels in this tumor model, were not investigated and should be assessed in future work.

The number of adaptive immune cells surrounding the tumor was not found to be affected in Chy mice. This finding is in concordance with previous observations in Chy mice, that humoral immune responses were not affected on the trunk of Chy mice although the lymphatic density was significantly reduced in trunk skin (Platt et al., 2013). However, this observation was apparently contradictory to our observations in the K14-VEGFR3-Ig model (paper II), where all immune cell lines were affected by the halted lymphangiogenesis. Importantly, these are two different genetic models, where the K14-VEGFR3-Ig mice exhibit a stronger phenotype with respect to the lack of skin lymphatics than the Chy model. The Chy model carries a heterozygous mutation, which naturally results in a mixed phenotype, as demonstrated in the paper by Platt et al. (Platt et al., 2013). At the same time, the Chy mutation has a better resemblance with human lymphedema, and is therefore probably a better translational model (Karkkainen et al., 2001). In conclusion, the differences between the chosen models could possibly explain the discrepancies between our observations.

Another possible explanation for the reduced macrophage population in Chy mice in paper I, is that lymphatic endothelial cells, together with neutrophils, are the major producers of M-CSF (CSF-1) (www.immgen.org), a key stimulator of macrophage proliferation and survival (Otero et al., 2009). Indeed, M-CSF was one of the proteins with the lowest expression in whole tumor lysates from C3HBA tumors in Chy mice. It is possible that the reduced level of lymphatic endothelium in this mouse model leads to low levels of CSF-1 and subsequently impairs macrophage survival.

The exact origin of macrophages found in the tumor microenvironment is still an open question. It has recently been demonstrated that tissue macrophages mostly originate from yolk sac progenitors, while macrophages involved in pathogen responses are recruited from bone marrow or spleen derived monocytes (Noy & Pollard, 2014). In our review (paper IV), we point to the possible contribution of
adipose tissue macrophages (ATM) to both TAM populations and otherwise to cancer progression through inflammatory mechanisms. Wagner et al. have previously reported accelerated tumor growth and increased infiltration of macrophages with TAM characteristics when tumors were implanted next to adipose tissue deposits (Wagner et al., 2012). Interestingly, it has been shown that tissue resident macrophages and TAMs may respond differently to anti-macrophage treatment in brain tumors (Pyonteck et al., 2013), which further emphasize the importance of understanding how macrophage are recruited, and function, in the tumor microenvironment.

To summarize, macrophages were found in reduced number in our Chy mouse model, either as a result of halted recruitment or a shortened tissue life span, subsequent to impaired lymphangiogenesis. We also suggest that local factors, e.g. elevated IL-6 levels as shown in paper I, in the C3HBA tumor microenvironment tended to skew the present macrophages toward a M2-like phenotype, which again enhanced primary tumor growth. This further underpins the complexity of targeting lymphangiogenesis in different cancer models and the need for individual adjustments in a potential clinical setting.

5.4 Assessment of the tumor microenvironment through interstitial fluid (I + III)

Our overall aim was to study interactions between the lymphatic compartment and tumor microenvironment in solid cancer and as well as leukemia. As briefly discussed in section 2.4, several factors contribute to an altered composition of interstitial fluid during cancer development. Further knowledge of these local changes may therefore provide improved understanding of cancer in general, but also identify potential biomarkers of disease development (Wagner & Wiig, 2015). In line with this, Haslene-Hox et al. published a proof-of-concept study that demonstrated drastically elevated protein levels of the ovarian biomarker CA-125 in TIF when
compared with plasma, a level which correlated with tumor stage (Haslene-Hox et al., 2014).

In paper I, we found elevated expression of IL-6 together with reduced levels of inflammatory proteins, in whole C3HBA tumor lysates. We wanted to assess the concentration of the same proteins in the interstitial fluid phase, since cytokines might bind to ECM proteins (Hynes, 2009). Although IL-6 was upregulated also in the interstitial fluid phase, there were indeed some discrepancies in concentration between whole tumor lysate and tumor interstitial fluid for the selected proteins in our study. Thus, there could be significant differences between total protein expression in a tumor (intracellular and extracellular) and the protein concentration in the fluid bathing the tumor microenvironment, which is important to keep in mind when the results are interpreted.

In paper III, collection and analysis of spleen interstitial fluid was the basis of our study design. We cannulated efferent spleen lymphatic vessels in leukemic and control rats to examine differences in intracellular lymphocyte signaling and proteomic profile. It is generally accepted that prenodal lymph can be considered as representative of true interstitial fluid, whereas lymph can be modified when passing through the lymph node (Wiig & Swartz, 2012). Prenodal lymph vessels from the spleen have previously been deemed impossible to cannulate (Semaeva et al., 2010), but during the preparations for our study, we were able to cannulate and harvest both prenodal and postnodal spleen lymph from one control rat. The proteomic profile of these two samples was compared using the proteomic software Progenesis, and we were able to show that the protein variations between these samples were minor.
Fig 3. Proteins from prenodal and postnodal lymph, correlation coefficient: $r^2=0.99$,

*From paper III*

As summarized in section 4, we found significant differences in both intracellular signaling and protein abundance in leukemic and healthy spleen lymph. Our study therefore provides additional proof-of-concept evidence that the interstitial fluid can be an important tool in detecting local changes in a microenvironment during cancer development.

5.5 Tumor-associated inflammation

Inflammation is a physiological response, triggered by either pathogens or cellular damage, subsequently activating the immune system to repair and remove the underlying cause. Immune cells have complex regulatory mechanisms to terminate this response, and in the absence of negative feed-back, acute inflammation can develop into a chronic condition. The potential link between inflammation and cancer was pioneered by R. Virchow in the mid-19th century (Plytycz & Seljelid, 2003).
Since then, substantial research work has confirmed the mechanistic association between inflammation and cancer development and progression, and has led to the conclusion that inflammation is an enabling hallmark of cancer (Hanahan & Weinberg, 2011).

In paper II, we report that lymphatic vessels are essential for the tumor-associated inflammatory response. Inflammation might have multiple consequences in the cancer setting; chronic inflammation can initiate cancer development, the tumor inflammatory response can further instigate tumor progression, immunosuppression and invasiveness (Hanahan & Weinberg, 2011; Shalapour & Karin, 2015). In line with these observations, we report reduced metastatic dissemination in the mice with a lower inflammatory tumor response, that also lack lymphatic vessels in the skin.

Similarly, in our screening of melanoma patients; the gene expression of inflammatory cytokines correlated with lymphatic vessel density. Apparently the density of tumor lymphatic vessels determines not only the level of immune infiltrate, but also the degree of inflammatory response in the tumor. Although we had indications from our animal model that the lymphatic vessel density promoted a more malignant phenotype, a potential correlation with overall survival could not be examined in our human data, as that information was not available from the genome atlas we used.

In paper I, we also observed lower expression of inflammatory cytokines, with the exception of IL-6, in Chy mice with reduced macrophage infiltration and impaired lymphangiogenesis. In this study, however, we observed an enhanced primary tumor growth in the mice with the lowest level of inflammatory cytokines. This differs somewhat from what we found in paper II, where the reduced expression of inflammatory markers was associated with weakened metastatic propensity. However, macrophages are able to eliminate tumor cells at an early stage of primary tumor development, in a process known as tumor surveillance and elimination (Feng et al., 2015). Thus, the enhanced tumor growth seen in Chy mice could be the result of impaired surveillance mechanisms, due to altered macrophage subpopulations, and
not necessarily linked to the expression of inflammatory cytokines. Additionally, although most of the inflammatory cytokines were downregulated in Chy mice, IL-6 was significantly elevated. IL-6 is known to promote cancer aggressiveness (Chang, Daly, & Bromberg, 2014), and the increased IL-6 expression was different from our findings in the melanoma mouse model (paper II), which further emphasizes the differences between the two mice models we used.

Finally, in paper IV, we outline the importance of macrophages as instigators of inflammation. We also propose that adipose tissue macrophages are major contributors to the chronic low-grade inflammation seen in obesity, and that this cell population is a possible link between inflammation, obesity and cancer.

In summary, our studies show an association between lymphangiogenesis and tumor inflammatory response. Our results also add to the assumption that the tumor inflammatory response is closely related to the level of immune cell populations recruited to the tumor site, where we have focused on tumor associated macrophages as part of the innate immune response. Since inflammation is known to be closely linked to cancer progression, our work adds to current knowledge by pointing to the potential immune-related consequences of targeting lymphangiogenesis in cancer therapy. Moreover, with respect to the enhanced primary tumor growth in paper I, we point to the possible unexpected consequences of targeting VEGFR3, and that anti-lymphangiogenic therapy not only could have different outcomes in various cancer types, but also depend on which stage of the cancer disease that is targeted.
6. Future perspectives

The last decades have revealed a plasticity of the tumor microenvironment that mirrors to a large extent the complexity of normal human biology. Although our knowledge on signalling pathways and promoting factors in cancer is rapidly increasing, we are only at the starting point of how to translate our knowledge into improved therapeutic strategies.

Both our mouse studies show an association between lymphatic vessels and immune cell infiltrate in the tumor microenvironment. In melanoma, impaired lymphangiogenic capacity reduced metastatic dissemination, while in breast cancer and sarcoma we observed an enhanced primary tumor growth. A remaining question is which types of cancer will benefit from such therapeutic approach, and also, at which stage of disease development this treatment could provide the best patient outcome.

Our acute myeloid leukemia study demonstrates that interstitial fluid is a compartment that can be analysed to reveal cancer specific proteins and cytokines. In the search of new biomarkers, interstitial fluid could therefore be a potential substrate to identify substances secreted from the tumor, and subsequently examine if these markers are detectable in plasma, and whether they correlate with the stage of disease.

Anti-macrophage treatment is already in clinical trials, and it is crucial to enhance our knowledge on the different populations of macrophages that have been detected within various tumor microenvironments. It is likely that this cell population will respond diversely to treatment due to their plasticity, but also as a consequence of different origins and characteristics.

Cancer is an assembly of diseases, and the heterogeneity between different cancer types, and even between individual patients, represents a great challenge for researchers and oncologists. This diversity makes it highly unlikely that one strategy
will be effective alone or fit all patients. Thus, it is important that we, in our search to improve cancer therapy, preserve a willingness to explore different approaches on how to target tumor progression and also how we can combine different modalities of treatment to benefit each individual patient.
References


transgenic mice expressing soluble VEGF receptor-3. Nat Med, 7(2), 199-205. doi:10.1038/84651


functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. Cancer Res, 70(14), 5728-5739. doi:10.1158/0008-5472.can-09-4672


Impaired lymphatic function accelerates cancer growth

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ABSTRACT

Increased lymphangiogenesis is a common feature of cancer development and progression, yet the influence of impaired lymphangiogenesis on tumor growth is elusive. C3HBA breast cancer and KHT-1 sarcoma cell lines were implanted orthotopically in Chy mice, harboring a heterozygous inactivating mutation of vascular endothelial growth factor receptor-3, resulting in impaired dermal lymphangiogenesis. Accelerated tumor growth was observed in both cancer models in Chy mice, coinciding with reduced peritumoral lymphangiogenesis. An impaired lymphatic washout was observed from the peritumoral area in Chy mice with C3HBA tumors, and the number of macrophages was significantly reduced. While fewer macrophages were detected, the fraction of CD163+ M2 macrophages remained constant, causing a shift towards a higher M2/M1 ratio in Chy mice. No difference in adaptive immune cells was observed between wt and Chy mice. Interestingly, levels of pro- and anti-inflammatory macrophage-associated cytokines were reduced in C3HBA tumors, pointing to an impaired innate immune response. However, IL-6 was profoundly elevated in the C3HBA tumor interstitial fluid, and treatment with the anti-IL-6 receptor antibody tocilizumab inhibited breast cancer growth. Collectively, our data indicate that impaired lymphangiogenesis weakens anti-tumor immunity and favors tumor growth at an early stage of cancer development.

INTRODUCTION

The lymphatic system is a common and early route of metastasis, where the occurrence of lymph node metastasis is a negative prognostic marker. Increased peritumoral lymphatic vessel density (LVD) correlates with increased frequency of lymph node metastasis and worse prognosis in various types of cancer [1–5], pointing to lymphatic vessels as a potential therapeutic target in the treatment of cancer. Various pharmacological inhibitors of lymphangiogenesis are currently investigated clinically [6–8], and the consequences of targeting tumor lymphangiogenesis therefore needs to be explored both in the early and advanced cancer setting. We addressed this issue by studying the early stages of cancer progression in Chy mice, which harbor a heterozygous inactivating point mutation of the tyrosine kinase domain of VEGFR-3 [9]. Importantly, the same mutation has been found in patients with hereditary lymphedema, and this model is therefore of particular interest in a translational context [10, 11]. Chy mice are characterized by impaired lymphangiogenesis in the dermis [10, 12], and accordingly, malignant tumors were implanted orthotopically in the subcutis to evaluate how cancer progression would be affected in a tumor microenvironment where the VEGFR-3 expression level is decreased.

We postulated that impaired lymphangiogenesis could increase the interstitial fluid pressure (IFP) and thus inhibit tumor growth by affecting the blood supply. Alternatively, impaired lymphangiogenesis could prevent tumor antigens from reaching lymph nodes, causing a reduced immune response and weakened tumor immunity. Interestingly, we found accelerated primary tumor growth of C3HBA breast cancer and...
KHT-1 sarcomas in Chy mice, which was associated with decreased lymphatic washout and a reduced number of peritumoral macrophages. Apart from IL-6, reduced levels of all macrophage-associated cytokines were observed in C3HBA tumors in Chy mice, indicating an impaired innate immune response and potentially influencing the early stages of breast cancer progression.

RESULTS

Accelerated tumor growth and impaired peritumoral lymphangiogenesis in Chy mice

Accelerated tumor growth of orthotopically implanted C3HBA breast cancer and KHT-1 sarcoma was observed in Chy mice with impaired dermal lymphangiogenesis compared with wt littermates (Figure 1A-1D). Metastases were not seen neither in regional lymph nodes nor in internal organs, when assessed macroscopically and microscopically (data not shown). To detect single-cell metastases, RNA was extracted from lung and liver tissues of mice included in the tumor growth trials, and RT-PCR for eGFP positive C3HBA tumor cells demonstrated transgene expression in the liver tissue of two tumor-bearing wt mice, whereas Chy mice had no transgene expression in these organs (Figure 1E). Thus, primary tumor growth was increased but an enhanced metastatic potential was not observed in the Chy model.

Next, we stained the tumor and peritumoral area with a LYVE-1 antibody to assess the lymphatic vessel density in Chy mice. Chy mice had no discernable lymphatics present in the peritumoral area in neither of the two tumor models. Wt mice had on average 30 and 8 LYVE-1 positive lymphatic vessels per hot spot around C3HBA and KHT-1 tumors, respectively (Figure 1F). Apart from a few lymphatic vessels embedded in the outer tumor rim of wt mice, lymphatics could not be identified inside the tumor tissue.

Based on the strong tendency for lymphatic metastasis in the initial stages of breast cancer progression in humans, we assessed whether the missing lymphatics around C3HBA tumors affected lymph flow, measuring washout of labeled albumin by optical imaging [13]. The lymphatic drainage, assessed as washout of Alexa 680-albumin, was significantly lower in the skin overlying C3HBA tumors in Chy mice, compared to wt mice (Figure 1G). The percentage removal of albumin per min from the peritumoral skin of wt mice was: -0.42 ± 0.05 % min⁻¹, and Chy mice: -0.18 ± 0.08 % min⁻¹ (p=0.005). This demonstrates that lymphatic drainage was strongly impaired in the peritumoral area of C3HBA tumors growing in Chy mice, potentially reducing the drainage of tumor antigens to regional lymph nodes and migration of tumor cells out of the primary tumor bed [14, 15].

We measured tumor IFP by the micropuncture technique [16] in the outer tumor rim to assess whether the impaired peritumoral lymphangiogenesis affected the intratumoral pressure, but there was no significant difference in IFP between wt and Chy mice, neither in C3HBA nor KHT-1 tumors (Figure 1H). Since there may also be a pressure gradient from central to peripheral

Figure 1: A–D. Tumor growth of C3HBA breast cancer and KHT-1 sarcoma in Chy and wt mice. A-B. Tumor growth curves depict the mean tumor volume ± SEM per group, from the day of measureable tumors in the mice. C–D. Days for each tumor to reach 2250 mm³ (tumor growth time). Bars display the mean TGT ± SEM per group, demonstrating accelerated tumor growth in Chy mice. C3HBA: n=12 mice/group. KHT-1: n=11 and n=6 in wt and Chy mice respectively.*p<0.05. (Continued)
tumor areas, we measured IFP in the tumor center with the wick-in-needle (WIN) technique [17]. Again we found no significant difference between tumors in wt and Chy mice (Figure 1I). Accordingly, the impaired lymphatic drainage from Chy mice tumors was not caused by changes in intratumoral interstitial fluid pressure.

**Tumor blood vessels and perfusion unaltered by the Chy mutation**

Based on previous reports, we examined how heterozygous VEGFR-3 inactivation in Chy mice influenced tumor angiogenesis [18]. CD31 staining demonstrated no difference in blood vessel density (BVD) when C3HBA tumors in Chy and wt mice were compared (Figure 2A). To assess the number of perfused blood vessels within Chy and wt C3HBA tumors, lectin was injected through the tail vein and the perfused areas were compared, but no significant difference was found (Figure 2B). Thus, the accelerated primary tumor growth observed in the Chy model was not caused by increased tumor angiogenesis nor increased number of perfused blood vessels.

The influence of VEGFR-3 heterozygous inactivation on ligands of the VEGF family was assessed by protein analysis. VEGF-C was upregulated in C3HBA tumors in Chy mice (Figure 2C), which is likely the result of reduced VEGFR-3 expression in this mouse model. The other VEGFR ligands were not significantly altered (Figure 2C).
Decreased inflammatory response and macrophage infiltration in Chy mice tumors

In our assessment of peritumoral lymphatics by LYVE-1 or VEGFR-3 staining (not shown), we observed scattered LYVE-1⁺ or VEGFR-3⁺ cells outside the lymph vessels. Based on the known expression of LYVE-1 or VEGFR-3 by macrophages [19, 20], immunohistochemistry was used to characterize this cell population further. We observed a reduced number of peritumoral F4/80⁺ macrophages in Chy mice and the total number of leukocytes (CD45⁺ cells) (Figure 3A) around C3HBA tumors was significantly reduced in Chy compared to wt mice. For KHT-1 tumors, a reduced number of peritumoral macrophages was observed in Chy mice, whereas the CD45⁺ cell count was no different from wt siblings (Figure 3B). To further assess the innate immune response, staining was performed with a CD11b antibody, which is a well-known marker of myeloid cells, including macrophages. A significant reduction in CD11b⁺ cells in Chy tumors was detected, both within the tumor (Figure 3D) and in the peritumoral area (Figure 3C), in accordance with the F4/80 staining.

The macrophages were further characterized by staining for CD163, a well-known marker of M2 (alternatively activated) macrophages [21]. However, the number of CD163⁺ cells was not significantly different between Chy and wt mice (Figure 3E) and since the total

**Figure 2:** A. Immunohistochemistry for CD31 demonstrates no difference in C3HBA intratumoral blood vessel density (BVD) between Chy and wt mice. Scale bars: 100 μm. Bar graph depicts the mean BVD ± SEM, n=3 per group. B. Tumor perfusion assessed by i.v. injection of TRITC-conjugated lectin demonstrated no difference between C3HBA tumors in Chy mice and wt mice. Scale bars: 100 μm. Bar graph depicts the mean number of perfused vessels per field-of-view ± SEM, n=7 per group. C. Western blot analysis of VEGF family ligands in whole tumor lysate from C3HBA tumors (30 μg protein/lane). VEGF-C is significantly upregulated in Chy compared to wt mice. Densitometry of western blots presented as the ratio of protein of interest over actin expression. Bars depict the mean ± SEM, n=3 per group. *p<0.05.
number of macrophages (both M1 and M2) was reduced in Chy mice, this implies that less M1 macrophages were present in the peritumoral tissue of Chy mice, causing a shift towards a higher M2/M1 ratio.

To test the influence of tumor-associated macrophages (TAMs) on early cancer development, wt mice with C3HBA breast cancers were treated with liposomal clodronate before and during tumor initiation to deplete TAMs. Liposomal clodronate treatment inhibited tumor growth, compared to sham treatment (Supplementary Figure S1A), and F4/80 staining demonstrated a reduced number of macrophages (Supplementary Figure S1B). While this points to a pro-tumorigenic role of TAMs in general, liposomal clodronate treatment kills all macrophage subtypes unselectively [22], and do not reflect the Chy phenotype with a skewed M2/M1 ratio. In line with previous data [23], liposomal clodronate treatment reduced tumor angiogenesis (Supplementary Figure S1C), which may have contributed to the impaired tumor growth in this experiment. At the same time, tumor angiogenesis was not affected in Chy mice, demonstrating that liposomal clodronate treatment did not recapitulate the findings in these mice.

Further, we wanted to assess whether the reduced lymphatic washout and increased M2/M1 ratio was associated with altered secretion of macrophage-associated cytokines in Chy mice tumors. A panel of pro- and anti-inflammatory cytokines was examined by protein analysis of whole C3HBA tumor lysates (Figure 3F), and apart from IL-6 all the pro- and anti-inflammatory cytokines were significantly downregulated in Chy tumors. Taken together, our results show a downregulated innate immune response in these mice.

Based on recent data alluding to the important role of tumor infiltrating lymphocytes (TILs) in breast

**Figure 3:** A–B. Immunohistochemistry for F4/80 and CD45 in C3HBA breast cancers and KHT-1 sarcomas. (A) Significantly less macrophages (F4/80) and leukocytes (CD45) were observed in the peritumoral area of C3HBA tumors in Chy compared to wt mice. (B) In KHT-1 tumors a significantly reduced number of macrophages, but not leukocytes in general was observed in Chy compared to wt mice. Scale bar: 50 μm. C–D. Immunohistochemistry demonstrates significantly fewer peritumoral CD11b+ cells surrounding C3HBA (C) and KHT-1 tumors (D) in Chy than in wt mice. Scale bars: 100 μm. (Continued)
cancers [24], we examined the adaptive immune response by immunostaining for CD3 (T-lymphocytes), CD 20 (B-lymphocytes) and FOX P3 (regulatory T-lymphocytes; Tregs) in C3HBA tumors (Supplementary Figure 2SA-S2C). However, the recruitment of these cell populations was not affected by the Chy mutation, which was also the case for CD3 staining in KHT-1 tumors (Supplementary Figure S2D).

Increased tumor IL-6 levels in Chy mice and the influence of anti-IL-6 treatment

Since macrophage-associated cytokines were profoundly influenced by the Chy mutation in tumor protein lysates, we evaluated how this affected inflammatory mediators in the tumor interstitial fluid. Multiplex ELISA demonstrated that high concentrations of IL-6 and VEGF were present in the interstitial fluid of C3HBA tumors, with a significantly higher IL-6 level in C3HBA tumors in Chy mice than in wt mice (Figure 4A), thus confirming the western blot analysis for this cytokine. While the western blots measured the total protein content of cytokines within the tumors, the ELISA measured cytokine levels exclusively in the extracellular fluid phase. Thus, the discrepancy between western blots and ELISA for the remaining cytokines assessed may be the result of differential binding of secreted cytokines to extracellular matrix proteins or intracellular sequestration of cytokines that is not detected in the interstitial fluid phase [25, 26]. In KHT-1 sarcomas no difference was detected in IL-6 levels between Chy mice and wt siblings (Figure 4B), which could be due to a high content of necrotic tissue in these tumors, causing spill-over of proteins between the intracellular and extracellular compartments.

Based on the elevated IL-6 levels in C3HBA tumors in Chy mice, both intracellularly and in the tumor interstitial fluid, we assessed the influence of the IL-6 receptor antibody tocilizumab on tumor growth. Treatment with tocilizumab significantly reduced tumor growth both in Chy and wt mice when compared to sham treatment (Figure 4C). Western blot analysis of phosphorylated STAT3 (Ser727) confirmed that tocilizumab blocked IL-6 downstream signaling within the tumors (Figure 4D). This demonstrates that IL-6 is important to C3HBA tumor growth, both in Chy mice and wt siblings, although the profound difference in IL-6 can not alone explain the different growth kinetics.

DISCUSSION

Increased lymphatic vessel density is associated with enhanced metastatic potential and poor prognosis [1–3]. Interestingly, we found accelerated tumor growth in mice with impaired lymphangiogenesis implanted orthotopically with C3HBA breast cancer or KHT-1 fibrosarcoma. In addition to reduced lymphatic drainage from the C3HBA breast cancers, Chy mice had a reduced number of macrophages,
and in particular M1 macrophages recruited to the peritumoral area, indicating a weakened anti-tumor immune response. It is well established that tumor associated macrophages (TAMs) of the M2 subtype contribute to malignant progression and are associated with poor prognosis in patients with advanced cancer [27] whereas our results suggest that M1 macrophages could protect against tumor progression in the early stages of cancer development as seen in mice without the Chy mutation. Importantly though, these findings should be confirmed in Chy mice with spontaneous cancers in the future.

**Figure 4:**

A. Multiplex ELISA of tumor interstitial fluid from C3HBA tumors demonstrating significantly higher IL-6 level in Chy compared to wt mice. Bars depict the mean cytokine concentration ± SEM, n=8 per group (except GM-CSF: n=3 per group). *p<0.05.

B. ELISA of tumor interstitial fluid from KHT-1 tumors demonstrating no significant difference in IL-6 levels between Chy and wt mice. Bars depict the mean cytokine concentration ± SEM, n=5 per group.

C. Tumor growth of C3HBA breast cancer in Chy and wt mice treated with either IL-6 receptor antibody (tocilizumab) or placebo (saline) i.p for 21 days. The graphs depict the mean tumor volume ± SEM, n=7 per group. Arrows indicate when the treatment started. Tocilizumab significantly reduced tumor growth both in Chy and wt mice. *p<0.05.

D. Western blot analysis demonstrating reduced STAT3 phosphorylation (Ser727) in C3HBA tumors when IL-6 signaling is inhibited with tocilizumab. Densitometry of western blots presented as the ratio of protein of interest normalized to STAT3 and actin expression. Bars depict the mean ± SEM, n=3 per group. *p<0.05.
While the impaired lymphangiogenesis in Chy mice did not affect interstitial fluid pressure inside C3HBA and KHT-1 tumors, lymphatic drainage from the peritumoral area was reduced. This is in line with a previous report in non-tumor bearing Chy mice [28]. Lymphatic peritumoral drainage transports cytokines and antigens to the lymph nodes to elicit an immune response [29], and this process is impaired in mice with reduced lymphangiogenesis [15]. In our study, less macrophages and leukocytes were recruited to the tumor vicinity together with a significant downregulation of macrophage-associated cytokines, pointing to a weakened innate anti-tumor immune response. This is in accordance with recent data demonstrating augmented melanoma and lymphoma growth, as well as decreased local lymph node inflammation, in kCVC mice with reduced lymphangiogenesis [15]. It has been established previously that the humoral immune response on the trunk is not influenced by the Chy mutation [28], and our current finding of no difference in the number of peritumoral adaptive immune cells between Chy and wt mice is in line with these observations.

Macrophages are immune cells characterized by heterogeneity and plasticity. The extremes of macrophage polarization have traditionally been simplified into M1 (classically activated) and M2 (alternatively activated), but the nomenclature in this field is rapidly changing [30]. Evidence from both preclinical and clinical research present a more complex picture of macrophage activation, where macrophage function and balance is skewed depending on the surrounding stimulus [31]. TAMs, which exhibit M2-like features, are known inducers of cancer progression by stimulating angiogenesis, promoting invasion, migration and suppressing antitumor immunity [32, 33]. CD163 is a macrophage-specific scavenger receptor that is upregulated by anti-inflammatory inducers and is therefore associated with macrophages bearing M2 features [34]. We established that the number of CD163 positive macrophages in Chy mice was no different from wt littermates. However, the total number of TAMs was significantly lower in Chy mice, implying a reduced number of anti-tumorigenic M1-activated macrophages to facilitate tumor progression. If tumor macrophage numbers were reduced therapeutically with liposomal clodronate, decreased tumor growth was observed, indicating a pro-tumorigenic effect of macrophages. However, the unselected killing of TAMs with this treatment [22] does not recapitulate the skewed M2/M1 ratio in Chy mice.

In our study, we examined cytokines related to inflammation from two different compartments within the tumor. Whereas the western blot analysis examined the total level of cytokines within the tumor tissue, the assessment of tumor interstitial fluid mirrors the secreted cytokines mediating signals between cells in the extracellular tumor microenvironment. Among seven cytokines analyzed in the C3HBA tumor interstitial fluid, IL-6 was the only cytokine found to be significantly higher in the extracellular microenvironment in Chy tumors. This increase appears to be breast cancer-specific since IL-6 levels were equal in serum and interstitial tissue of non-tumor bearing Chy and wt mice [12], and in KHT-1 tumors with or without the Chy mutation. There are several cell types in the tumor microenvironment that potentially could secrete IL-6; stromal cells (e.g fibroblast, adipocytes) and cancer cells [35, 36]. Indeed, Chy mice are characterized by more subcutaneous adipose tissue than their wt littermates [10]. It has been previously reported that IL-6 is significantly upregulated in peritumoral adipose tissue [37] which could explain a higher IL-6 levels in these mice. The inflammatory microenvironment created by excess adipose tissue has lately received increasing attention, and one of the key cytokines in the link between obesity and cancer is in fact IL-6 [38]. In addition to IL-6 being deposited by the increased adipose tissue, the impaired lymphatic washout in Chy mice could potentially reduce the washout of locally produced cytokines from the tumor microenvironment, thus contributing to the increased IL-6 levels in Chy tumors.

Inflammation is recognized as an enabling characteristic in cancer development [39] and IL-6 is one of several cytokines (IL-1, TNFa, IL-23) found to be essential in inflammatory processes and cancer growth [40, 41]. It has been reported previously that IL-6 induces endothelial cell proliferation [37], but we did not observe increased tumor angiogenesis in Chy tumors. IL-6 further stimulates myeloid-derived suppressor cells (MDSC) [42] and anti-tumor T-cell activity [43]. Furthermore, IL-6 mediates monocytes-to-macrophage differentiation [44, 45], while activating several known macrophage attractants (e.g CCL2, CXCL-12) [46], improving macrophage survival after recruitment [47] and polarizing them into M2 macrophages [47–49]. In the current report, inhibition of IL-6 signaling with tocilizumab yielded significant growth retardation, in orthotopic breast cancer, implicating IL-6 as a key mediator of tumor progression. While tocilizumab treatment inhibited C3HBA tumor growth both in Chy and wt mice, the substantially higher IL-6 level in Chy tumors indicates that this cytokine is relevant for the accelerated tumor growth observed.

Various therapeutic strategies aimed at tumor lymphangiogenesis are currently being tested both preclinically and clinically [6, 8]. In this regard, a pharmacological downregulation of VEGFR-3 would simulate the Chy phenotype, and should be tested in the early cancer setting experimentally. Our results indicate that reduced VEGFR-3 levels could have a detrimental effect on the immunological anti-tumor response in an early stage of primary tumor growth, through impaired lymphangiogenesis and IL-6 elevation. Interestingly, the IL-6 receptor antibody tocilizumab could be employed in this setting to counteract the progression of breast carcinomas.
MATERIALS AND METHODS

Mice

Breeding, maintenance and genotyping of heterozygous VEGFR-3 mutant mice (Chy mice) were performed as described previously [10]. The mice were maintained on a C3H background and the breeding yielded either Chy mice (VEGFR3“Chy”) or wildtype (wt) siblings (VEGFR-3“wt”). The mice were anesthetized with a mixture of ketamine (12.2 mg/ml; Ketalar, Pfizer) and metetomidine (24.3 µg/ml; Domitor, Orion Pharma) during micropuncture and wick-in-needle experiments, and with 1 % isoflurane in a combination with O2 and N2 during all other procedures. Animals were euthanized with CO2. All animal experiments were conducted in accordance with the regulations of the Norwegian State Commission for Laboratory Animals, which are consistent with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and Council of Europe (ETS 123). Experiments were performed with the approval from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited Animal Care and Use Program at University of Bergen.

Cell lines and tumor growth experiments

The C3HBA breast cancer cell line was purchased from the NCI-Frederick Cancer DCT Tumor Repository (Frederick, MD), whereas the KHT-1 sarcoma was a kind gift from Professor Michael Horsman (Aarhus, Denmark). The tumor cells were grown at 37°C in 5% CO2 in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with non-essential amino acids, 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml of streptomycin and 400 µM L-glutamine (all products: Lonza).

For implantation of mouse tumors, a tumor tissue piece of 1 mm3 was excised from an euthanized animal and implanted in the mammary fat pad (C3HBA) or s.c in the dorsal neck region (KHT-1). Female mice of fertile age were used for the C3HBA breast cancer experiment, and male and female mice of fertile age were used for the KHT-1 trial. The C3HBA and KHT-1 tumors are both syngeneic to C3H mice. For the metastasis study, liposomal clodronate and IL-6 analysis.

To assess the functional importance of IL-6, Chy and wt mice were given daily intraperitoneal injections for 21 days with either 200 µl saline (0.9% NaCl) (controls) or 200 µl of an anti-IL-6 receptor monoclonal antibody tocilizumab (RoActemra, Roche) (500 µg/ml) [35].

Lectin perfusion

Blood vessel perfusion was assessed by i.v. injection of TRITC-conjugated lectin, as described elsewhere [51]. Briefly, 100 µl (1 mg/ml) of TRITC-conjugated Lecopersicon esculentum lectin (Vector Laboratories) was injected through the tail vein in anesthetized mice. Ten minutes later, the mice were euthanized by cervical dislocation and perfused with 40 ml phosphate-buffered saline (PBS, Sigma). Tumors were harvested and snap-frozen in OCT medium in liquid nitrogen. Tumor perfusion was analyzed microscopically by measuring the area of lectin positive vessels in three vascular hot spots per tumor at 200x magnification.

eGFP transfection of the C3HBA breast cancer cell line

The pWPI plasmid (Addgene), containing the green fluorescent protein (eGFP) gene was produced in One Shot® Stbl3™ Chemically Competent E. coli (Invitrogen), under growth selection of ampicillin (Pentrexyl, Bristol-Myers Squibb), and positive clones were verified by sequencing. HEK293FT cells (Invitrogen) were co-transfected with envelope plasmid pMD2.g, packaging plasmid psPAX2 (Addgene) and the pWPI plasmid, using Lipofectamine 2000 (Invitrogen). The fully competent lentivirus was harvested and used for transduction of C3HBA cells with pWPI. The in vitro transduction efficacy was assessed based on eGFP protein expression using the NucleoCounter® NC-3000TM (Chemometec), and the cells were sorted using a BD FACSARIA (BD Biosciences) at the Flow Cytometry Core Facility (University of Bergen) to enrich for eGFP+ cells.
RNA extraction and cDNA production

RNA extraction was performed using the mirVana™ kit (Life Technologies). After DNase treatment (DNA-free™ kit, Ambion), the RNA concentrations were measured using a NanoDrop spectrophotometer (Thermo Scientific), and cDNA was made from 500 ng RNA per sample, using the qScript cDNA Supermix (Quanta BioSciences). The cDNA was then treated with 1 μl RNAse H (Invitrogen) to remove remaining RNA. RT-PCR was done using AmpliTaq Gold polymerase, and PCR reactions were run with 35 cycles and the appropriate temperature settings. A β-actin PCR reaction was undertaken to ensure equal cDNA content in the samples. All PCR products were checked for specificity by Sanger sequencing.

Primers:
- eGFP F2: GAGCTGGACGCGACGTAAC
- eGFP R2: CACGAACTCCAGGACGACCATG
- β-actin_F1m: TGGCATTGTTACCAACTGGG
- β-actin_R1m: AGTTTTCATGGATGCCACAGG

Lymphatic drainage

Lymphatic drainage from the skin directly overlying the tumor was assessed by optically monitoring the depot clearance of near-infrared labelled albumin as described previously [13]. Briefly, 0.5 μl Alexa 680-conjugated bovine serum albumin (Invitrogen) was injected intradermally with a graded Hamilton syringe (34 G), and the mice were optically imaged every 60 min for a 6 hour period using an Optix® MX system (GE Healthcare). Mice were anesthetized (1% isofluorane) only during imaging and remained awake in between measurements. Images were analyzed using eXplore Optix Optiview software (GE Healthcare), and the number of counts calculated for each region of interest. For calculations of depot clearance rates (k) the natural logarithm of the fractional amount of counts remaining at the injection site was plotted against time. The resultant monoexponential curves were fitted with linear regression, and k found as the slope of each curve.

Tumor interstitial fluid pressure

Tumor interstitial fluid pressure (IFP) was measured in wt and Chy mice using the micropuncture and wick-in-needle (WIN) techniques, as described previously [17]. Briefly, the IFP in superficial areas (0-1 mm depth) was recorded in anesthetized mice using a sharpened micropipette (diameter; 4-7 μm), connected to a servo-controlled counter-pressure system that was inserted through intact skin (18). To assess the IFP in deep intratumoral areas, a 23 G needle with a 2-4 mm side hole and filled with nylon fibers and saline (WIN technique), was inserted into the central part of the tumor and connected to a transducer dome through a saline filled PE-50 catheter [17].

Primary antibodies

Immunohistochemistry/immunofluorescence


Western blots

Rabbit anti-actin (Sigma), rat anti-IL1-beta/IL-1F2 (R&D), rabbit anti-TGF-beta (Nordic Biosite), rat anti-GM-CSF (Abcam), rat anti-IL10 (Abcam), goat anti-M-CSF (Abcam), goat anti-VEGFD (Abcam), rabbit anti-VEGF (Abcam), rabbit anti-VEGFC (Abcam), rabbit anti-PIGF (Abcam), rabbit anti-IL-6 (Abcam), rabbit anti-phospho (Ser727) STAT3 (Cell Signaling), rabbit anti-phospho, (Tyr705) STAT3 (Cell Signaling), rabbit anti-STAT3 (Cell Signaling). Anti-GM-CSF, IL-10 and IL-1beta were monoclonal antibodies, all other antibodies were polyclonal.

Immunohistochemistry of paraffin embedded tissue sections

Tissues were fixed in formalin, paraffin embedded and 4 mm sections prepared. Sections were deparaffinized and rehydrated, before antigen retrieval at 98°C for 1 hour in 0.01 M citrate buffer (pH 6.0). After blocking with diluted serum from the secondary antibody host for 30 min, the slides were incubated overnight (+4°C) with the primary antibody. After blocking endogenous peroxidase activity for 20 min with 3% hydrogen peroxide (Sigma), a biotinylated anti-rat or anti-rabbit secondary antibody (Vector Laboratories) was applied for 30 min as appropriate. The antigen-antibody complex reaction was augmented with avidin-biotin-peroxidase (ABC) for 30 min according to the manufacturer’s instructions (Vectastain® ABC Kit, Vector), and stained for 2-10 min with diamino-benzidine tetrahydrochloride (DAB, Vector). The sections were then counterstained with haematoxylin (Fisher), dehydrated and mounted with Entellan (Electron Microscopy Services). Parallel sections were run for all the experiments without primary antibody, to assure the specificity of the immunoreactions.

Blood vessel density (BVD) was assessed by immunolabeling for CD31, using the microvessel density (MVD) method [52]. Briefly, the mean BVD in three vascular hot spots per tumor was assessed at 200x magnification.

Intratumoral macrophages were quantified by immunolabeling with a CD11b antibody, which was the only antibody that yielded acceptable staining within the tumor tissue, while peritumoral macrophages were quantified by immunolabeling with CD11b, F4/80 and a CD163 antibody. CD3, CD20 and FOXP3 antibodies were used to quantify adaptive immune cells in C3HBA and
KHT-1 tumors, but extensive necrotic areas within KHT-1 tumors yielded too much unspecific staining for these parameters to be quantified. The number of cells per field-of-view was counted in three hot spots at 200x, 400x or 600x objective magnification.

**Western blot**

Cells and tissues were homogenized and lysed in a custom made total protein lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) containing a protease and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Protein concentrations were measured by a bicinchoninic acid (BCA) assay (Pierce), and 30 mg protein was loaded per lane for all the immunoblots. The protein lysates were fractionated by electrophoresis using NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen) and transblotted to nitrocellulose membranes using the XCell II Blot Module (Invitrogen). Adequate sample transfer was confirmed by staining the blot with Ponseau S solution (Sigma). Thereafter the membranes were blocked with 5% bovine serum albumine (Sigma) for 60 min, before immunoblotting overnight with the primary antibody. The immobilized antibody was detected using a horseradish peroxidase-conjugated secondary antibody and stained with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The immunoreaction was visualized using a LAS-3000 imaging system (FujiFilm, Tokyo, Japan). Immunoblots for actin were made for all samples to assure equal protein loading. The western blot protein bands were compared by automated densitometry using Image J software (NIH, USA).

**Sampling and ELISA of tumor interstitial fluid**

Interstitial fluid was sampled from tumors in Chy and wt mice, as described previously [53]. Briefly, the tumor tissue was put on a mesh before centrifugation at 424 g for 10 min, yielding 10-50 ml of interstitial fluid. The collected fluid samples were stored at -80°C prior to analysis. The interstitial fluid was assessed for a panel of common inflammatory cytokines using a multiplex fluorescent bead immunoassay kit (Millipore) and an IL-6 ELISA kit (R&D systems), according to our previous protocol [12]. Samples were diluted with serum matrix diluent and run according to the manufacturers’ instructions. Total surface fluorescence was measured with a flow-based dual laser system (Luminex100, Luminex Corporation). Cytokine concentrations were automatically calculated based on standard curve data.

**Statistics**

Data were compared using the nonpaired Student’s t-test unless otherwise specified. p <0.05 was considered statistically significant. In the tocilizumab treatment study, data were analyzed using two-way analysis of variance (ANOVA).

**ACKNOWLEDGMENTS**

The C3H101H-Flt4<Chy>/H mice (repository number is EM:00068) were obtained from the MRC-Harwell which distributes this strain on behalf of the European Mouse Mutant Archive (EMMA: www.infrainfrontier.eu). Chy mice used in preliminary experiments were a kind gift from Professor Kari Alitalo. We gratefully acknowledge Rolf Bjerkvig for providing us with immunohistochemistry resources used in the present work. We also thank Åse Rye Eriksen for technical assistance and Helene Stuart and Gry Bernes for assistance in the animal facility.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

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**Author contributions**


**REFERENCES**


through downregulation of PlGF. Cancer cell. 2011; 19:31-44.


Impaired lymphatic function accelerates cancer growth

**SUPPLEMENTARY FIGURES**

**Supplementary Figure S1:**

**A.** Growth of C3HBA breast cancers in wt mice, demonstrating significant tumor inhibition during treatment with liposomal clodronate, compared to sham treatment. Treatment commenced the day before C3HBA was injected into the mammary fat pad. Graphs depict the mean tumor volume ± SEM, n=8 per group.

**B.** Immunohistochemistry for F4/80 demonstrates significantly less peritumoral macrophages in Chy than in wt mice. Scale bars: 100 μm. Bars graphs depict the mean number of cells per field-of-view ± SEM, n=3 per group. *p<0.05. **p<0.01.

**C.** Immunohistochemistry for CD31 demonstrates significantly less intratumoral blood vessels in Chy than in wt mice. Scale bars: 100 μm. Bar graph depicts the mean blood vessel density (BVD) ± SEM, n=3 per group. *p<0.05. **p<0.01.
Supplementary Figure S2: A-D. Immunohistochemistry for adaptive immune cells by CD3, CD20 and FOXP3 antibodies. There was no significant difference in the number of intratumoral CD3⁺, CD20⁺ or FOXP3⁺ cells between Chy and wt mice. Bar graphs depict the mean number of cells per field-of-view ± SEM, n=3 per group. Scale bars: 100 μm.
Lymphatic vessels regulate immune microenvironments in human and murine melanoma

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Lymphatic remodeling in tumor microenvironments correlates with progression and metastasis, and local lymphatic vessels play complex and poorly understood roles in tumor immunity. Tumor lymphangiogenesis is associated with increased immune suppression, yet lymphatic vessels are required for fluid drainage and immune cell trafficking to lymph nodes, where adaptive immune responses are mounted. Here, we examined the contribution of lymphatic drainage to tumor inflammation and immunity using a mouse model that lacks dermal lymphatic vessels (K14-VEGFR3-Ig mice). Melanomas implanted in these mice grew robustly, but exhibited drastically reduced cytokine expression and leukocyte infiltration compared with those implanted in control animals. In the absence of local immune suppression, transferred cytotoxic T cells more effectively controlled tumors in K14-VEGFR3-Ig mice than in control mice. Furthermore, gene expression analysis of human melanoma samples revealed that patient immune parameters are markedly stratified by levels of lymphatic markers. This work suggests that the establishment of tumor-associated inflammation and immunity critically depends on lymphatic vessel remodeling and drainage. Moreover, these results have implications for immunotherapies, the efficacies of which are regulated by the tumor immune microenvironment.

Introduction

The aberrant growth of regional lymphatic vessels — often referred to as lymphangiogenesis or lymphatic hyperplasia — is associated with enhanced locoregional metastasis and poor outcome in many solid tumors including melanoma (1). Lymphatic vessels contribute to tumor progression at least in part by actively facilitating metastatic dissemination to sentinel lymph nodes through a variety of mechanisms, both in primary tumors as well as at distal sites (2). However, sentinel lymph nodes are also sites where antitumor immune responses can be generated, and thus lymphatic drainage from the tumor likely plays multiple and complex roles in tumor progression. To date, however, there is little understanding of how this communication pathway between tumors and sentinel lymph nodes contributes to the host immune response to the tumor and its progression.

Lymphatic vessels carry fluid and immune cells from peripheral tissues to draining lymph nodes (dLNs), where both components help shape immunity and maintain tolerance to self-antigens (3–5). Without local lymphatic vessels and their associated drainage, peripherally activated dendritic cells (DCs) cannot traffic to the dLNs to activate immune responses (6), and LN resident immature DCs are not exposed to lymph-borne self-antigens released from extracellular proteases and apoptotic cells for tolerogenic presentation to autoreactive T cells (7, 8). Additionally, lymphatic endothelial cells (LECs) themselves can contribute to regional immunity in other ways, including active regulation of fluid drainage (9), direct modulation of DC trafficking and activation (10, 11), cellular egress leading to immune resolution (12, 13), and direct suppression of lymphocyte activation through steady-state presentation of endogenous self antigens (14) or cross-presentation of draining exogenous antigens (7, 15, 16). Importantly, lymphangiogenesis is seen in a host of inflammatory situations, including melanoma and other cancers (1, 17). However, the role of lymphangiogenesis in inflammation and immunity remains unclear. The abundant clinical and experimental evidence correlating lymphangiogenesis with tumor progression contrasts with reports that lymphangiogenesis in tissue transplantation can promote graft rejection (18), and further confounding the issue are reports suggesting that lymphangiogenesis promotes immune resolution in chronic inflammation (12, 13). Therefore, it is likely that lymphatic vessels may serve multiple and complex roles in both the induction and resolution of local immune responses in acute versus chronic inflammation (19).

Inflammation and immunity can play important roles in the initiation, promotion, and metastatic progression of many types of solid tumors. Tumors establish mechanisms to counteract host immunity, and it is the balance between pro- and antitumor inflammatory mediators that likely dictates tumor progression (20, 21). While different types of inflammation can either pro-

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rather than passive conduits for tumor metastasis, lymphatic vessels are likely context-dependent regulators of local inflammation and immunity. We recently reported that in a model of murine melanoma, lymphangiogenesis resulted in impaired adaptive immune responses through direct suppression of CD8+ T cells (16). On the other hand, as stated above, lymphatic vessels communicate information and transport immune cells, antigens, and signals from the periphery to the dLN, implying that lymphatic vessels must be required for initiating an immune response against a growing tumor. In this work, we explore the relationship between local lymphatic vessels and immune infiltration in melanoma. In human metastatic melanoma, we found strong correlations between the expression levels of lymphatic genes (PDPN, LYVE1, and VEGFC) and inflammatory cell markers and cytokines. In murine melanoma, we asked whether tumors developing in the absence of local lymphatics or suppress tumor progression in different cancers (21), successful immunotherapy directs productive immune-mediated tumor killing and regression (21–23). Current clinical trials testing checkpoint blockade strategies (e.g., anti-CTLA-4 and anti-PD-1) in metastatic melanoma are demonstrating improved survival in a subset of patients (24, 25). Interestingly, those patients that respond to such strategies appear to stratify by the preexistence of immune cell infiltration (26–28), particularly CD8+ T cells. The finding that some patients lack tumor-infiltrating lymphocytes and consequently demonstrate poor response to immunotherapy (28, 29) indicates that endogenous mechanisms regulating immune induction in the tumor may be responsible for therapeutic resistance (4). While lymphatic vessels and their associated drainage function facilitates communication between tumors and the adaptive immune response, very little is known about how their remodeling may influence the subsequent antitumor immune response. Rather than passive conduits for tumor metastasis, lymphatic vessels are likely context-dependent regulators of local inflammation and immunity. We recently reported that in a model of murine melanoma, lymphangiogenesis resulted in impaired adaptive immune responses through direct suppression of CD8+ T cells (16). On the other hand, as stated above, lymphatic vessels communicate information and transport immune cells, antigens, and signals from the periphery to the dLN, implying that lymphatic vessels must be required for initiating an immune response against a growing tumor. In this work, we explore the relationship between local lymphatic vessels and immune infiltration in melanoma. In human metastatic melanoma, we found strong correlations between the expression levels of lymphatic genes (PDPN, LYVE1, and VEGFC) and inflammatory cell markers and cytokines. In murine melanoma, we asked whether tumors developing in the absence of local lymphatics...
lymphatic vessels, and therefore without communication with the
dLN, can acquire an inflammatory microenvironment that is typi-
cally seen otherwise. We found that in mice lacking dermal lym-
phatic capillaries, intradermally implanted B16 melanomas were
substantially less infiltrated by immune cells, contained markedly
reduced levels of inflammatory cytokines, and failed to induce
cellular adaptive immune responses. Given the importance of
endogenous immune recognition for successful immunotherapy,
lymphatic vessels may provide an intriguing control point for reg-
ulating antitumor immunity and response to therapy.

Results
In human metastatic cutaneous melanoma, lymphatic gene expres-
sion correlates with immune parameters. Lymphatic vessel density
is associated with melanoma stage and locoregional metastasis (1),
but it is unknown how it correlates with tumor immune infil-
tration. First, we categorized 266 human metastatic cutaneous
melanoma samples (The Cancer Genome Atlas, TCGA) (30)
to determine whether correlations could be found between the
expression levels of lymphatic genes with genes associated with
immune infiltration. Of note, in the tumor microenvironment,
none of the most common LEC markers — podoplanin (PDPN),
LYVE1, and PROX1 — are completely specific to LECs; for exam-
ple, some tumor-associated macrophages can express LYVE1 (31),
and stromal fibroblasts may express PDPN (32). Furthermore,
we found that human metastatic melanoma can substantially express
PROX1 (Supplemental Figure 1A; supplemental material available
online with this article; doi:10.1172/JCI79434DS1). Therefore,
we first examined which of these LEC genes correlated most strongly
with the lymphatic growth factor VEGFC. Interestingly, we found
strong correlations among the gene expression levels of PDPN,
LYVE1, and VEGFC, but no correlation between any of these and
PROX1, VEGFD, VEGFA, or VEGFB (Supplemental Figure 1B).
We therefore defined a lymphatic score (LS) to represent the relative
expression levels of PDPN, LYVE1, and VEGFC in each sample
(see Methods) and further segregated samples into 3 groups repres-
enting low (LS0), intermediate (LS=0), and high (LS6) degrees of
lymphatic vessel involvement such that 50% of the patients were

Figure 2. Impaired tumor drainage and DC trafficking
to local lymph nodes in K14-VEGFR3-Ig mice. B16F10
tumors were implanted intradermally into WT or K14-
VEGFR3-Ig transgenic (Tg) mice and excised at day 9.
(A) Peritumoral area from WT or Tg mice stained for
lymphatic endothelial cells (LYVE1; n = 3; n.d., not detect-
ed) (arrowhead). Scale bar: 50 μm. (B) Lymphatic vessel
density quantified as LYVE1 structures per 0.04 mm².
Data were compared using Student’s unpaired t test.
**P < 0.01. (C) Intratumoral area stained for blood endo-
thelial cells (CD31, n = 3) (arrowhead). Scale bar: 50 μm.
(D) Blood vessel density quantification as CD31 struc-
tures per 0.04 mm². (E) Tumor growth profiles in WT and
Tg mice over 13 days. (F) Fluid drainage from the tumor to
the draining lymph node (dLN, brachial) assessed 30 min-
utes after intratumoral injection of 70-kDa FITC-dextran
and plotted as fluorescence intensity in arbitrary units
(A.U.) per LN normalized to WT (n = 7). (G) DC trafficking
from the tumor to the dLN, determined by the quanti-
fication of CD11c+MHCII+FITC+ cells in the dLN 24 hours after
intratumoral injection of 0.5-μm FITC-labeled latex beads
and representative flow cytometry dot plots (n = 5). (H) Quantification of bead+ DCs in the LN. (I) Comparison of relative B cell (B220+) and T cell (CD3ε+) populations in the
dLNs to nondraining lymph nodes (ndLN) (n ≥ 4). Data are
represented as the mean ± SEM. Statistical analysis with
Mann-Whitney U test. *P < 0.05, **P < 0.01.
Dermal lymphatic vessels are required for LN responses to intra-
dermally implanted murine melanoma. Given the positive correla-
tion between LS and immune cell markers in human metastatic
melanoma, we wanted to experimentally probe the role of lym-
phatic vessels in the generation of inflammatory and immune
responses against melanoma. We implanted B16F10 melanomas
into the dermis of transgenic K14-VEGFR3-Ig mice (referred to in
the figures as Tg mice); these mice lack dermal lymphatic vessels
due to the expression of a soluble VEGFR3-Ig under the control of
the epithelial keratin 14 (K14) promoter, which effectively ‘traps’
VEGFR3 ligands (VEGF-C and VEGF-D) to prevent local VEGFR3
signaling (33). Histological analysis of tumor tissue demonstrated
maintenance of the lymphatic-free phenotype in melanomas in
LS^{hi} (within 2/3 of the SD around the mean) and 25% were above
and below (Supplemental Figure 2A). This cumulative score cor-
related very strongly with VEGFC, PDPN, and LYVE1, but again
not with VEGA, VEGFB, or VEGFD (Supplemental Figure 2B).

Using this data set, we correlated LS with immune markers
to evaluate the relationship between lymphatic involvement and
immune infiltration. We observed strong positive correlations
between LS and a variety of immune cell–specific genes including
CD45, CD11B, F4/80, KLRB1, CD3D, CD8A, CD4, and FOXP3 (Fig-
ure 1A). Furthermore, LS^{lo} patients consistently exhibited higher
expression of these immune markers than LS^{hi} and LS^{lo} (Figure
1B), indicating strong correlations between lymphatic vessel genes
and immune infiltrates in human metastatic cutaneous melanoma.

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Figure 3. B16F10 melanomas implanted in K14-VEGFR3-Ig mice lack a local inflammatory infiltrate. The inflammatory infiltrate was determined on day 9 in B16F10 tumors grown in WT and K14-VEGFR3-Ig (Tg) mice. (A) Immunohistochemical analysis of tumor immune infiltrates in WT and Tg mice. Scale bars: 50 μm (CD11b, F4/80, and CD3ε) and 100 μm (MHCII). (B–G) Analysis of immune cell populations in the tumor (B–D) and spleen (E–G) by flow cytometry. (B and E) Total leukocytes (CD45+), (C and F) Treg cells (CD3ε+CD4+CD25+FoxP3+), and (D and G) inflammatory monocytes (CD11c+CD11b–F4/80–Ly6c–Ly6g–). n ≥ 4. Statistical analysis with Mann–Whitney U test. *P < 0.05, **P < 0.01.

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K14-VEGFR3-Ig mice (Figure 2, A and B). No significant changes were observed in blood vessel densities as analyzed by numbers of CD31+ structures per area (Figure 2, C and D); however, the percentage area stained by CD31 was decreased (Supplemental Figure 3), perhaps reflecting smaller vessels as previously reported (34). The lack of dermal lymphatic vessels did not affect tumor growth rates (Figure 2E), and did not appear to affect local collagen density (by picrosirius red staining) or fibroblast phenotype (by α-smooth muscle actin (α-SMA) immunohistochemistry) in either normal skin or tumor stroma (Supplemental Figure 4).

To determine the degree of disrupted communication between the tumor and dLN due to missing dermal lymphatic vessels, we quantified the total amount of fluorescently labeled dextran (70 kDa) in the dLNs (brachial) 30 minutes after injection into tumors at day 9. Not unsurprisingly, we found a roughly 5-fold decrease in the amount of dextran contained in the dLNs of K14-VEGFR3-Ig mice compared with WT mice (Figure 2F). The axillary LNs of WT mice also contained dextran but to a much lesser extent compared with the brachial, and we found no evidence of dextran in any other LNs including the inguinal (data not shown). We also quantified DC trafficking following intratumoral injection of FITC-labeled latex beads (0.5 μm), which are too large to flow into lymphatic vessels and instead must be taken up by DCs in the tumor before being transported to the dLN (35). As expected, DC trafficking was drastically reduced in K14-VEGFR3-Ig mice (Figure 2, G and H), whose LNs had demonstrated lower total cellularity, but a significantly higher percentage of CD3ε+ T cells before tumor implantation than those in WT mice (Supplemental Figure 5 and Figure 2I).

In WT mice, LNs draining implanted B16 melanomas were enlarged compared with nondraining LNs, with increased fractions of T cells, reflecting T cell expansion (Figure 2I). Given the importance of lymphatic transport of fluid, antigens, and DCs in mounting such immune responses, we predicted that these LN changes would be absent in tumor-bearing K14-VEGFR3-Ig mice. Indeed, we found that relative lymphocyte fractions were similar in tumor-draining versus nondraining LNs in transgenic mice (Figure 2J), suggesting a lack of immune response to the growing tumor.

Reduction of tumor-infiltrating leukocytes and inflammatory cytokines in mice lacking dermal lymphatic vessels. Next, we analyzed immune cell infiltration in B16F10 tumors implanted in K14-VEGFR3-Ig and WT mice. Immunohistochemical staining for CD11b, F4/80, MHCII, and CD3ε revealed reduced numbers of intratumoral leukocytes (Figure 3A). Flow cytometric analysis of B16F10 tumors demonstrated a 3-fold reduction of CD45+ cells in tumors growing in K14-VEGFR3-Ig compared with WT mice (Figure 3B). Further breakdown of the CD45+ population demonstrated a broad reduction of the most dominant immune subsets, including B cells (B220+), antigen-presenting cells (CD11b+ and CD11c+) and CD3ε+ T cells as well as Treg cells (CD3ε+ CD4+ CD25+ FoxP3+) and immature inflammatory monocytes (CD11c Cd11b+F4/80 MHCIi Ly6g Ly6c+) (Figure 3, B–D, and Supplemental Figure 6). These differences were due to immune infiltration into the tumor, since no differences were observed in homeostatic cutaneous and splenic immune populations (Supplemental Figure 7). Similarly, no differences were found between the systemic leukocyte populations (spleen) in tumor-bearing WT and K14-VEGFR3-Ig mice (Figure 3, E–G).

Mirroring the decrease in immune cell infiltrates, we found substantial decreases in the most abundant inflammatory cytokines in tumors growing without lymphatic capillaries (Figure 4A and Supplemental Figure 8, A–C). Similarly, in human cutaneous metastatic melanoma, gene expression of inflammatory cytokines correlated with LS (Supplemental Figure 8D). Again, stratification of patients...
into LS\textsuperscript{hi}, LS\textsuperscript{mid}, and LS\textsuperscript{lo} populations predicted high, intermediate, and low expression of each inflammatory factor, respectively (Figure 4B), suggesting that LS strongly correlates with leukocyte infiltration and cytokine expression in human melanoma.

We confirmed this phenotype in a second model of lymphatic vessel dysfunction (Chy mice, C3H background). Chy mice harbor a heterozygous inactivating mutation in the tyrosine kinase domain of VEGFR3 (36), causing a decrease in lymphatic transport from skin (37). Notably, in this model, dermal lymphatic vessels have been shown to be mostly absent in extremities (i.e., forelimb and ear skin), while trunk skin contains sparse residual lymphatic capillaries able to support cellular egress from tissue (6). We therefore implanted syngeneic C3HBA breast carcinoma cells into ear dermis of Chy mice and WT littermates. Tumors demonstrated enhanced growth in Chy mice (data not shown) and surprisingly, demonstrated some LYVE1\textsuperscript{+} structures surrounding tumors, although at a significantly lower density than tumors in WT littermates (Supplemental Figure 9). This decrease in lymphatic vessels corresponded to decreased CD45\textsuperscript{+} leukocytic infiltration and a notable lack of CD3\textsuperscript{ε+} T cells in and around the tumor (Supplemental Figure 9). Although the differences in leukocytic infiltration in tumors growing in Chy mice were not as pronounced as those in tumors in K14-VEGFR3-Ig mice, this result is consistent with the decrease in lymphatic density being less pronounced as well.

Given the presence of soluble VEGFR3 within the adult K14-VEGFR3-Ig mice, we sought to verify that our results were due to absence of a lymphatic vasculature rather than inhibition of local VEGFR3 signaling during tumor development. To test this, we implanted tumors into WT C57BL/6 mice and administered a VEGFR3-blocking antibody at the time of tumor implantation and every 3 days thereafter (Supplemental Figure 10A). The inhibition of VEGFR3 signaling during tumor growth did not significantly alter fluid drainage out of tumors to dLNs (Supplemental Figure 10B), indicating that preexisting lymphatic vessels were functionally in maintaining communication with the dLN. Additionally, no differences were observed within leukocyte populations in either the tumors (Supplemental Figure 10, C and D) or spleens (Supplemental Figure 10, E and F).

**Decreased metastatic dissemination in K14-VEGFR3-Ig mice.** Following sacrifice at later time points, we quantified the incidence of spontaneous lung metastases. In WT mice, 10 of 16 implanted tumors metastasized and colonized lungs, while only 2 of 15 tumors implanted in K14-VEGFR3-Ig mice successfully colonized the lung (Figure 5, A and B). To rule out the possibility that circulating tumor cells in transgenic mice had decreased potential to extravasate into and colonize lung tissue, we injected tumor cells intravenously and found no differences in either incidence of lung colonization or percentage of lung area covered by metastatic tumor cells between WT and transgenic mice (Figure 5, C–E). Considering that the lym-
phatic vasculature in the lung is intact in K14-VEGFR3-Ig mice, we also analyzed macrophage infiltration into these metastatic lung nodules and found no differences in macrophage infiltration compared with WT mice (Figure 5, F and G).

**Dermal vaccination does not control primary tumor growth in the absence of lymphatic vessels.** We previously reported evidence that ovalbumin-expressing (OVA-expressing) B16F10 (B16F10.OVA) tumors respond to prophylactic vaccination against OVA (16), where intradermal vaccination resulted in potent activation of immune cells within the dLN, and activated CD8+ T cells homed to tumors and controlled primary tumor growth. We applied this model vaccine to our K14-VEGFR3-Ig mice to determine whether immune activation would be delayed in these animals. Ten days before tumor implantation, naïve mice were vaccinated against OVA (10 μg) with LPS (10 μg) intradermally in forelimbs. Subsequently, a vaccine boost was given 5 days after implantation and tumor growth was monitored for 1 week (Figure 6A). Interestingly, while vaccination controlled tumor growth in WT mice, it had no effect on tumors in K14-VEGFR3-Ig mice (Figure 6B). The altered responsiveness to the vaccine correlated with an impaired number of circulating antigen-specific CD8+ T lymphocytes as detected by H-2Kb SIINFEKL pentamer staining on day 12 (Figure 6C and D). Representative flow cytometry plots showing intracellular cytokine staining for IFN-γ and quantification of IFN-γ+ CD8+ T cells following peptide restimulation of tumor-draining LN cells on day 12 (Figure 6E and F). Representative flow cytometry plots (Figure 6G and H) quantification of tumor-infiltrating antigen-specific CD8+ T cells detected by H-2Kb SIINFEKL pentamer staining on day 12 (n = 5, in at least 2 separate experiments). Box and whisker plots show data from min to max. Statistical analysis with 1-way ANOVA. *P < 0.05.

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Figure 6. K14-VEGFR3-Ig mice demonstrate impaired antitumor immunity in response to dermal vaccine delivery. (A) Experimental schematic. WT or K14-VEGFR3-Ig (Tg) mice were vaccinated intradermally (i.d.) in the forelimbs with either 10 μg ovalbumin (OVA) and 10 μg LPS, or 10 μg LPS alone. After 10 days, B16F10.OVA tumor cells (0.5 × 10⁶) were injected i.d. in the back skin and the vaccine was boosted, at the same dose, on day 15. Mice were sacrificed on day 22 (12 days after tumor implantation). (B) Tumor growth was slower in vaccinated vs. control mice for melanomas in WT (left), but not Tg (right) mice. P values determined by regression analysis. *P < 0.05. (C) Representative plots and (D) quantification of circulating antigen-specific CD8+ T lymphocytes as detected by H-2Kb SIINFEKL pentamer staining on day 12. (E) Representative flow cytometry plots showing intracellular cytokine staining for IFN-γ and (F) quantification of IFN-γ+ CD8+ T cells following peptide restimulation of tumor-draining LN cells on day 12. (G) Representative flow cytometry plots and (H) quantification of tumor-infiltrating antigen-specific CD8+ T cells detected by H-2Kb SIINFEKL pentamer staining on day 12 (n = 5, in at least 2 separate experiments). Box and whisker plots show data from min to max. Statistical analysis with 1-way ANOVA. *P < 0.05.
mice (Figure 6, G and H). These findings are consistent with our previous findings that K14-VEGFR3-Ig mice respond in a delayed manner to vaccination (8).

**Tumors established in the absence of lymphatic vessels are more vulnerable to transferred T cells.** Although K14-VEGFR3-Ig mice were unable to induce a potent adaptive T cell response against B16F10.OVA tumors expressing a model antigen, the absence of tumor-infiltrating Treg cells and inflammatory monocytes indicates a more permissive microenvironment for effector T cell function. We activated antigen-specific CD8+ T cells (OT-I) ex vivo with SIINFEKL-loaded splenic DCs and IL-2 (10 U/ml) for 4 days. Five days after implantation, 10^6 activated CD8+ T cells were transferred intravenously and tumor growth was monitored for 9 days (Figure 7A). As expected, transferred, activated CD8+ T cells significantly controlled growth of B16F10.OVA cells in WT mice (Figure 7B). Interestingly, regression of B16F10.OVA growth in K14-VEGFR3-Ig mice was significantly increased when compared with littermates, with respect to both level of regression and time to onset (Figure 7, B and C).

Total circulating CD45+ leukocytes (Figure 7D), antigen-specific T cells (Figure 7E), or functional IFN-γ production by CD8+ T cells in spleen following restimulation (Figure 7F) were equal in both strains. Within tumor microenvironments, however, a significant infiltration of CD45+ cells was observed in K14-VEGFR3-Ig mice over controls (Figure 7G). This corresponded with an increase in tumor-infiltrating antigen-specific CD8+ T cells (Figure 7H). To test CD8+ T cell functionality within tumor microenvironments, we performed an in vivo brefeldin A assay for intracellular staining of IFN-γ. Enhanced IFN-γ production was observed in T cells infiltrating tumors lacking a lymphatic compartment (Figure 7I).

**Discussion**

Melanoma is a particularly attractive target for immunotherapy given its inherent immunogenicity and high frequency of infiltrating intratumoral lymphocytes in a major subset of patients (38, 39). Furthermore, several immunotherapeutic strategies to treat metastatic melanoma appear to be dependent upon preexistence of an immune infiltrate (26, 27, 40). It remains unclear, however, what determines the propensity of an individual patient to generate an endogenous response against their tumor. In this study, we examined the role that lymphatic vasculature plays in generation of endogenous inflammatory and adaptive immune responses within and against a developing malignancy. Using a cohort of 266 cutaneous metastatic melanoma patients, we established an LS based on expression of the lymphatic vessel markers PDPN and LYVE1. These markers significantly correlated with each other as well as with VEGFC. Interestingly, LS did not correlate with oth-
er VEGFs, indicating specificity to the VEGF-C/VEGFR3 signaling axis. Patients who scored high for LS consistently had higher expression of immune and inflammatory markers, whereas those who scored low presented with decreased expression. To the best of our knowledge, this study provides the first human data that indicates that expression of lymphatic vessel markers may be predictive of immune microenvironments in metastatic melanoma.

To model the role of lymphatic vessels in the induction of tumor inflammation and immunity, we used K14-VEGFR3-Ig mice in which transgenic expression of the VEGFR3-Ig fusion protein under control of the K14 promoter effectively inhibits dermal lymphatic vessel formation (33). This phenotype persists in skin throughout adulthood, while systemic lymphatic networks, though aberrant early, are restored over time (i.e., heart, diaphragm) (33). A decrease in tumor incidence and delayed onset in response to chemical carcinogenesis was recently reported in that model when compared with controls (34), which was notably in a different background than ours (FVB vs. C57BL/6). Initiated tumors, however, progressed normally in transgenic mice with no difference in tumor growth observed, and decreased rates of initiation correlated with decreased local immune populations in skin of transgenic mice. Importantly, the authors observed decreased homeostatic myeloid populations in skin as well as changes in immune infiltrate in and around developing tumors and suggested that impaired local inflammation contributes to impaired initiation. Here, in the C57BL/6 background, we did not observe differences in steady-state cutaneous immune populations in transgenic mice and suggest that in this background the absence of dermal lymphatic vessels impaired local inflammation in response to tumor implantation. Lymphoid populations were, however, altered at homeostasis, likely due to disrupted immune architecture and reduced cellularity already reported in these mice (8).

Lymphatic vessels and their associated fluid drainage thereby play a critical role in initiating host responses to a developing tumor, and while completely absent in dermis of our experimental model, it is reasonable to imagine that significant remodeling of local lymphatic vessels, which will functionally perturb and alter fluid flow rates, may similarly influence inflammation and host antitumor immunity. Consistent with this, a 60% reduction of lymphatic vessels in Cby mice resulted in a more modest 40% reduction in CD45+ infiltrate. The direct mechanism by which lymphatic vessels and their associated transport of fluid and cells impact local inflammation remains an interesting question. The current paradigm suggests that chemokines and cytokines released by tumor cells induce immune infiltration, sequentially leading to production of inflammatory mediators, tumor cell killing, activation of DC homing to LNs, and induction of adaptive immunity. Our data may indicate that in the absence of important feed-forward mechanisms leading to adaptive immunity, the growing tumor fails to facilitate the accumulation of inflammatory mediators over time, and thus the reduced levels noted in our study. These observations are consistent with a role for lymphatic vessels in immune resolution where cell egress mediates resolution of inflammation in skin models (12), but indicates that with limited lymphatic drainage, in the context of a growing tumor, inflammation is not initiated and therefore resolution responses are not required.

While our results demonstrate that induction of local inflammation requires functional lymphatic drainage, systemic leukocyte populations and accumulation of Treg cells and inflammatory monocytes were similar in both strains. Inflammatory monocytes expanded in the spleens of both K14-VEGFR3-Ig and WT mice but did not infiltrate the tumor microenvironment. Consistently, splenic inflammatory monocyte populations have been reported to be a marker of tumor progression, yet are not predictive of infiltration and function within tumor microenvironments (41, 42). As such, our data indicate a separation of locoregional inflammation and systemic responses. Our work interestingly may support the concepts that targeting a sentinel LN is a viable option for optimal induction of local immunity (43) and local biomarker validation in tumor microenvironments may provide enhanced value over those found in blood.

In addition to lymphatic vessels, certain types of macrophages can express VEGFR3 and consequently their signaling may be affected in this model. On the other hand, VEGFR3 expression was previously not observed on cutaneous macrophages in either untreated, DMBA-TPA–treated skin, or squamous cell carcinomas (34). We provide specific experimental evidence, however, to demonstrate that active VEGFR3 signaling during tumor development does not mimic the observations found in mice completely lacking dermal lymphatic vessels. Importantly, this experiment also makes the distinction between lymphangiogenic or hyperplastic lymphatic networks and functionality of an existing network. Administration of an anti-VEGFR3 antibody importantly inhibits lymphangiogenic responses and other VEGFR3-mediated biology (macrophage and angiogenesis), but does not affect the existing vasculature. Using this model, we observed no significant change in any cell population, including macrophages, as a result of this VEGFR3 inhibition and furthermore, immunohistochemical analysis of lung nodules following intravenous tumor cell administration revealed equivalent amounts of macrophage infiltration within the same genetic background. Consequently, impairment of immune infiltration was restricted to regions of high transgene expression that lack lymphatic vessels, and was not a result of decreased signaling during tumor development.

Angiogenic blood vessels regulate lymphocyte entry into tissues through upregulation of adhesion molecules in response to local inflammation (44) and may utilize VEGFR3 during angiogenic sprouting (45). This raises the possibility that subtle changes in vascular endothelium may contribute to the observed decrease in immune cell infiltration observed in transgenic mice rather than a direct effect of impaired lymphatic drainage. The density of CD31+ structures in B16F10 tumors was similar in WT and transgenic mice, though we did observe a decrease in the percentage area stained with our CD31 marker. However, it is difficult to separate vasculature changes that may result from soluble VEGFR3 and those that would result from altered local inflammation, which is clearly altered in our model. Conversely, altered tumor vasculature and resulting hypoxia may also influence inflammation and progression, highlighting the complex interactions that are at play within tumor microenvironments. However, in healthy skin, K14-VEGFR3-Ig animals demonstrated robust T cell infiltration following dermal challenge in a contact hypersensitivity assay (8). Similarly, we observed increased immune cell infiltra-

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tion following intravenous transfer of effector CD8+ T cells, both of total CD45+ cells and antigen-specific CD8+ T cells, indicating that tumor-associated endothelium is permissive of infiltration.

In addition, previous reports have described dermal thickening in transgenic mice (34), which, if fibrotic, may influence immune cell infiltration and function. Other architectural changes within skin present in the K14-VEGFR3-Ig mice may also influence immune infiltration in the tumor context or metastasis. Subcutaneous lipid deposition is not elevated in K14-VEGFR3-Ig mice, unlike the Chy mutant mice (37). Chy mice, but not K14-VEGFR3-Ig mice, exhibit increased dermal collagen content, and K14-VEGFR3-Ig mice demonstrate enhanced hydraulic conductivity as compared with WT littermates (37). Consistent with this, no difference between collagen content or α-SMA was detected in skin of transgenic mice, either at steady state or in association with an implanted tumor. In the context of both of these tissue microenvironments, however, we observed reduced leukocytic infiltration into intradermal tumors. Interestingly, in Chy mice we only observed a 60% reduction in lymphatic vessel density in the ear and yet this was sufficient to alter the local infiltrate. These data are consistent with the human data in which the extent of lymphatic vessel gene expression was predictive of local infiltration.

Interestingly, systemic metastasis was reduced in K14-VEGFR3-Ig mice compared with WT mice. This was not a result of an impaired microenvironment within the lung itself, as intravenously administered tumor cells were able to effectively colonize lungs in both strains. In our case, LN metastasis may be a route of systemic dissemination, as tumor cells move through collecting lymphatic vessels remaining in subcutaneous tissue (37) and eventually re-enter circulation via the thoracic duct (17). While direct evidence for lymphatic vessel-mediated systemic spread is lacking and the efficacy of sentinel LN resection as a means of preventing metastatic spread is controversial (46, 47), these data indicate that lymphatic vessels contribute to a more metastatic phenotype within primary tumor microenvironments. In fact, impaired local inflammation that results from loss of lymphatic vessels may itself alter metastatic phenotype, as immune cells play an important role in directing tumor cell exit via hematogenous vasculature through secretion of matrix metalloproteinases, growth factors, and chemokines (48). In PyMT mice with a recessive null mutation in the Csf1 gene, macrophage recruitment was impaired, leading to delayed progression to malignancy and lung metastasis (49). Furthermore, macrophages are often found tethered to blood vessels where they facilitate the exit of metastatic tumor cells (50). We observed reduction in both inflammatory cells, including macrophages, as well as their soluble mediators, suggesting that mechanisms of metastasis would be impaired in our model. Whether the change in metastatic potential observed in K14-VEGFR3-Ig mice results from the lack of direct lymphatic access to circulation or the altered immune microenvironment within tumors and sentinel LNs remains difficult to uncouple.

In our OVA vaccination model, effective CD8+ T cell immunity was not induced against tumors following intradermal administration of vaccine. In healthy K14-VEGFR3-Ig mice, systemic T cell responses to dermal vaccination were robust as measured by IFN-γ production by CD8+ T cells in spleens 21 days after vaccination when compared with WT littermates (8). T cell kinetics were delayed, however, with activation primarily occurring in the spleens rather than dLNs. This is consistent with T cell responses observed in plt (Ccl119+/Ccl21a−/−) mice (51), a model of impaired DC trafficking from skin due to a deficiency of homing CCR7 ligands, and Cer7−/− mice (52). Again, in plt mice, spleens appear to be the main site of activation, as splenectomized mice fail to mount T cell responses to immunization (51). In our model, we did not observe significant levels of circulating antigen-specific T cells at time of sacrifice, indicating that at least during the experiment we did not generate sufficient adaptive immune responses against implanted, cutaneous tumors. Our observation of decreased fluid drainage and cellular egress to dLNs in K14-VEGFR3-Ig mice supports the model that antitumor CD8+ T cells are primed in the dLN rather than systemically or within tumor microenvironments. While soluble protein and adjuvants might gain access to circulation, implanted tumor cells and associated debris would be restricted to cutaneous tissue and drained either actively or passively to the dLN. As a consequence, adaptive immunity against tumors may be impaired in the absence of lymphatic vessel communication with dLNs. These results corroborate recent work in another model of lymphatic dysfunction in which the Kaposi’s sarcoma-associated herpesvirus latent-cycle gene, k-cyclin, is expressed under the control of the VEGFR-3 promoter (53). In these mice, antigen presentation in dLNs was impaired and CD8+ T cells demonstrated suppressed cytotoxicity (54). This highlights the important role of dLNs in antitumor immunity (22, 43).

Potent adaptive immunity drives compensatory regulatory mechanisms in tissue that impair therapeutic efficacy (40). In the absence of induced adaptive immunity we asked whether mechanisms of immune suppression typically associated with this tumor model were also affected. While we were able to detect inflammatory monocytes and Treg cells in spleen, 2 cell types associated with CD8+ T cell suppression, they were completely absent from tumor microenvironments in K14-VEGFR3-Ig mice. As a result, adoptive T cell transfer therapy, which is already quite effective in our model, was more efficacious in tumor microenvironments lacking dermal lymphatic vessels. Importantly, we demonstrate enhanced functionality of effector T cells within tumor microenvironments. In this study, we modeled impaired inflammation and immunity in the absence of dermal lymphatic vessels, thus mimicking patient subsets without detectable systemic immunity. However, patients can also present with detectable peripheral immune responses but still exhibit impaired lymphocyte infiltration within the tumor microenvironment (55). Consequently, additional mechanisms are at play in the clinical setting that limit local antitumor immunity including, for example, impairment of lymphocyte homing due to changes in the tumor-associated vasculature (44) as well as local dysfunction and exhaustion of infiltrating lymphocytes (56). Furthermore, the observation of both inflamed and noninflamed lesions within a single patient, with a single systemic immune response (55), supports the hypothesis that the distinct tumor microenvironments of each lesion as well as tumor cell heterogeneity (29) may act as local regulators of lymphocyte homing, retention, and function. Regional differences in lymphatic vessel function, for example preexisting lymphatic vessel dysfunction and decreased lymphatic vessel density...
in regions of chronic sun exposure and severe solar elastosis (57), may be one mechanism distinguishing inflamed and noninflamed lesions. The specific role of lymphatic vessels and their associated drainage in regulating regional differences in cutaneous immune responses remains an open and exciting question.

Our understanding of the role of lymphatic vessels in the induction and resolution of an antitumor immune response is clearly still in its infancy. While we recently demonstrated an immunosuppressive role for lymphatic vessels in murine melanoma (16), with these current data we also suggest that lymphatic function contributes to local inflammation and induction of antitumor adaptive immune responses. While patients do not present with a complete absence of dermal lymphatic vessels, our transcriptional analysis of human tumors along with the wealth of histological lymphatic vessel density analyses indicates a spectrum of lymphatic vessel involvement, which may correlate with the heterogeneity in immune infiltrate also observed clinically. As a consequence, we propose that the contribution of the lymphatic vasculature and its functional drainage to local immunity may be both a prognostic and targetable feature of tumor microenvironments.

**Methods**

**TCGA analysis and LS.** Level 4 gene expression data were downloaded for metastatic skin cutaneous melanoma from TCGA, which were processed by the Broad Institute’s TCGA workgroup (release date 20130523), with no further selection. Of the 266 samples, 212 were from regional sites (160 from LNs and 52 from skin or other soft tissue) and 35 were from distant sites (30). The RNA-Seq level 4 gene expression data contain upper quartile-normalized and log2-transformed RSEM (RNA-Seq by expectation maximization) values summarized at the gene level (58). The tumor staging had been performed by the TCGA consortium and every patient used in the study had metastatic disease at the time of biopsy (stage II-IV). All available metastatic cutaneous melanoma samples were analyzed and segregated into 3 groups representing low, mid, and high degrees of lymphatic involvement within the tumor according to their LS (29). To determine LS for each sample, expression levels of the 2 lymphatic markers PDPPN and LYVE1, which correlated strongly with each other as well as with the expression levels of VEGFC, were first each normalized to their mean expression levels by subtracting the average value and dividing by the SD, such that the average value of the normalized data was zero and the SD was 1. Then, the average normalized values of PDPPN, LYVE1, and VEGFC were added together to form the LS. In this way, the average LS score was zero, and negative values represented below-average lymphatic involvement. Finally, we grouped the patients into LS<sub>std</sub> (representing LS values within 2/3 of the SD around the mean), LS<sub>hi</sub> (> mean + 2/3 SD), and LS<sub>lo</sub> (mean - 2/3 SD); 50% of patients were LS<sub>std</sub>, while 25% fell into each of the other 2 groups.

**Mice and tumor transplantation.** K14-VEGFR3-Ig male mice described elsewhere (33) were maintained on a C57Bl/6 background and crossed with WT C57Bl/6 female mice. Ten- to twelve-week-old K14-VEGFR3-Ig mice and WT littermates of both sexes were used in all experiments. Cby mice, described previously (36), were obtained from MRC-Harwell distributing mice on behalf of the European Mouse Mutant Archive (C3H1O1H-F144-Chy/H mice, repository number EM:00068), and were maintained on a C3H background. TCR-Tg OT-I mice were purchased from Harlan Laboratories and maintained in house. WT C57Bl/6 mice were purchased from Harlan Laboratories. Animals were housed in pathogen-free facilities. B16F10 (ATCC), B16F10.OVA (gift of Bertrand Huard, University of Geneva, Geneva, Switzerland) and C3HHB (NCI-Frederick Cancer DCT Tumor Repository) cells were grown at 37°C in 5% CO<sub>2</sub> in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich), supplemented with nonessential amino acids and 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 400 μM L-glutamine (all from Lonza). Cells were detached with trypsin-EDTA (Sigma-Aldrich) and passaged every 3 days. B16F10 cells (0.5 × 10<sup>6</sup>) in 50–100 μl saline were inoculated intradermally and dorsolaterally. Tumor growth was measured daily using calipers. For intradermal ear injections, animals were anesthetized with 1% isoflurane in combination with O<sub>2</sub> and N<sub>2</sub>, and 20 μl of cell slurry was injected into the dermis of the ear. In some mice, 500 μg anti-VEGFR3 neutralizing antibody (mF4-31C1, Eli Lilly and Company) was administered on days 0, 3, and 6 after tumor inoculation. For metastasis experiments, B16F10 cells (5 × 10<sup>6</sup>) were introduced via tail vein injection and animals were sacrificed 14 days later. In some cases, 6 hours prior to sacrifice, mice were given 250 μg brefeldin A i.p. in preparation for intracellular cytokine staining.

**Quantification of metastasis.** For the quantification of metastatic melanomas, lungs isolated from mice after s.c. or i.v. injection of B16F10 melanoma cells were paraffin embedded and microsectioned at 3 different planes (~350 μm apart) and stained with hematoxylin and eosin. Histological slides (3 per mouse) were then examined blindly for metastatic melanomas. The quantification of tumors in each image is relative to the total tissue area and is presented as a percentage: tumor-to-lung ratio = tumor area / total lung area × 100.

**Fluid drainage and DC trafficking.** Either 20 μl 0.5-μm FITC-conjugated latex microspheres (Polysciences, diluted 1:25 in sterile saline) or 20 μl FITC-dextran was injected into the tumor with a 23-gauge needle. After 30 minutes (for dextran) or 24 hours (for microspheres), tumors and LNs (brachial) were harvested. For dextran drainage, LNs were homogenized and fluorescence quantified by plate reader (Safire<sup>2</sup>, TECAN). For DC trafficking, single-cell suspensions were prepared and analyzed by flow cytometry for CD11c<sup>+</sup>MHCII<sup>+</sup> FITC<sup>-</sup> DCs.

**Prophylactic vaccination.** Mice received 10 μg LPS as a negative control or 10 μg LPS plus 10 μg OVA (grade IV, Sigma-Aldrich) in 2 intradermal doses of 25 μl per foreleg. Ten days later, 5 × 10<sup>6</sup> B16F10. OVA or OVA/VEGF-C tumor cells in 50 μl were inoculated intradermally and dorsolaterally, and a vaccine boost (identical to the first) was administered again on day 15.

**Adaptive T cell transfer.** Splenic CD8<sup>+</sup> T cells and CD11c<sup>+</sup> DCs were isolated by positive magnetic cell sorting (Miltenyi Biotech). Purified CD8<sup>+</sup> OT-I and splenic DCs were cocultured at a ratio of 10:1, supplemented with 1 nM SIINFEKL (GenScript) and 10 U/ml IL-2 (Roche). Cells were collected after 4 days and injected i.v. into tumor-bearing mice (1 × 10<sup>6</sup> cells in 200 μl).

**Immunohistochemistry.** Tumor samples were fixed in 4% paraformaldehyde, paraffin-embedded, and cut into 4-μm sections. For immunolabeling, paraffin-embedded tissue sections were deparaffinized and rehydrated before antigen retrieval at 98°C for 1 hour in 0.01 M citrate buffer (pH 6.0). Antigen retrieval for F4/80 staining was performed with Proteinase K (Dako) treatment according to the manufacturer’s instructions. After blocking with diluted serum from the secondary antibody host for 30 minutes, the slides were incubated overnight (4°C) with the primary antibody. Endogenous peroxidase activity was
blocked for 20 minutes with 3% hydrogen peroxide (Sigma-Aldrich) and a biotinylated anti-rat, anti-rabbit, anti-hamster, or anti-goat secondary antibody (Vector Laboratories) was applied for 45 minutes. The anti-antibody complex reaction was augmented with avidin-biotin-peroxidase for 45 minutes according to the manufacturer’s instructions (Vectorstain ABC Kit, Vector) and stained for 1–10 minutes with diamobenzidine tetrahydrochloride (DAB, Vector). The sections were then counterstained with hematoxylin (Thermo Scientific), dehydrated, and mounted with Entellan (Electron Microscopy Services). Primary antibodies used were: rabbit anti-LYVE1 (1:100, catalog ab14917, Abcam), rabbit anti-CD11b (1:200, catalog ab75476, Abcam), rat anti-F4/80 (1:50, catalog ab6640-200, Abcam), rat anti-MHCII (I-A/I-E) (1:100, catalog 11-5322-82, eBioscience), hamster anti-CD3e (1:100, catalog 550277, Abcam), rabbit anti-alpha SMA (1:100, catalog ab5694, Abcam), goat anti-CD3 (1:100, catalog sc1506, Santa Cruz Biotechnology), and rat anti-CD45 (1:250, clone 30-F11, BD Pharmingen).

**Immunohistochemical staining of human tissue.** Human tissue was obtained by surgical excision from a patient with primary melanoma, and used for research upon informed consent at the Ludwig Institute for Cancer Research (Canton of Vaud University Hospital) as a part of a phase 1 vaccination trial (LUD 00-018 study, ClinicalTrials.gov Identifier NCT00112229). The tissue was formalin fixed and paraffin embedded and 4-µm sections were prepared. Antigen retrieval for melan-A and PROX-1 staining was performed with Tris-EDETA (pH 9) for 15 minutes. Primary antibodies used were: mouse anti-human melan-A (1:50, clone A103, Dako) and mouse anti-human PROX1 (1:100, catalog AF2727-SF, R&D Systems). Alexa Fluor-labeled secondary antibodies were incubated for 1 hour at room temperature (1:400, Invitrogen). Sections were counterstained with DAPI (Lucerna Chem AG) and coverslipped with fluorescence mounting medium (S3023, DAKO).

**Picrosirius red staining.** Paraffin-embedded sections were deparaffinized and hydrated in xylene and ethanol. The slides were then stained with picrosirius red solution composed of picric acid and Direct Red 80 (both Sigma-Aldrich) according to the manufacturer’s instructions and dehydrated in ethanol and xylene. Sections were washed in 2 changes of acidified water after picrosirius red staining.

**Western blot and cytokine array.** Tumor tissues were homogenized and lysed in a custom-made total protein lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100) containing protease and phosphatase inhibitors (Roche). Protein concentration was measured using a bicinchoninic acid (BCA) assay (Pierce) and 30 µg protein was loaded per lane for all immunoblots. The protein lysates were resolved using NuPAGE Novex 4%–12% Bis-Tris Gels (Invitrogen). Protein transfer was assessed by Ponceau staining (Sigma-Aldrich), and then membranes were blocked with 5% fat-free dry milk for 1 hour. Immobilized antibody was detected using the horseradish peroxidase–conjugated secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). The immune reaction was visualized with an LAS-3000 imaging system (FujiFilm). Primary antibodies used were: rat anti-γCSF (1:500, catalog ab13789-100, Abcam), rabbit anti-MCP-1 (1:2,000; catalog NB11-2000; Novus Biologicals), rabbit anti-IFN-γ (1:500, catalog AAM29, AbD Serotec), rabbit anti-TNF-α (1:100, catalog ACC-250844, BioSite), rabbit anti-NOS2 (1:1,000; catalog 29828; Cell Signaling Technology), goat anti–IL-1β (1:500, AF-401-NA, R&D Systems), rat anti–IL-10 (1:500, catalog ab33471, Abcam), rabbit anti-VGF (1:500, catalog ab46154, Abcam), goat anti-MCSF (1:500, catalog af4616-sp, Novus Biologicals), rabbit anti–TGFP-β (1:500, catalog AB-100NA, R&D Systems) and rabbit anti–β actin (1:500, catalog ab8227, Abcam).

The relative levels of 22 different cytokines and chemokines in tumor lysates were evaluated using a Mouse Cytokine Antibody Array Kit (ab33993, Abcam) according to the manufacturer’s protocol. Immunoblot images were captured and visualized using the LAS-3000 imaging system and spot intensity was analyzed using ImageJ v1.49 software (NIH). The values are shown as relative intensity of expression compared with an internal control (biotinylated IgG).

Cytokines detected by the antibody array kit were: G-CSF, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p40/p70, IL-12p70, IL-13, IFN-γ, MCP-1, MCP-5, RANTES, stem cell factor, soluble TNF receptor I (TNF-RI), TNF-α, thrombopoietin, VEGF.

**Flow cytometry.** Tumors and LNs (draining and nondraining) were incubated in collagenase D (1 mg/ml in HBSS with 2% FBS) for 1 hour at 37°C followed by 100 mM EDTA and then pushed through a 70-µm strainer to create a single-cell suspension. The following anti-mouse antibodies were used for flow cytometry: CD45-APC, CD45-Pacific blue, or biotinylated CD45 (clone 30-F11); CD3-Pacific blue (clone 17A2) or CD3-PerCP/Cy5.5 (clone 145-2C11); CD4-PECy7 or CD4-PE (clone GK1.5); CD8a-PECy7 or CD8a-APC-Alexa 780 (clone 53-6.7); F4/80-PE (clone BM8); CD25-FTTC (clone 3C7); FoxP3-PerCP/Cy5.5 (clone FJK-16B); CD11c-Alexa 647 (clone N418); MHCIIF-FTTC (clone M5/114.15.2); biotinylated Gr1 (clone RB6-8C5); CD45.2-Pacific blue (clone 104); IFN-γ-APC (clone XMG1.2); and CD11b-PECy7 (clone M1/70) (all from eBioscience). Pentamer staining for Trp2 H-2Kb-PE (ProMimyme) and H-2Kb-SIINFEKL-PE (ProMimyme) was performed according to the manufacturers’ guidelines. Cell viability was determined using LIVE/DEAD Fixable Dead Cell Stain Kits or propidium iodide (both Invitrogen).

**Statistics.** Unless stated differently, statistical significance was analyzed by unpaired, 2-tailed Student’s t test using Prism software (GraphPad Inc.); P < 0.05 was considered to be statistically significant. Mann-Whitney U tests were performed on in vivo data unless the data were confirmed to fulfill the criteria of normal distribution and equal variance, after which t tests and ANOVA were performed. Regression analysis was performed to compare slopes of tumor growth curves (GraphPad Inc.).

**Study approval.** All animal experiments were conducted in accordance with the regulations of the Norwegian State Commission for Laboratory Animals and the Swiss Animal Protection Act, which are consistent with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and Council of Europe (ETS 123), and with approval from the AAA-LAC International accredited Animal Care and Use Program at University of Bergen and the Cantonal Veterinary Office of Canton de Vaud, Switzerland.

**Author contributions**

AWL, MW, HW, and MAS conceived the ideas and designed the experiments. AWL, MW, MF, ESS, and MAB performed experiments and analyzed the data. SS and TFG provided normalized TGCA dataset. KA provided the K14-VEGFR3-Ig model. HPE aided data acquisition and interpretation. AWL, MW, HW, and MAS wrote the paper.
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Supplemental Figure 1. Correlation of lymphatic markers from human metastatic melanoma samples. A. Immunofluorescence detection of PROX1^+Melan-A^+ cells in primary human cutaneous melanoma. Nuclear PROX-1 staining consistent with lymphatic vessel staining (Melan-A, arrow) and cytoplasmic staining in Melan-A^+ cells (*). PROX1, red; Melan-A, green; DAPI, blue. Scale bar = 50 µm. B. Correlation of lymphatic markers (LYVE1, PDPN, PROX1) with each other and with vascular endothelial growth factor genes (VEGFA, VEGFB, VEGFC, VEGFD). 266 metastatic cutaneous melanoma samples from the Broad Institute’s TCGA database. Pearsons correlation coefficient (r).
Supplemental Figure 2. The lymphatic score correlates with the lymphangiogenic growth factor VEGFC and not VEGF A,B or D. A. Distribution of lymphatic scores. B. Correlation of lymphatic score (LS) with VEGFC, PDPN, LYVE1, VEGFA, VEGFB, and VEGFD expression in 266 metastatic cutaneous melanoma samples. Pearsons correlation coefficient (r).
Supplemental Figure 3. Quantification of percent area of CD31 staining. A. Quantification of percent area of CD31+ pixels quantified over total image area from immunohistochemical staining in paraffin sections. Data represented as mean ± SEM, n=3, **p<0.05.
Supplemental Figure 4. K14-VEGFR3-Ig mice do not exhibit enhanced cutaneous fibrosis as compared to wildtype littermates. 

A. Picosirius Red staining was performed on skin taken from naive wildtype (WT) and K14-VEGFR3-Ig (TG) mice. Collagen fibers are stained red; scale bar = 100µm. 

B. Immunohistochemistry for α-SMA in naïve skin from WT and TG mice (scale bar = 50µm) and 

C. quantification of number of α-SMA⁺ cells per 0.04mm²; n=3. Data represented as mean ± SEM, n=3. 

D. Picosirius Red staining in B16F10 tumor implanted intradermal in WT and TG mice. Scale bar = 100µm; “T” marks tumor.
Supplemental Figure 5. Lymph node cellularity in wildtype and K14-VEGFR3-Ig mice. Brachial lymph node cellularity (naïve) in WT and TG mice, n=4. P-values were obtained with Student’s unpaired t test.
Supplemental Figure 6. Gating scheme for analysis of infiltrating immune populations by flow cytometry. Multi-color flow cytometry gating scheme to quantify immune populations in tumor, spleen and draining lymph node. Representative plots from the tumor of wildtype mouse.
Supplemental Figure 7. Homeostatic cutaneous immune populations in wildtype and K14-VEGF3-Ig mice. Naïve skin and spleens were digested and analyzed by flow cytometry for immune populations from wildtype (WT) and K14-VEGFR3-Ig (TG) mice. A. Total CD45^+ cells as a percent of live cells and B. CD3ε, CD11c and CD11b cells as a percent of CD45^+ cells are quantified from the skin and C and D. spleens of mice. E. Representative plots of Ly6c^+ and Ly6g^+ populations in spleens of naïve and tumor-bearing mice and F. quantification of % Ly6c^+Ly6g^- inflammatory monocytes in naïve spleens, n≥4. Quantification of tumor-bearing mice in Figure 3. Gated on CD45^-CD11c^-CD11b^-F4/80^- inflammatory monocytes.
Supplemental Figure 8. Correlation between lymphatic involvement and intratumoral cytokine expression. A. Representative blot (above) and key (below) from cytokine array, n=7. Black squares indicate control IgG spots used for relative intensity calculations. Quantification presented in Figure 3. B. Representative western blots on tumor lysate and C. quantification of optical density normalized to WT (n=3); data are represented as mean ± SEM. P-values were obtained with Student’s unpaired t tests of values. *p<0.05, **p<0.01. D. Representative plots correlate expression of key cytokines (CCL2, TGFB, MCSF, IL1B) with lymphatic score in 266 cutaneous metastatic melanoma samples from the Broad Institute TCGA database. Pearsons correlation coefficient (r).
Supplemental Figure 9. Chy mice demonstrate decreased dermal lymphatic vessel density and leukocytic infiltrate. C3BHA syngeneic breast carcinoma cells were injected intradermal (n=5) into ears of Chy mice harboring an inactivating mutation in the tyrosine kinase domain of VEGFR3. Lymphatic vessel density (LYVE1; 20x scale bar=100µm) and leukocytic infiltrate (CD45 and CD3ε; 40x scale bar=50µm) were evaluated by immunohistochemistry (left) and quantified as number per area (right). P-values were obtained with Student’s unpaired t tests of values. **p≤0.01 ***p<0.001.
Supplemental Figure 10. Inhibition of VEGFR3 signaling during tumor progression is insufficient to suppress local inflammation in wildtype mice. A. B16F10 tumors were implanted into wildtype mice and treated with either VEGFR3 neutralizing antibody (mF4-31C1, Eli Lilly and Company, 500µg) or rat IgG isotype control on day 0, 3 and 6 to inhibit active VEGF-C signaling during tumor development. B. Fluid drainage was assessed by intratumoral injection of 70kDa FITC dextran and analysis of tumor draining lymph node 30 min later. (n=5) Relative numbers of tumor-associated C. CD45+ cell populations and D. inflammatory monocytes (CD11c+CD11b−F4/80−Ly6cLy6g), quantified as a percent of live cells in the tumor. Relative numbers of splenic (E) CD45+ cell populations and F. inflammatory monocytes (CD11c+CD11b−F4/80−Ly6cLy6g) (n=5). Data represented as mean ± SEM.
Isolation of lymph shows activation of STAT3 and CREB pathways in the spleen during AML development in a rat model.

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Abstract:
Acute myeloid leukemia (AML) is a heterogeneous malignant condition characterized by massive infiltration of poorly differentiated white blood cells in the blood stream, bone marrow and extramedullary sites. During leukemic development, hepatosplenomegaly is expected to occur as large blood volumes are continuously filtered through these organs. We asked whether infiltration of leukemic blasts initiated a response that could be detected in the interstitial fluid phase of the spleen and used the BNML rat model, a model known to mimic human AML in growth characteristics and behavior. By cannulating efferent lymphatic vessels, we were able to monitor the response of the spleen microenvironment during AML development. By flow cytometric analysis of lymphocytes isolated from spleen lymph, we found increased STAT3 and CREB signaling, and proteins related to these pathways were identified with a different profile in leukemic when compared with control spleen lymph, even at an early time-point of disease development. Additionally, SPARC-like 1 protein, recently identified as a promoter of AML cell growth and a biomarker of patient outcome, was locally produced in the spleen and upregulated in the leukemic setting. Thus, interstitial fluid, and its surrogate efferent lymph, can be used to provide unique information about spleen responses during AML progression. Consequently, the presently demonstrated access to this compartment can offer new insight into organ-specific processes in leukemogenesis.
Introduction:

Acute myeloid leukemia (AML) is a heterogeneous malignant condition that arises from the myeloid compartment, characterized by massive infiltration of abnormally differentiated white blood cells in the bone marrow, blood stream and in extramedullary sites (Kennedy & Barabe, 2008; Solh, Solomon, Morris, Holland, & Bashey, 2016). Although treatment of childhood leukemia has been a great success in terms of overall survival (Athale, Gibson, Bradley, Malkin, & Hitzler, 2016), only 35-40% of adult AML patients < 60 years survive the disease, and for patients > 60 years of age, the prognosis of survival is only 5-15% (Dohner, Weisdorf, & Bloomfield, 2015). Due to infiltration of leukemic blasts in the bone marrow, normal hematopoiesis and immune cell function is dramatically halted, and patients often present with bleeding, anemia, fatigue, infections, bone or joint pain or hepatosplenomegaly (Lowenberg, Downing, & Burnett, 1999). The enlargement of the liver and spleen is secondary to infiltration of leukemic cells (Lowenberg et al., 1999) as large blood volumes are filtrated through these organs every day (Eipel, Abshagen, & Vollmar, 2010; Mebius & Kraal, 2005).

The spleen is the largest lymphatic organ in the body and, unlike lymph nodes, is directly exposed to pathogens in the blood stream, which gives this organ a unique position in the body as an immunological monitor of the hematological compartment (Mebius & Kraal, 2005). It is also known to participate in fluid volume regulation (Kaufman & Deng, 1993), remove damaged erythrocytes, recycle iron, execute both innate and adaptive immune functions (Mebius & Kraal, 2005), take part in systemic inflammation (Huston et al., 2006; Semaeva et al., 2010) and contribute to extramedullary hematopoiesis (Kiel & Morrison, 2008).

We have previously demonstrated that the spleen generates inflammatory mediators and mobilizes T-cells in response to lipopolysaccharide (LPS) exposure (Semaeva et al., 2010),
and that splenic efferent lymph can be used to detect locally produced proteins in the spleen microenvironment during systemic inflammation (Oveland et al., 2012). These studies showed that the spleen lymph cannulation technique gives unique access to the interstitial fluid phase of the spleen. Thus, we asked whether perturbations forced upon this microenvironment during leukemic blast infiltration, could affect the intracellular phosphorylation status of splenic lymphocytes and also be reflected in the leukemic spleen lymph proteome.

To address these questions, we used the well-established BNML rat model of acute myeloid leukemia, an animal model that is known to resemble human AML in regard to growth characteristics and therapeutic response (Iversen, Sorensen, & Benestad, 2002; Martens, van Bekkum, & Hagenbeek, 1990), and studied the cellular elements and proteins in spleen lymph.

**Methods**

**Experimental animals and housing.**

The experiments were performed in female Brown Norwegian rats that were fed a standard laboratory diet. All rats were exposed to light on a 12: 12 h cycle in humidity- and temperature controlled environment.

**Anesthesia and surgery**

Anesthesia was induced with pentobarbital sodium, 50mg/kg body weight given i.p. When anesthetized, the (rectal) body temperature was monitored and maintained at 37°C ±1°C using a heating pad and lamp. Polyethylene (PE-50) catheters were placed in one femoral vein for injection of saline during the experiment and in the contralateral artery for blood sampling. On termination of the experiment, the rat was euthanized by cardiac arrest induced under
anesthesia, with an i.v. injection of saturated potassium chloride. All experiments were conducted in accordance to the regulation of the Norwegian State Commission and with approval from the Local Ethical Committee at University of Bergen.

**BNML Leukemia**

BNML cells were derived from the leukemic spleen of a terminal stage leukemic BN animal, and the cells were kept on liquid nitrogen in freezing medium (50% FBS, 40% DMEM, 10% DMSO). 10-15 days prior to experiments, 5 female rats were injected i.v. through the tail vein with 20 x 10^6 BNML cells in 200 µl phosphate buffered saline. After injection, the rats were monitored every other day for signs of discomfort or infections.

**Lymph sampling**

To sample lymph from the spleen, we used the approach as earlier described (Semaeva et al., 2010). After anesthesia, the rat was laparotomized and the spleen was carefully laid against the stomach, exposing its dorsal side and vessels. The spleen, liver and intestines were covered with bandages soaked in saline to prevent drying during the procedure. Several afferent lymphatic vessels were usually identified, and with one exception, these vessels were deemed impossible to cannulate. The afferent vessels drained to a lymph node station with 3-4 lymph nodes were one node was significantly larger than the others. Surprisingly, we found that the afferent vessel draining to the large lymph node drained directly from the liver and that this lymph node was the first node to receive lymph from the liver. Because the lymph node station proximal to the spleen received lymph from both the liver and spleen, we started to ligate the afferent liver lymph vessel to assure that the lymph we collected derived from spleen only. When a proper spleen lymph vessel was identified, this was ligated using an ethicon 7-0 surgical non-absorbable suture. Subsequent to ligation, the vessel swelled, a small
incision was made and a polypropylene tube (0.2 mm in outer diameter) was inserted and fixed into the lymph vessel. The polypropylene tube drained into heparinized microhematocrit tubes. After the lymph vessel was successfully cannulated, lymph was collected for a total of 3-4 hours. The lymph was transferred to Eppendorf tubes and frozen at -20 °C. An arterial blood sample was taken 30 minutes after the lymph vessel cannulation and the rat was given 1 ml bovine serum albumin (20 mg/ml) i.v. every hour for the duration of the experiment.

**Histology-verification**

Spleens sections (4µm) were cut from formalin fixed tissue blocks and stained with hematoxylin for 5 min, washed in tap water for 5 min and dehydrated in 70% and 95% ethanol for 2 x 1 min. The slides were subsequently stained with eosin for 20 s, dehydrated in 70% and 95% ethanol for 2 x 3 min, Xylene 2 x 5 min and mounted in Entellan before left to dry in room temperature. We sectioned totally 9 spleens and leukemic infiltration was diagnosed by an experienced pathologist in n=4 spleens.

**Determination of single-cell phosphosignaling**

Single-cell phosphoflow cytometry of blood and spleen lymphocytes was performed as previously described (Irish et al., 2004). Briefly, 150 µl spleen lymph was collected and fixed in 850 µl 2% paraformaldehyde (5 min) and centrifuged at 3000 rpm (5 min). The supernatant was removed and the cells were re-suspended in PBS before stored at -80°C. Collected blood samples were added into lysis buffer (BD Biosciences) at 37°C (10 min) and centrifuged at 496 g for 8 min, supernatant was removed and the cells were washed twice in PBS before re-suspended in PBS and stored at -80°C.

Cells were permeabilized with 1 ml methanol (0-4°C) before washed twice in PBS with 2% BSA, and lymph and plasma samples were divided in 2 tubes; 1 tube for STAT3/STAT5
staining and 1 tube for CREB/p38 staining. Conjugated antibodies were added in optimized concentrations and incubated in the dark for 30 min at room temperature. Cells from spleen lymph and plasma were stained with: p-STAT3 Alexa 647 (BD Bioscience, Cat. 562071, 3 µl per sample), p-STAT5 Pacific Blue (BD Bioscience, Cat. 560311, 20 µl per sample), p-CREB Alexa 647 (BD Bioscience, Cat. 558434, 20 µl per sample) and p-p38 Pacific Blue (BD Bioscience, Cat. 560313, 20 µl per sample).

Samples were run on a BD LSR Fortessa with 4 lasers (488nm, 561nm, 635nm and 407nm) and Diva software version 8.0. BD Cytometry Setup and Tracking beads were used for standardization of application setup.

**Size exclusion chromatography of plasma and lymph**

Size exclusion chromatography was performed as previously described (Semaeva et al., 2010), using two 4.6 mm ID × 30.0 cm TSKgel Super SW3000 columns coupled in series (Tosoh Biosciences, Stuttgart, Germany) with an optimal separation range for globular proteins of 10–500 kDa. The protein concentration in the elution fluid was measured by UV detection at 220 nm on an Ettan LC System (GE Healthcare Europe, Hillerød, Denmark) and the buffer/mobile phase was 0.1 M Na₂SO₄ in 0.1 M phosphate buffer pH 6.7–7.0.

**Preparation for mass spectrometry**

The protein concentration in each sample was measured using a BCA protein assay. Aliquots of 1 µg, 5 µg, 10 µg or 15 µg protein were reduced using dithiothreitol (DTT; Amersham Biosciences) and alkylated in the dark using iodoacetamide (IAA; Sigma Aldrich, St. Louis, MO, US), followed by digestion using porcine trypsin (Promega, Fitchburg, MO, US). The samples were desalted using Oasis HLB µElution Plate 30 µm (Waters, Milford, MA, US), as
described by Kroksveen et al. (Kroksveen et al., 2013).

**Liquid Chromatography – Mass Spectrometry (LC-MS) analysis**

Peptides were separated during a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 280 nl /min) on the analytical column (Acclaim PepMap 100, 15 cm x 75μm i.d. nanoViper column, packed with 2μm C18 beads). Solvent A and B was 0.1% FA (vol/vol) with 2% ACN or 90% ACN (vol/vol) respectively. From 0-6 min the gradient was 5%B, at from 6-6.5 increase to 8%B. The gradient composition was 8–38%B from 6.5-67 min, then 38–90%B from 67-70 min. Elution of very hydrophobic peptides and conditioning of the column were performed between 70-75 minutes with isocratic elution with 90%B, ramp from 90-5%B from 75-78 min and hold at 5%B until 90 min.

The eluting peptides were ionized in the electrospray and analyzed by the LTQ-Orbitrap Velos Pro. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition.

Survey full scan MS spectra (from 300 to 2000 m/z ) were acquired for 80 min in the Orbitrap with a resolution R = 60000 at 400 m/z (after accumulation to a target value of 1E6 in the linear ion trap with maximum allowed ion accumulation time of 500 ms). The 7 most intense eluting peptides above an ion threshold value of 1000 counts, and charge states 2 or higher, were sequentially isolated to a target value of 1E4 and fragmented in the high-pressure linear ion trap by low-energy CID (collision-induced-dissociation) with normalized collision energy of 40% and wideband-activation enabled. The maximum allowed accumulation time for CID was 200 ms, the isolation width maintained at 2 Da, activation q = 0.25, and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with the secondary electron multipliers. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 20 s. Lock-mass internal calibration was not enabled.
Proteomic data analysis and statistics

The acquired raw data files were analyzed in a label-free manner using both Progenesis LC-MS (version 4.0, Nonlinear Dynamics, UK) and MaxQuant (version 1.5.3.17) as described in (Cox et al., 2014; Vethe et al., 2015). Extracted MS/MS spectra were searched against a concatenated target/decoy version of the UniProtKB *Rattus Norvegicus* database. In the Progenesis analysis, this was performed using SearchGUI (version 2.0.5) (Vaudel, Barsnes, Berven, Sickmann, & Martens, 2011) with OMSSA and X!Tandem (Craig & Beavis, 2004) algorithms selected. Identified peptides were assigned to proteins using PeptideShaker (version 1.0.2) (Barsnes et al., 2011), and validated peptide-spectrum matches (PSM) were reimported into Progenesis. In MaxQuant, peptide and protein identifications were performed using the built-in Andromeda search engine (Cox et al., 2011). Fixed and variable modifications were carboxymethylation of cysteine (+57.021464 Da) and oxidation of methionine (+15.994915), respectively. MaxQuant also include N-terminal acetylation (+42.010565) as a variable modification, by default. Precursor mass tolerance was 10 ppm (20 ppm in MaxQuant) and fragment mass tolerance 0.5 Da searching for b and y ions. Two missed cleavages were allowed using trypsin as enzyme. Identification matches were validated using a stringent 1% false discovery rate threshold estimated using the target/decoy approach (Elias & Gygi, 2010). Perseus (version 1.5.0.0) was used for data analysis of the MaxQuant output (see www.coxdocs.org → Perseus → “use_docs” for detailed information).

Statistical methods

All values are means ± SEM, unless otherwise stated. Differences between multiple groups were tested with one-way ANOVA, followed by Tukey post-hoc test or two-way ANOVA when two groups were compared. p < 0.05 was considered statistically significant.
Results and discussion:

Leukemic development of BN rats and characterization of pre- and postnodal lymph

We chose the well-established BNML rat model to mimic AML in our study (Kennedy & Barabe, 2008; McCormack, Bruserud, & Gjertsen, 2008). As the disease progresses in this model, the rats develop an increasing tendency of bleeding and in the end stage; weight loss, dehydration, hind limb paralysis and lethargy (McCormack, Skavland, Mujic, Bruserud, & Gjertsen, 2010). Efferent lymph cannulation of the spleen is technically demanding even in the normal in vivo setting, due to the size of the vessels and movement of intraabdominal organs because of respiration. Consequently, the time-point of harvesting spleen lymph needed to be balanced between sufficient leukemic infiltration of the spleen and a tolerable level of intraabdominal bleeding for the procedure to be technically possible, and was in preliminary experiments found to be 1-2 weeks after leukemic transplantation. Reported results are from AML-affected spleens that were harvested on day 10-15 post-injection of BNML cells.

To assess whether the spleen was infiltrated by leukemic blasts at the time of lymph cannulation, the organ was harvested, weighed and snap frozen immediately after the rat was euthanized, and later processed for regular H&E staining. Leukemic infiltration of the spleen was verified by a trained pathologist in n=4 rats (Fig 1A). As AML advances in the BNML rat model, splenomegaly is expected to develop (McCormack, Bruserud, & Gjertsen, 2005), and although the organs were harvested on a relatively early time point of disease development, there was a clear tendency of spleen enlargement, evaluated by weight (p=0.0693) (Fig 1B).

Phosphosignaling in spleen lymph and plasma during AML development

We have previously reported accelerated efflux of T-lymphocytes in spleen lymph and elevated phosphosignaling in spleen lymphocytes after LPS exposure (Semaeva et al., 2010).
Our hypothesis was that leukemic infiltration would affect the local spleen microenvironment that might be detected in e.g. cell signaling. We composed a panel of markers known to be associated with several major signal transduction pathways (signal transducer and activator of transcription protein 3 (STAT3), signal transducer and activator of transcription protein 5 (STAT5), cAMP response element binding protein (CREB) and p38 mitogen-activated protein kinase (p38)) and assessed whether the intracellular phosphorylation status of these markers was altered in splenic and peripheral lymphocytes. And indeed, the phosphorylation levels of STAT3 (Fig 2A) and CREB (Fig 2B) were significantly upregulated in AML spleen lymphocytes compared with peripheral lymphocytes in control rats, and in control rats and leukemic rats, respectively.

Our observation of increased STAT 3 phosphorylation correlates well with studies showing constitutive STAT3 signaling in leukemic blasts from AML patients (Benekli, Baumann, & Wetzler, 2009) and an association with poorer overall survival in leukemia patients (Benekli et al., 2002). A selective STAT3-inhibitor induced apoptosis in several AML cell lines and in cells harvested from pediatric AML patients, pointing towards STAT3 as a potential target in leukemic therapy (Redell, Ruiz, Alonzo, Gerbing, & Tweardy, 2011). One of the new experimental approaches of AML treatment is concomitant use of tyrosine kinase inhibitors (TKI) (Medinger, Lengerke, & Passweg, 2016), and blocking STAT3 signaling subsequently enhanced leukemic cell death in vitro during TKI treatment (Bewry et al., 2008). Even though some clinical trials of STAT3 inhibition have reported conflicting results (Bruserud, Nepstad, Hauge, Hatfield, & Reikvam, 2015), STAT3 still remains an intriguing potential target in AML treatment, partly due to the heterogeneity of this disease (Bruserud et al., 2015; Sakamoto et al., 2015).

The other target with a significant upregulation of phosphorylation in splenic lymphocytes was CREB, which has been found to promote leukemic cell survival in vitro and to be
upregulated in leukemic blasts from bone marrow of AML patients (Crans-Vargas et al., 2002; Shankar et al., 2005). CREB target genes are essential in e.g. cell proliferation, apoptosis, differentiation and hematopoiesis which make CREB signaling pathways interesting targets in AML therapy (Cho, Mitton, & Sakamoto, 2011; Sakamoto et al., 2015). A recently published study propose that the ability of CREB to upregulate CCAAT-enhancer-binding protein-δ (C/EBPδ) is a critical step in leukemogenesis, as it subsequently blocks myeloid terminal differentiation, an arrest that is reversed when the CREB-C/EBPδ pathway is silenced (Tregnago et al., 2016).

Collectively, the increased intracellular phosphosignaling pattern of STAT3 and CREB in lymphocytes harvested from spleen lymph, markers shown to be strongly associated with leukemogenesis, suggests that the infiltration of AML blasts is affecting the local cell signaling of the spleen.

**Upregulation of proteins involved in STAT3 and CREB pathways in spleen lymph**

Next, we asked whether we could identify patterns of STAT3 and CREB activation in the proteome of the leukemic spleen lymph. Four lymphatic samples from leukemic spleens and control spleens were immunodepleted by removal of the seven most abundant plasma proteins, trypsinized and analyzed on a LC-ion-trap mass spectrometer. Plasma samples from the same animals were pooled into a leukemic and control pool, and prepared and analyzed in the same run on the mass spectrometer. To identify proteins in pathways that involved STAT3 or CREB, we used the Perseus interface (Aasebo, Berven, Selheim, Barsnes, & Vaudel, 2016), and 19 and 32 unique proteins in STAT3 and CREB regulated pathways, respectively. When the level of these proteins in lymph was compared, there was a significant upregulation of STAT3 related proteins (Fig 3A). Surprisingly, contrasting the flow cytometry data, we found a downregulation of CREB associated proteins in the leukemic spleen lymph (Fig 3B),
still, however, suggesting involvement in spleen AML development. When comparing flow cytometry and proteomic data, we must bear in mind that intracellular phosphorylation of proteins and extracellular expression may not be directly comparable. Clearly, to be detected in lymph it is required that proteins are secreted to the interstitium, where they may also be sequestered in extracellular matrix proteins or be degraded by proteolytic enzymes, e.g. (Clause & Barker, 2013; Hynes, 2009), that might explain the diverging results for CREB in the intracellular and extracellular compartment.

**Increased levels of leukemia-associated proteins in spleen lymph**

In a previous study, we reported local spleen production of inflammatory cytokines and proteins reflected in spleen lymph and interstitial fluid as a response to systemic inflammation (LPS) (Oveland et al., 2012). We wanted to use a similar approach to assess whether we could identify leukemia-associated proteins that were upregulated as a result of AML infiltration. The LC-ion-trap mass spectrometer identified 338 unique proteins in immunodepleted lymph and plasma from leukemic and control rats. During steady state conditions, the interstitial concentration of any protein deriving from plasma only, will be lower than in plasma, whereas locally produced proteins will have a lymph to plasma (L/P) ratio >1.0 (Michel & Curry, 1999). This concentration and ratio can be found by assaying prenodal lymph (Wiig & Swartz, 2012). Even though all our lymph samples were postnodal, we were able to harvest prenodal spleen lymph in one control rat. Progenesis software identified 203 proteins and when normalized intensity of proteins from these samples were assessed, linear regression analysis gave a regression coefficient of $r^2=0.994$. The proteins from pre- and postnodal lymph were therefore found to be practically identical with respect to protein concentration and protein distribution (Fig 1C). Thus, with the assumption that in our case the sampled lymph was ~prenodal, we defined proteins as locally produced in the spleen when they had a
lymph/plasma (L/P) ratio ≥ 1.5. We identified 59 unique proteins in control spleen lymph, 14 proteins in leukemic spleen lymph and 17 proteins present in both groups, that all had a L/P ratio ≥ 1.5 (Fig 4A).

We further assessed whether proteins known to be associated with AML could be detected in leukemic spleen lymph and selected five proteins that are known from literature to be related to AML development; aminopeptidase N (Bouchet, Tang, Fava, Legrand, & Bauvois, 2014; Piedfer et al., 2011), tyrosine-protein kinase receptor (Doepfner, Boller, & Arcaro, 2007; Rushworth, Murray, Zaitseva, Bowles, & MacEwan, 2014), SPARC-like protein 1 (Alachkar et al., 2014; Nian et al., 2014), insulin-like growth factor 1 (Doepfner, Spertini, & Arcaro, 2007; Karmali et al., 2015) and proteasome subunit alpha type (Ganesan et al., 2016; Larrue et al., 2016). Indeed, all these proteins were found to be elevated in leukemic spleen lymph, when compared to control spleen lymph and control plasma (p=0.0480) (Fig 4B). Of particular interest in our material was SPARC-like protein 1, a protein that recently has been linked to leukemic cell growth in vitro and proposed as a possible marker of patient outcome (Alachkar et al., 2014). SPARC-like protein 1 was identified with a L/P ratio of 2.652 and 2.079 in leukemic rats and control rats, respectively, while the plasma pool levels remained constant between the groups (Fig 4C). This indicates that the protein is locally produced in the spleen in both health and disease, and further suggests an upregulation of protein secretion during AML development. Thus, leukemia-associated proteins were detected with increased levels in leukemic spleen lymph when compared with control lymph, and SPARC-like protein 1 seems to be upregulated in the spleen subsequent to AML blast infiltration. This further implicates the spleen as an active participant and possible promoter of the leukemogenic process, and that there is an interaction between the bone-marrow compartment and the spleen during AML development.
To summarize; by cannulating efferent lymphatic vessels we were able to monitor the response on the spleen microenvironment of leukemic cell invasion that is occurring during AML development. We found increased signaling for key leukemia target molecules in splenic lymphocytes and alterations in the proteomic profile of spleen lymph, even at an early time-point of leukemic development. Thus, interstitial fluid, and its surrogate efferent lymph, can be used to provide unique information about spleen responses during AML progression. Consequently, the presently demonstrated access to this compartment can offer new insight on organ-specific processes in leukemogenesis.

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References:


Semaeva, E., Tenstad, O., Skavland, J., Enger, M., Iversen, P. O., Gjertsen, B. T., & Wiig, H. (2010). Access to the spleen microenvironment through lymph shows local cytokine production, increased cell flux, and altered signaling of immune cells during...
lipopolysaccharide-induced acute inflammation. J Immunol, 184(8), 4547-4556. doi:jimmunol.0902049 [pii]
10.4049/jimmunol.0902049
**Figure 1.** Leukemic development in experimental animals. 

A, Normal spleen tissue (left), arrows point toward leukemic blasts infiltration in the spleen (right). Scale bar 100 µm. 

B, Spleen weight (grams) of control (n=5) and AML rats (n=4), harvested on day 10-15 after transplantation of 20 x 10⁶ BNML cells, shows a clear tendency of spleen enlargement, t-test, p = 0.0693. Values are mean ± SEM. 

C, Normalized intensities of proteins from pre- and post nodal lymph, n=1 rat (control). Linear regression analysis (log-log), r²=0.99.
Figure 2. Spleen lymph lymphocytes respond to AML infiltration. A, Spleen lymph from control and leukemic animals analyzed for phosphorylation of STAT3, STAT5, CREB and p-38. Significant difference of phosphoprotein response in AML lymph for STAT3 compared with control plasma, and for CREB in AML lymph compared with control and AML plasma. Leukemic animals (n=3) for spleen lymph and plasma, control animals n=6 in STAT3 signaling, and n=4 in CREB signaling. Unsuccessful attempts of retrieving CREB and p38 signaling from lymph and plasma in n=2 animals in the control group. One-way ANOVA with Tukey post-hot test, *p < 0.05. Values are mean ± SEM
Figure 3. STAT3 and CREB activation evaluated by proteomic profile. A, Proteins related to STAT3 regulated pathways upregulated in the leukemic spleen lymph (p = 0.0202), n=4 in both groups. B, CREB associated proteins downregulated when compared with control spleen lymph (p = 0.0207), n=4 in both groups. Two-way ANOVA, *p < 0.05. Values are mean ± SEM.
Figure 4. Proteins related to AML leukemogenesis upregulated in leukemic spleen lymph. 

A, Assessment of locally produced proteins defined as L/P ratio ≥ 1.5 identified 59 unique proteins in control spleen lymph, 14 proteins in leukemic spleen and 17 proteins in both groups, n=4 in both groups. 

B, Proteins related to AML leukemogenesis found upregulated in leukemic spleen lymph (p = 0.0480), when compared to both control lymph and plasma, n=4 in both groups, one-way ANOVA with Tukey post-hoc test. 

C, SPARC-like protein 1 identified as a locally produced protein linked to leukemogenesis and upregulated in leukemic lymph compared with control lymph, reflected as L/P ratio of 2.652 and 2.079, respectively. n=4 in both groups.
Adipose tissue macrophages: the inflammatory link between obesity and cancer?

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Introduction: Obesity has increased dramatically over the last three decades. Thus, epidemiological evidence linking obesity and cancer has ignited our interest in the relationship between adipose tissue mass and cancer development. Obesity is defined as an excess of adipose tissue that is typified by a chronic, low-grade inflammatory response instigated by macrophage infiltration. Therefore, in this review, we will discuss the putative causal relationship between obesity-induced chronic inflammation and cancer with particular focus on adipose tissue macrophages.

Areas covered: Chronic, low-grade inflammation has long been associated with cancer initiation, promotion and progression. Therefore, signals derived from adipose tissue macrophages may play a significant role in carcinogenesis. In this review we will discuss the molecular mechanisms of cancer development in obesity and highlight possible therapeutic strategies aiming at adipose tissue macrophages.

Expert opinion: The strong correlation between tumor-associated macrophage infiltration and tumor growth and progression emphasizes the value of macrophages as an effective therapeutic target. It remains to be deciphered to what extent adipose tissue macrophages contribute to these processes, especially in tumors growing within or adjacent to adipose tissue. More effort should also be placed on elucidating macrophage differences between humans and mice that may lead to the development of more effective diagnostic and therapeutic strategies.

Keywords: adipose tissue, cancer, inflammation, macrophage, obesity

1. Introduction

The adipose tissue represents an intricate, vital and highly active metabolic and endocrine organ. Yet, until recently, the adipose tissue has been considered as an inert tissue viewed solely as a storage depot of energy in the form of triglycerides [1]. Adipose tissue biology has unfolded, particularly in the last three decades, in the limelight of obesity rise worldwide. Obesity, defined as a body mass index (BMI) ≥ 30 kg/m², has increased dramatically with 671 million adults being classified as obese, according to the Global Burden of Disease Study 2013 [2]. The condition is characterized as an excess of adipose tissue mass associated with metabolic dysregulation leading to insulin resistance, chronic inflammation and altered secretion of adipokines [3,4]. Additionally, metabolic dysregulation often manifesting in metabolic syndrome (defined as a combination of abdominal obesity, hypertension, hypertriglyceridaemia, low HDL – cholesterol, and hyperglycemia) is an established risk factor for many cancers [5-7]. Indeed, obesity is associated with an increased mortality from cancer of the prostate and stomach in men, breast (post-menopausal), uterus, cervix, and ovaries in women, and esophagus, liver, gallbladder, pancreas, colon and kidney in both genders [6].
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**Article highlights.**

- Obesity has been associated with an increased mortality from several cancer types, including those of esophagus, liver, gallbladder, pancreas, colon and kidney in both men and women.
- In obesity, chronic inflammatory response instigated by adipose tissue macrophages creates a mutagenic and growth-promoting environment that potentiates the acquisition of oncogenic mutations.
- Chronically activated macrophages within adipose tissue produce a wide range of cytokines, chemokines and growth factors that stimulate tumor growth and progression.

This box summarizes key points contained in the article.

It is now well established that the immune system interacts with metabolic processes both in the physiological and pathological states, a crosstalk often referred to as immunometabolism [3]. This liaison might be viewed as a key homeostatic mechanism, dysfunction of which can lead to a group of chronic metabolic disorders. For example, low-grade chronic inflammatory response associated with macrophage accumulation within adipose tissue is a critical component in the development of obesity-associated comorbidities such as cardiovascular diseases, type 2 diabetes mellitus and the focus of this review, cancer [4,7]. Within the pro-inflammatory microenvironment of the obese state, crosstalk between adipocytes, macrophages and epithelial cells may enhance cancer risk and/or progression. Apart from directly initiating the formation of malignant cells, chronic inflammation might also act as a tumor promoter by triggering infiltration of initiated tissues by immune cells [8].

Research over the last two decades has solidified the concept that chronic inflammation provides the underpinnings for cancer development [9,10]. Several inflammatory conditions precede malignant changes, including gastritis for gastric cancer, pancreatitis for pancreatic cancer and inflammatory bowel disease for colorectal cancer [9,10]. In this review we will discuss the reflexive relationship between chronic inflammatory response within adipose tissue and cancer with particular focus on adipose tissue macrophages (ATMs). Additionally, we aim to provide a logical framework for understanding the inflammatory connection between obesity and cancer.

### 2. Adipose tissue and macrophages

The adipose tissue has long been thought of as being composed of adipocytes only, that is, specialized cells designed for the storage of energy in the form of triglycerides. To fulfill its role in maintaining energy homeostasis, the adipose tissue responds rapidly and dynamically to any alterations in the balance between energy intake and expenditure. When energy intake derived from food outpaces energy expenditure (the sum of the basal metabolic rate, the diet-induced thermogenesis and the energy expended in physical activity) the adipose tissue expands by enhancing adipocyte proliferation (hyperplasia) and/or enlarging adipocyte size (hypertrophy). Although acute changes in energy intake can be easily handled by adipocytes, a chronic increase of energy imposes substantial metabolic stress on cells crammed full of lipids. Indeed, chronic and excessive expansion of adipose tissue is associated with a smoldering inflammatory response, typified by an elevated expression of pro-inflammatory cytokines and chemokines such as TNF-α, IL-6 and monocyte chemotactic protein (MCP)-1 (also referred to as chemokine (C-C motif) ligand 2 (CCL2)) [11,12]. The recent discovery that macrophages within hypertrophied adipose tissue are the major effectors of inflammation and secrete a slew of pro-inflammatory cytokines highlights the fact that the adipose tissue consists of more than just lipid-storing cells [13-15]. Apart from adipocytes, the adipose tissue is composed of cells embedded in the connective tissue matrix and referred to as the stromal vascular fraction, including preadipocytes at various stages of differentiation, fibroblasts, vascular endothelial cells and a heterogeneous population of immune cells. Among immune cells, ATMs are the most abundant and their number increases commensurate with adipose tissue mass. For example, in lean healthy mice ATMs comprise 10–15% of stromal cells whereas in obesity their number soars up to 45–60%. The same is true in humans where a three fold increase in the number of ATMs has been observed in severely obese subjects with BMIs ~ 50 kg/m² [13,16].

Adipose tissue hypertrophy has been attributed to hypoxia, adipocyte necrotic death, and increased secretion of numerous cytokines, chemokines, growth factors and hormones, as well as dysregulated release of free fatty acids (FFAs), which independently contribute to macrophage recruitment [17,18]. As other types of macrophages, ATMs can be recruited from the reservoirs of monocytes in blood, spleen and bone marrow, and possibly from tissue-resident progenitors [16]. Additionally, recent data indicate that a significant fraction of ATMs undergoes cell division locally, as determined by Ki67 expression and 5-ethynyl-2-deoxyuridine (EdU) incorporation [19,20].

#### 2.1 Macrophage polarization

A characteristic of the ATMs in obesity is their pro-inflammatory phenotype [15,21]. Macrophages are heterogeneous and remarkably plastic cells capable of assuming a range of different phenotypes in response to a barrage of cues derived from microbes, damaged cells or activated lymphocytes [22,23]. Based on in vitro studies, macrophages have been classified into two polarization states – a classically activated (M1) and an alternatively activated (M2) phenotype, mirroring the Th1 and Th2 lymphocytes. According to this classification, IFN-γ alone or in combination with microbial products, such as lipopolysaccharide (LPS), or TNF-α induce the M1 form of polarization. Conversely, IL-4 and IL-13 impose the alternatively activated (M2) phenotype, a
M1 macrophage polarization is mediated mainly through the NF-κB and signal transducer and activator of transcription (STAT)-1. In contrast, STAT-6 activation predominates in M2-polarized macrophages. Several other factors are involved in M2 macrophage polarization, such as PPAR-γ, Krüppel-like factor – 4 and interferon regulatory factor (IRF)-4 [23,24]. Furthermore, various stimuli, including IL-10, TGF-β and glucocorticoids (GCs) induce ‘M2-like’ form of polarization that shares some, but not all, the signature features of the alternatively activated (M2) macrophages, a finding often observed in vivo (e.g., in cancer). Thus, M1- and M2-polarized macrophages represent two extremes in the spectrum of possible macrophage polarization states [22-24].

In general, M1 macrophages secrete high levels of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) and produce reactive oxygen and nitrogen intermediates. M1 macrophages express higher levels of MHC class II molecules participating as inducers on effector cells in polarized Th1 responses. They are microbicidal and tumoricidal. In contrast, M2 macrophages secrete high levels of anti-inflammatory cytokines (e.g., IL-10 and TGF-β) and express high levels of scavenger, mannose and galactose-type receptors. M2 macrophages are associated with polarized Th2 responses, anti-inflammatory and homeostatic functions, tissue repair, angiogenesis and tissue remodeling [22,23].

During the last years, a large body of evidence has accumulated suggesting that metabolism is important in shaping the functional phenotype acquired by macrophages in response to distinct microenvironmental cues [25]. Additionally, macrophages, through release of soluble mediators, orchestrate various aspects of metabolism as illustrated by the development of metabolic diseases when maladaptive. In terms of glucose metabolism, classically activated (M1) macrophages display a metabolic shift towards the anaerobic glycolytic pathway [25]. Enhanced glycolysis involves a switch in the expression of 6-phospho-fructo-2-kinase / fructose-2,6-bisphosphatase (PFK2) from the liver-type PFK2 (L-PFK2) to the more active ubiquitous PFK2 (uPFK2) isoform that maintains higher fructose-2,6-bisphosphate concentrations due to a reduced bisphosphatase activity [26]. An anaerobic process such as glycolysis in M1 macrophages is fitted to meet their rapid energy requirements associated with microbicidal activity as well as to cope with a hypoxic tissue microenvironment. Interestingly, accumulation of succinate, an intermediate metabolite of the tricarboxylic acid cycle, stabilizes hypoxia-inducible factor (HIF)-1α, leading to activation of many genes, including those encoding IL-1β as well as genes involved in glycolysis [27]. Conversely, oxidative glucose metabolism typifies M2 macrophages, which are mostly involved in homeostatic processes that require sustained supply of energy. Also, amino acid metabolism is intimately associated with the functional phenotype of macrophages as illustrated by 1-arginine (1-Arg). M2 macrophages stimulate 1-Arg metabolism leading to the production of ornithine and polyamines, precursors necessary for collagen synthesis, cell proliferation and tissue remodeling via arginase 1 (ARG1). In contrast, M1 macrophages produce nitric oxide (NO•) that is crucial for their microbicidal activity and L-citrulline via nitric oxide synthase 2 (NOS2 or iNOS). Additionally, the host protective function against bacterial infection in M1 macrophages is supported by increased expression of ferritin involved in iron sequestration. Conversely, M2 macrophages release iron, an essential element required for cell proliferation, due to increased expression of ferroportin and thus favor tissue repair and remodeling [25].

The findings presented above indicate that metabolic adaptation is an integral aspect of macrophage polarization and their functional heterogeneity. Given their various functions, it is not surprising that dysregulation of macrophages is linked with a broad spectrum of disorders, ranging from allergy and asthma, autoimmunity and fibrosis to atherosclerosis, metabolic diseases and cancer [16].

2.2 Adipose tissue and inflammation

The first evidence indicating a putative causal relationship between macrophage infiltration and chronic inflammation in adipose tissue of obese mice stems from work carried out more than a decade ago [13,28]. The adipose tissue has been typified by an increased number of ATMs expressing CD11c as indicative of their M1 polarization state. As judged by immunohistochemical analysis, the majority of ATMs have been found surrounding moribund or dead adipocytes, forming so-called crown-like structures [29]. Furthermore, electron microscopy analysis revealed necrotic features of adipocyte cell death, such as ruptured membranes, cellular debris within extracellular microenvironment as well as the presence of small cytoplasmic lipid droplets [29]. Because necrotic cells loose membrane integrity, they rapidly discharge their intracellular components, some of which may trigger an inflammatory response. Indeed, expression analysis of ATMs within adipose tissue of obese mice has demonstrated augmented pro-inflammatory activity manifested by increased expression of TNF-α, IL-6 and iNOS. Conversely, solitary macrophages within adipose tissue of lean mice have been found expressing IL-10 and ARG1 characteristics of M2 macrophages [30,31]. In vitro studies have shown that LPS-stimulated macrophages decrease insulin sensitivity of adipocytes via downregulation of glucose transporter type 4 (GLUT4) and insulin receptor substrate – 1 [32], in both a cell-contact-dependent and -independent manner. Additionally, LPS-stimulated macrophages that have been found to produce TNF-α and IL-12 impair adipogenesis whereas their IL-4 stimulated counterparts have little or no effect [21]. Thus, the switching from anti-to pro-inflammatory phenotype has been attributed to insulin resistance observed in obese animals. Indeed, conditional ablation of CD11c+ cells expressing the diphtheria toxin receptor led to a marked decrease in pro-inflammatory cytokines and to...
rapid normalization of insulin sensitivity in obese mice [33].

Many clinical and pre-clinical studies have reinforced the evidence for a role of macrophages in the development of metabolic diseases. For example, an integrative genetics analysis of human adipose tissue identified a gene expression signature associated with metabolic dysfunction that was enriched for gene sets strongly associated with macrophages and inflammation (referred to as the macrophage-enriched metabolic network or MEMN) [34-36]. This suggested that macrophages and inflammation are important cells and processes affecting the adipose tissue involved in the development of metabolic diseases, consistent with a body of related literature using the mouse model systems.

3. Adipose tissue macrophages and cancer initiation and promotion

Obesity-associated chronic inflammation, aberrant production of adipokines and insulin resistance are likely to play a role in carcinogenesis. In recent years, the endocrine function of adipocytes has received increasing attention due to the possible associations between secreted adipokines and the development of cancer. For example, increased proliferation of colon, ovarian, breast and prostate cancer cells has been observed upon stimulation with the satiety hormone leptin [37-39]. Furthermore, induced invasive potential of B16BL6 melanoma cells through the stimulation of epithelial-to-mesenchymal transition and downregulation of the adhesive protein E-cadherin and metastasis suppressor Kiss1 has been shown following culture with adipocyte-conditioned medium [40]. Given the tumor-promoting role of adipocytes, it was surprising to find an accelerated tumor growth in A-Zip/F1 mice devoid of adipose tissue as well as with undetectable levels of leptin, adiponectin and other adipokines [41,42]. Therefore, the role of adipokines in enhancing tumor growth and progression has been belittled [42]. Interestingly, A-Zip/F1 mice were diabetic [43]. If sustained, insulin resistance increases insulin and IGF-1 appear to offer the most plausible mechanistic explanation. Both insulin and IGF-1 are mitogenic (but not mutagenic) and both are present at high levels in insulin-resistant patients [44]. Even though increased cell proliferation by itself may not be mutagenic, obesity-associated chronic inflammation potentiates the acquisition of oncogenic mutations. Interestingly, receptors for insulin and IGF-1 have been found over-expressed on the surface of certain cancer cells [44,45]. Insulin resistance will not be discussed further in this review, but the relationship between insulin signaling and cancer has been extensively studied elsewhere (for an excellent review, see Pollak et al.) [45].

3.1 Inflammation and cancer

The functional relationship between inflammation and cancer was first proposed in the nineteenth century, on the basis of observations that tumors arose at sites of chronic inflammation, and that a cellular inflammatory infiltrate was always present in human tumor biopsies [10,46]. Inflammation is defined as a physiological process composed of dynamic, interwoven and histologically apparent cytological changes with cellular infiltrate and inflammatory mediators present in the affected tissue as a result of an injury or aberrant stimulation with physical, chemical or biological agents. It involves localized reactions leading to morphological changes (i.e., tissue remodeling and angiogenesis) aiming at tissue repair and healing. Inflammation is divided into acute and chronic forms. Acute inflammation is characterized by a rapid onset, is short-lived, lasting usually a few days with neutrophil accumulating first and later followed by monocytes. If not resolved, prolonged chronic inflammation ensues, lasting for several months or even years, and is characterized by a progressive shift in the composition of the cellular infiltrate, with neutrophils being completely replaced by monocytes and macrophages.

In obesity, the initial stimuli that cause chronic inflammation are not well defined but may involve hypoxia, adipocyte necrotic death, increased secretion of cytokines, chemokines, hormones and growth factors, and dysregulated FFA release (for a comprehensive review, see Dalmas et al.) [17]. For example, moribund adipocytes surrounded by macrophages in the adipose tissue of obese humans and mice may regulate their recruitment and activation [29]. Interestingly, a difference in terms of activation state, gene expression patterns and response to LPS has been found between ATMs from obese and lipodystrophic aP2-nSREBP-1c mice, characterized by an increased number of apoptotic, rather than necrotic, adipocytes [47]. Therefore, it has been proposed that macrophage recruitment and activation may vary in response to apoptotic and necrotic cells. Necrotic cell death, in the absence of pathogens, triggers sterile inflammation mediated by endogenous danger-associated molecular patterns (DAMPs) (also referred to as damage-associated molecular patterns) [48]. The DAMPs include high-mobility group box 1 protein
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(HMGB1), recently found to be extremely high in obese children, that binds to germline-encoded pattern recognition receptors present on macrophages and triggers expression of pro-inflammatory genes downstream of NF-κB [49,50].

If not quickly controlled, the sterile inflammation can become chronic and predispose cells for an oncogenic transformation via induction of genomic instability, alterations in epigenetic state and stimulation of cell proliferation. Among the molecular factors that contribute to inflammation-induced carcinogenesis are reactive oxygen and nitrogen species (ROS and RNS, respectively), abundant pro-inflammatory cytokines and chemokines as well as increased COX-2 and NF-κB expression. In the case of obesity that can last over years or even decades, chronic inflammatory response may contribute to increased DNA mutation rates and overall genetic instability. For example, high levels of macrophage-derived nitric oxide (NO•) via up-regulation of NOS2 can react with O2 or superoxide (O2•-) to generate additional reactive species such as nitrous anhydride (N2O3) and peroxynitrite (ONOO’), respectively. The latter one reacts with CO2 forming nitrosoperoxycarbonate (ONOOCO3−) and homolysis of the -O-O- bond within ONOOCO3− produces nitrogen dioxide (NO2•) and carbonate radical anion (CO3•−), of which CO3•− is capable of guanine oxidation in DNA (Figure 1) [51]. Interestingly, recent studies have identified AGC and TGC sequences in DNA as a major determinant of ONOOCO3− oxidation hotspots [52]. High levels of NO• lead to DNA strand breaks, point mutations and aberrant DNA cross-linking, thus causing genomic instability and contributing to carcinogenesis by mutating proto-oncogenes and tumor suppressor genes (Figure 1) [51]. Additionally, chronic inflammation reduces expression and enzymatic activity of mismatch repair (MMR) proteins such as mutL homolog 1 (MLH1) and mutS homologs 2 and 6 (MSH2 and MSH6) [53]. Inflammatory response also increases expression of DNA methyltransferase leading to global hyper-methylation of the genome, resulting in the promoter silencing of several genes, including the MMR gene hMLH1 [54]. Importantly, hyper-methylation of tumor suppressor genes such as APC, CDKN2, BRCA1, Rb and MDM2 has been implicated in the development of several cancers including those of breast and colon [55]. Together, these findings indicate that genomic instability and epigenetic changes are involved in inflammation-induced carcinogenesis. Interestingly, a recently performed epigenome-wide association study has revealed an association between BMI with methylation of HIF3A, a critical component of the transcriptional response to hypoxia, in blood and adipose tissue of adult subjects [56]. A strong foundation has therefore been provided for further exploration of the causal relationship between epigenetic modifications and regulation of BMI and the downstream detrimental effects of obesity.

In an extension to its physiologic role in maintaining tissue homeostasis, inflammatory response may also provide survival and proliferation signals to initiated tissues, thus leading to tumor promotion and progression (Figure 2). This holds particularly true for TNF-α and IL-6 that have been found abundantly expressed by ATMs in obese mice and humans. Upon stimulation with TNF-α and binding to its cognate receptor TNF-R1, NF-κB activation leads to up-regulation
of several negative regulators of apoptosis, such as cellular FLICE-inhibitory protein (c-FLIP) and cellular inhibitor of apoptosis protein (cIAP)1 [57]. Furthermore, the tumor-promoting activity of TNF-α has been demonstrated in various experimental cancers. For example, it has been shown that macrophage-derived TNF-α promotes Wnt/β-catenin signaling in gastric mucosa by inhibiting glycogen synthase kinase 3β (GSK3β), thus contributing to the tumor development [58]. Importantly, increased systemic levels of TNF-α, as found in obesity, have been correlated with higher risk of cancer-related death and, to a lesser degree, with overall cancer prevalence [59]. IL-6 most likely stimulates cell proliferation and survival through the Janus kinase (JAK) / STAT3 pathway [60]. Similar to TNF-α, circulating levels of IL-6 have been correlated with overall cancer death and increased risk of cancer events [59]. In addition to higher abundance of pro-inflammatory cytokines, excess adiposity is associated with increased levels of several hormones, particularly estrogens. Although mainly synthesized in the ovaries of pre-menopausal women, peripheral tissues, such as adipose tissue, serve as a critical source of estrogens after the menopause. Indeed, in post-menopausal women, levels of estrogens have been found elevated with increasing BMI [61]. Importantly, increased expression of aromatase, which catalyzes synthesis of estrogens from androgens, has been associated with an increased pro-inflammatory cytokine production, including TNF-α and IL-1β, in mammary ATMs that surround necrotic adipocytes in obese mice [62]. The association between BMI, inflammation and increased aromatase expression has been further confirmed in breast tissue of overweight and obese women [63].

4. Adipose tissue macrophages and cancer progression

As the adipose tissue, established tumors are composed of a heterogeneous population of cells and include both tumorigenic and non-tumorigenic stromal cells. It is now recognized that the collaborative networks between tumor and stromal cells provide the underpinnings for disease progression [64]. Among stromal cells are immune and inflammatory cells, cells of mesenchymal origin, including extracellular matrix-forming fibroblasts, and a vascular network of endothelial cells. Macrophages in tumors, alike those found in adipose tissue in obese mice and humans, may comprise a major component of the inflammatory infiltrate and in some cases even outnumber tumorigenic cells [65]. TAMs are derived from circulating monocytes that respond to a barrage of chemokines produced by stromal and tumor cells, particularly MCP-1. In this regard, high levels of MCP-1 have been correlated...
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with an increased number of TAMs leading to poor prognosis in certain cancers, including those of breast and bladder [16,66]. Although TAMs play a well-defined role in cancer progression, the importance of ATMs in this process is incompletely understood. The adipose tissue has been intimately associated with the microenvironment of many tumors and within this configuration, both tissues form a suitable environment for continuous and dynamic interactions. Indeed, we previously demonstrated that the adipose tissue juxtaposed to implanted B16F10 mouse melanoma tumors exhibited reduced adipocyte size, profound fibrosis, increased angiogenesis and abundant infiltration of macrophages that resembled TAMs [67]. In this regard, macrophage recruitment was associated with adipocyte necrosis, as judged by loss of perilipin staining and translocation of HMGB1 into the cytoplasm and extracellular space [68]. Interestingly, tumors implanted at a site distant from the adipose tissue depot were growth delayed, depleted with inflammatory cells and had fewer blood vessels, further supporting the idea that the adipose tissue fuels tumor growth by serving as a depot for M2-like macrophages [65,67]. Although it was initially believed that tumor cells were the principal source of angiogenic factors, it is now recognized that inflammatory cells, including macrophages, secrete an array of angiogenic factors such as VEGF, GM-CSF, IL-1ß, IL-6 and TNF-α [65,69].

Therapy based on bevacizumab, an antibody against VEGF and an inhibitor of angiogenesis, in combination with conventional chemotherapy, is considered a first-line treatment option for patients with colorectal cancer. However, diminished efficacy in obese subjects has been reported and hypothetically linked with elevated levels of pro-angiogenic factors, such as VEGF, derived from an increased adipose tissue mass [70]. Given their abundance in the adipose tissue, ATMs may play a crucial role in the angiogenic switch, necessary for tumor progression. Interestingly, ATMs derived from obese humans displayed the gene expression profile resembling that of TAMs [71]. In this regard, ATMs have been found producing growth factors, cytokines, chemokines and proteolytic enzymes involved in the regulation of tumor growth, angiogenesis, and invasion as well as promoting cancer metastasis.

Apart from stimulating angiogenesis, TAMs have also been found stimulating lymphangiogenesis, by producing large amounts of VEGF-C and VEGF-D. For example, in human cervical cancer, the density of TAMs expressing VEGF-C correlates with an increased number of peritumoral lymphatic vessels including those containing cancer cells, a prerequisite for subsequent metastatic lymph node (LN) dissemination [72]. Increased angiogenesis, lymphangiogenesis and LN metastasis attributed to the presence of macrophages have also been found in obese mice with implanted B16F10 mouse melanoma tumors [73]. In addition, the presence of lymphatic endothelium-specific hyaluronan receptor (LYVE)-1 has been observed on stabilin-1⁺, F4/80⁺, CD11b⁺ macrophages found mostly at the periphery of growing tumors in mice [74]. It has been therefore proposed that macrophages may transdifferentiate into lymphatic endothelial cells and incorporate into pre-existing lymphatic vessels [74].

5. Therapeutic perspectives

In obesity, the putative causal relationship between chronic inflammatory response and cancer qualifies ATMs as a potential therapeutic target. Although much remains to be deciphered, the understanding of the link between inflammation and cancer is emerging. Therapies directed at the chronic inflammatory response in general have already been proven successful in preventing cancer prevalence, as revealed by an increased efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) in reducing risk of colon adenomas, a precursor of colon cancer [75]. Although dampening the inflammatory and immune response could seem to be beneficial to prevent cancer initiation, an overall immunosuppression could prove detrimental as illustrated by an increased cancer incidence following inhibition or deletion of NF-κB in pre-clinical studies [76-80]. Furthermore, the increased risk of certain cancers has been observed within patients with AIDS or pharmacologically immunosuppressed following transplant surgery [75]. Therefore, although chronic inflammation promotes carcinogenesis under certain conditions, such as obesity, it seems unlikely that immunosuppression alone can accomplish beneficial results.

In the adipose tissue, the M2 macrophage polarization state of resident ATMs is perpetuated by cytokines, such as IL-4 and IL-13, produced by unique c-Kit⁺Sca-1⁺ lymphoid cells and eosinophils. Such cell recruitment is inhibited in the obese state [81,82]. Furthermore, the increased risk of certain cancers has been observed within patients with AIDS or pharmacologically immunosuppressed following transplant surgery [75]. Therefore, although chronic inflammation promotes carcinogenesis under certain conditions, such as obesity, it seems unlikely that immunosuppression alone can accomplish beneficial results.

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insulin sensitivity in WT mice, when compared to GPR120 knockout mice [85].

Unlike those in the initiating inflammatory microenvironment, macrophages in established tumors exhibit M2-like phenotype [86]. For example, refractoriness to anti-VEGF therapy has been associated with tumor-infiltrating cells of myeloid origin [87]. Thus, it has been proposed that anti-angiogenic therapies directed at VEGF may become more effective when combined with targeting the recruitment, infiltration and retention of myeloid cells. Indeed, by using anti-VEGF mAb in combination with mAb, which targets CD11b+Gr1+ bone marrow-derived myeloid cells, more efficient inhibition of refractory tumor growth has been observed than by using anti-VEGF mAb alone in pre-clinical studies [87,88]. The same holds true for radiotherapy, wherein myeloid cells recruit to the tumors grown in irradiated tissues, thereby restoring tumor growth. Indeed, significant enhancement of tumoricidal response to radiation has been observed following administration of CD11b mAb in pre-clinical studies [89]. Inflammatory immune component within the tumor microenvironment can originate from two distinct sources: nearby local tissues or bone marrow. The former one represents the most accessible option. Given that the adipose tissue serves as a rich source of macrophages, it remains to be deciphered to what extent ATMs contribute to radiation and chemotherapy resistance of primary tumors or metastasis to the lymph nodes, which are typically ensheathed by adipocytes.

As macrophages represent the first line of defense, their pro-inflammatory activation should suppress tumor formation or even stimulate tumor regression. However, in tumors, the role of inflammatory response is more intricate and sometimes contradictory. For example, macrophage-derived NO• production has been shown to inhibit DNA synthesis and mitochondrial respiration in L1210 mouse lymphocytic leukemia cells in a dose-dependent manner [90]. Furthermore, NOS2 expression in tumor-infiltrating macrophages has been demonstrated to directly inhibit tumor growth and metastasis [91]. However, phenotypic switching within tumor microenvironment might render them tolerant and / or tumor promoting. It has been suggested that in established tumors, diminished NO• production is due to the formation of tumor-derived suppressor molecules such as IL-10, TGF-β and prostaglandin E2 (PGE2) [92,93]. Indeed, macrophages isolated from tumor-bearing mice exhibited suppressed cytolytic activity and decreased NOS2 expression. Interestingly, macrophages from within the tumors revealed a more pronounced suppression when compared with more distally located peritoneal counterparts [94]. Moreover, tumoricidal or tumorigenic role of NO• may in part depend on the status of the tumor suppressor gene p53. NO• leads to increased p53 activity, which in turn causes apoptosis, cell cycle arrest or senescence in damaged cells whereas during its absence, NO• can lead to genotoxic stress and increased cell proliferation [75]. In this regard, NO• location, concentration, and timing placed in context with the tumor microenvironment are crucial in the development of strategies for cancer treatment and prevention. The same holds true for TNF-α originally identified as a factor that causes tumor necrosis at high concentrations [75]. As described above, TNF-α activity at moderate levels has a tumor-promoting role [75]. The possibility to skew macrophage polarization within established tumors from M2-like into M1 and / or to enhance their tumoricidal activity in a controlled manner could theoretically inhibit tumor progression or even stimulate tumor regression. Indeed, intra-tumor injection of GM-CSF has proven successful in inhibiting growth and metastasis of murine mammary tumors through TAMs re-polarization, as revealed by their reduced angiogenic potential [95].

6. Conclusion and expert opinion

Dysregulation of macrophages residing in the adipose tissue has been attributed to the development of low-grade chronic inflammatory response that potentiates the risk of obesity-associated comorbidities, including cancer. Additionally, macrophages from either obese or tumor-associated adipose tissue share some of the features with TAMs [67,71]. The strong correlation between TAMs infiltration and cancer progression, which has already been recognized in murine tumor models, highlights their value as an effective therapeutic target. Conversely, our paucity of knowledge about human TAMs is now becoming increasingly apparent. Although the correlation between TAM infiltration and poor prognosis has been reported in several publications, conflicting results have also been shown [66,96]. This discrepancy might be attributed to the technical limitations of obtaining fresh material for fluorescence-activated cell sorting and to macrophage immunohistochemical phenotyping with the cell surface antigen CD68 serving as the marker of choice for macrophage characterization. Owing to its inherent presence during both M1 and M2 polarization states, it is imperative that future work should aim at identification of markers that differentiate between macrophages of tumoricidal and tumorigenic activity. Furthermore, the importance of human ATMs in tumor growth and progression is far from being understood, mainly due to a limited assessment of tumor-associated adipose tissue, despite the fact that some tumors grow directly within or adjacent to adipose tissue, including those of breast and skin. Therefore, a better identification of macrophage subpopulations will provide a new prognostic and predictive tool in clinical practice. More importantly, it will help with the identification of molecular targets for therapies that aim at inhibiting tumor angiogenesis, lymphangiogenesis and metastasis. This search will most likely be based on the current knowledge of markers that distinguish macrophage subpopulations in mice. In addition, more effort should be placed on understanding effects of mixed M1 and M2 polarization stimuli on macrophage differentiation and activation, which more closely resembles in vivo situation. Given perceived differences between murine and human macrophages, macrophage gene
expression signature should also be identified within human tumor samples from individuals with different stages of cancer. This analysis will address the question whether it is the difference in gene expression or the total number of macrophages that ultimately matters. Additionally, not only the mere presence of macrophages but also their localization could become the part of the traditional classification schemes and would provide new prognostic and/or predictive tools in clinical practice. Recent evidence suggests that local proliferation can contribute to macrophage adipose tissue infiltration during obesity. Therefore, in order to develop therapeutic strategies, it is of crucial importance to determine whether ATM proliferation accounts for TAMs accumulation within tumors. If this turns out to be true, anti-proliferative drugs could limit TAM numbers and slow tumor growth. Although it is now well recognized that macrophages drive tumor growth and progression in general, it remains to be seen to what extent ATMs contribute to these processes.

**Declaration of interest**

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**Bibliography**

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

13. First paper demonstrating an increased number of macrophages within adipose tissue in obesity.


• Paper demonstrating that macrophages surround necrotic adipocytes forming crown-like structure.


44. Johnson JA, Gale EA. Diabetes, insulin use, and cancer risk: are observational studies part of the solution or part of the problem? Diabetes 2010;59(5):1129-31


60. Heinrich PC, Behrmann I, Zeleniuch-Jacquotte A, et al. Body mass index, circulating levels of sex-steroid index, circulating levels of sex-steroid...
Adipose tissue macrophages: the inflammatory link between obesity and cancer?


First paper demonstrating an increased number of macrophages within tumor-associated adipose tissue that exacerbate chronic inflammation and stimulate angiogenesis.


70. Aykan NF, Yildiz I, Sen F, et al. Effect of increased body mass index (BMI) on time to tumour progression (TTP) in unresectable metastatic colorectal cancer (mCRC) patients treated with bevacizumab-based therapy. Med Oncol 2013;30:679


77. Gapuzan ME, Yufit PV, Gilmore TD. Immortalized embryonic mouse fibroblasts lacking the RelA subunit of NF-kappaB demonstrate a growth inhibitory role for NF-kappaB. Proc Natl Acad Sci USA 1998;95:5307-12


84. Makki K, Frovigel P, Wolowczuk I. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. ISRN inflammation 2013;2013:139239

85. Oh DY, Taludar S, Bae EJ, et al. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensing effects. Cell 2010;142:687-98


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