The Immune Landscape in Breast Cancer - The Role of CD169+ Macrophages, Lymphoid Structures and Neutrophils

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The Immune Landscape in Breast Cancer

The Role of CD169^{+} Macrophages, Lymphoid Structures and Neutrophils

OSCAR BRIEM
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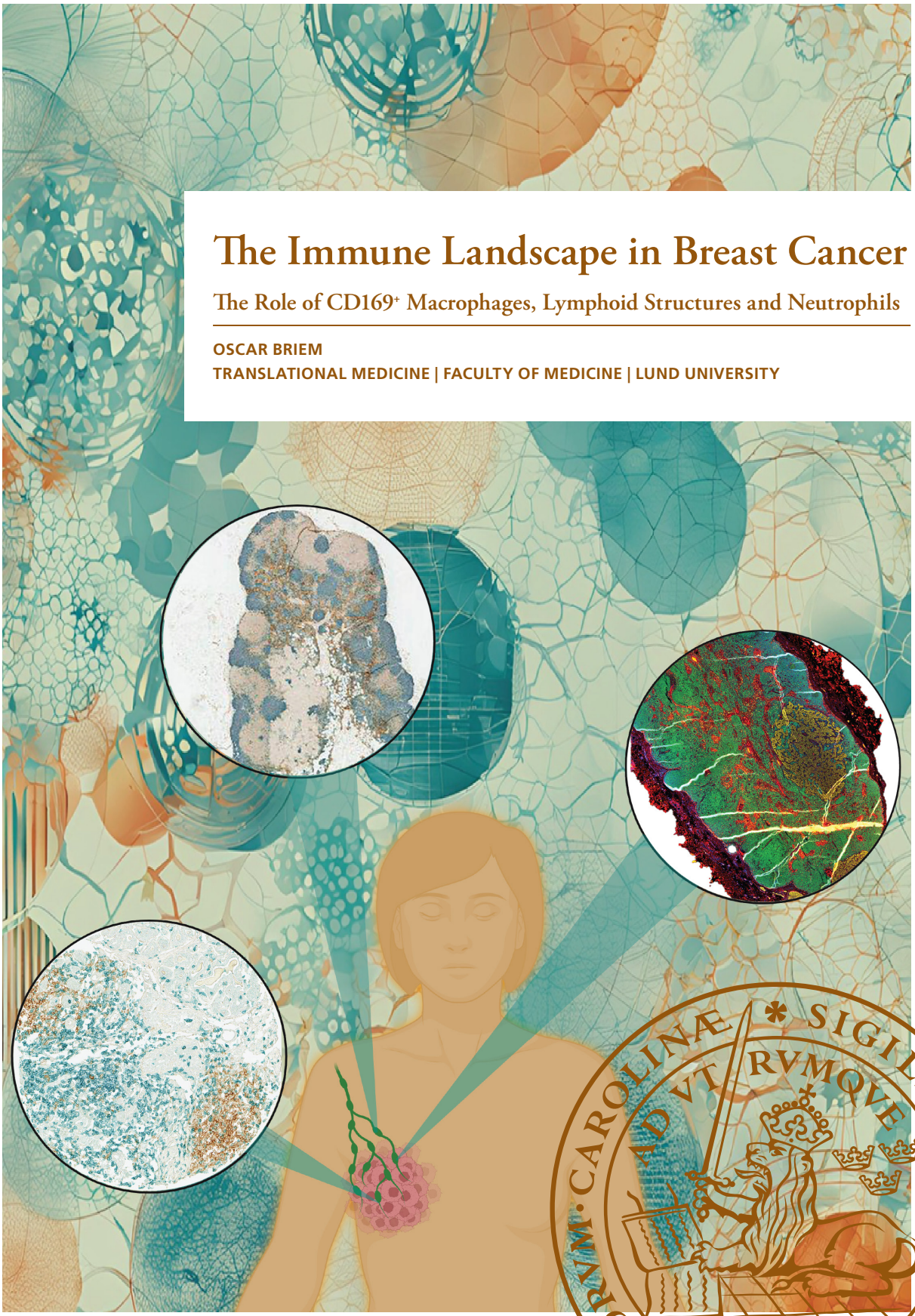

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About the author

Oscar Briem earned his Bachelor's and Master's degrees in Biomedicine from Lund University. He then pursued a PhD in Cancer Immunology within the same institution, at the Clinical Research Centre, Malmö. His research focused on the role of myeloid cells in breast cancer tumours and the impact of metastasis on various lymph node resident immune cells. His work has led to novel insights into biomarkers in breast cancer and to a deeper understanding of tumour-immune cell interactions.

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Oscar Briem

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University, Sweden

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Title and subtitle: The immune landscape in breast cancer – The role of CD169$^+$ macrophages, lymphoid structures and neutrophils

Abstract: Advancing immunotherapeutic strategies for breast cancer requires a deeper understanding of the tumour microenvironment (TME) and its role in tumour progression or elimination. This thesis explores myeloid-lymphoid cell interactions in both primary tumours (PTs) and lymph nodes (LNs), and further investigates whether lymph node metastases (LNM) influence local immune responses or merely serve as disease progression markers.

The first project examined CD169$^+$ macrophages and tertiary lymphoid structures (TLS), assessing their prognostic impact in LNs and breast tumours. Presence of CD169$^+$ macrophages and TLS in LNM correlated with better prognosis, whereas their presence in breast tumours was linked to a worse outcome, and an immunosuppressive TME containing regulatory B- and T-cells. The second project further characterised CD169$^+$ macrophages, revealing phenotypic similarities between lymph node-resident (SCS and MS macrophages) and tumour-associated populations. *In vitro* studies demonstrated that in relation to lymphoid cells, CD169$^+$ macrophages inhibit T-cell proliferation via ROS and PGE2 while they enhance B-cell activation leading to increased IL-6 and IgG secretion. Additionally, their chemokine and cytokine secretome included CXCL10, CCL2, IL-15, IL-6, CCL17, and IL-10, highlighting the diverse immunomodulatory roles of CD169$^+$ macrophages.

The third and fourth projects used spatial proteomics in paired uninvolved LN (UnLN) and LNM to investigate how LNM in breast cancer alter LN architecture and immune cell proteomes. The results showed the depletion of SCS CD169$^+$ macrophages in LNM compared to UnLN, likely due to Bcl-xL downregulation. Conversely, MS CD169$^+$ macrophages persisted but adopted an immunosuppressive phenotype with increased Arg1 expression. B-cell follicles exhibited a premature contraction signature, marked by FoxP3 and CD25 upregulation, while interfollicular T-cell regions displayed reduced activation and proliferation markers, likely driven by loss of SCS macrophage and a metastasis-induced immunosuppressive milieu. Additionally, neutrophils declined in LNM, and single-cell RNA sequencing identified neutrophil markers associated with T-cell-independent B-cell activation (NAMPT, BAFF, APRIL, LL-37 and HMGB1). Their depletion likely weakens adaptive immune responses in LNs.

Altogether, these findings highlight the role of CD169$^+$ macrophages, lymphoid structures and neutrophils in secondary lymphoid organs and primary breast cancers. Furthermore, LNM reshape the immune landscapes by altering myeloid cell populations and suppressing adaptive immunity. These findings provide insights for breast cancer progression and therapeutic strategies.

Key words: Breast cancer, Lymph node metastasis (LNM), CD169$^+$ Macrophages, Tertiary lymphoid structures (TLS), Neutrophils

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To my family and friends

Table of Contents

Abbreviations

ACT	Adoptive cell therapy
ADCC	Antibody dependent cellular cytotoxicity
ADCP	Antibody dependent cellular phagocytosis
APC	Antigen presenting cell
BCR	B-cell receptor
BCSS	Breast cancer-specific survival
CD	Cluster of differentiation
CSF	Colony stimulating factor
CSR	Class switch recombination
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
DCIS	Ductal carcinoma in situ
DSP	Digital spatial profiling
EC	Endothelial cell
ER	Oestrogen receptor
EFS	Event-free survival
FDC	Follicular dendritic cell
GC	Germinal centre
HER2	Human epidermal growth factor receptor 2
HEV	High endothelial venule
ICB	Immune checkpoint blockade
ICI	Immune checkpoint inhibition
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
iTIL	Intratumoral tumour infiltrating lymphocyte
LCIS	Lobular carcinoma in situ
LEC	Lymphatic endothelial cell
LN	Lymph node
LNM	Lymph node metastasis
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex

MS	Medullary sinus
MPO	Myeloperoxidase
NET	Neutrophil extracellular trap
NK cell	Natural killer cell
NLR	Neutrophil-to-lymphocyte ratio
PAMP	Pathogen-associated molecular pattern
PD-1	Programmed cell death protein 1
PFS	Progression-free survival
PR	Progesterone receptor
PRR	Pathogen recognition receptor
PT	Primary tumour
RFI	Recurrence-free interval
ROS	Reactive oxygen species
scRNA-seq	Single-cell RNA sequencing
SCS	Subcapsular sinus
SHM	Somatic hyper mutation
SIGLEC	Sialic acid-binding immunoglobulin-like lectin
SLN	Sentinel lymph node
SLO	Secondary lymphoid organ
sTIL	Stromal tumour infiltrating lymphocyte
TAM	Tumour associated macrophage
TCR	T-cell receptor
TD	T-cell dependent
TDLN	Tumour draining lymph node
T_{FH}	Follicular helper T-cell
TGF-β	Transforming growth factor beta
Th	T-helper cell
TI	T-cell independent
TIL	Tumour infiltrating lymphocyte
TLLS	Tertiary lymphoid like structure
TLR	Toll-like receptor
TLS	Tertiary lymphoid structure
TME	Tumour microenvironment
TNBC	Triple negative breast cancer
T_{reg}	Regulatory T-cell
UnLN	Uninvolved lymph node

Preface

Cancer is a disease that affects millions of people worldwide. My first encounter with its devastating impact was when my grandmother was diagnosed with pancreatic cancer, a particularly aggressive form of cancer often detected too late for effective treatment. While pancreatic cancer remains a major clinical challenge, breast cancer has benefited from earlier detection and extensive research, leading to significantly improved outcomes. Being the most common cancer in women, advancements in breast cancer treatment have the potential to save thousands of lives each year.

This thesis explores the intricate relationship between breast cancer and the immune system: how our body's defences can both hinder and promote tumour progression. The field of tumour immunology has expanded rapidly over the past decade, largely driven by breakthroughs in immunotherapy, which culminated in the Nobel Prize in Physiology or Medicine in 2018. My fascination with immunology began in high school, where an introductory biology course sparked my curiosity. This passion deepened during my biomedical studies, ultimately leading me to pursue a master's thesis in tumour immunology, a decision that shaped the course of my academic journey.

Herein I present my thesis. Summarising and representing the research from years of dedication, towards understanding cancer immunology. This thesis gathers the fundamental parts into understanding the concepts of tumour immunology and provides new insights into the field. While a cure for cancer remains elusive, I firmly believe that continued research in breast cancer will lead to earlier detection through cost-effective screening methods and personalised treatments. One day, cancer may no longer be a life-threatening diagnosis but a manageable condition. That future is built step by step, through fundamental discoveries and translational research within several fields, tumour immunology representing one of them.

Popular Science Summary

Breast cancer is a global health challenge, affecting millions of women annually. It is the most common cancer among women and, in some countries, surpasses lung cancer as the leading cause of cancer-related deaths. In Sweden, more than 200 women per 100 000 are diagnosed with breast cancer each year. Today, 9 out of 10 women diagnosed with breast cancer survive at least 10 years, a dramatic improvement compared to survival rates 40 years ago. These advancements are attributed to significant progresses in breast cancer diagnosis and the development of better treatment alternatives. However, the goal remains unchanged: to cure and improve the lives of all patients affected by this disease.

Cancer arises when certain cells in the body accumulate multiple mutations in their DNA. These mutations can occur due to environmental factors, such as the individual's lifestyle, but may also be inherited. Because the accumulation of DNA mutations is a gradual process, cancer is more prevalent as we age. Once a cell acquires enough mutations, it begins to divide uncontrollably, forming tumours and eventually spread to other organs, a process known as metastasis, which is the major cause of cancer-related deaths.

Every breast cancer patient has a unique set of DNA mutations, but identifying common patterns in tumours among patients is the key to developing preventive strategies and treatments. For example, hormone receptor-positive breast cancers are treated with therapies targeting those receptors. However, some subtypes, such as triple-negative breast cancer (TNBC) which lacks hormone receptors, have poorer outcomes due to limited treatment options and are primarily managed with chemotherapy. To expand treatment alternatives, the role of the immune system in cancer has been a research focus for the last two decades. This thesis focuses on this research area, known as tumour immunology, and explores how the body's immune cells interact with cancer cells.

Cancer can be viewed as a chronic inflammatory condition that triggers an immune response. The immune response involves two major arms:

1. The innate immune response, which provides the first line of defence.
2. The adaptive immune response, which is slower to develop but highly specific and efficient.

These immune responses can be targeted with Immunotherapy. These treatments are designed to enhance the immune system's ability to fight tumours, and have primarily focused on boosting T-cells, a key component of the adaptive immune response. However, tumours consist of diverse immune cell populations, each playing distinct roles. This thesis investigates the roles of macrophages, neutrophils, T- and B-cells in breast cancer progression.

In the first paper, the prognostic impact of a macrophage population identified as macrophage displaying CD169 ($CD169^+$ macrophages) was investigated together with their relation to immune cell aggregates called tertiary lymphoid structures (TLS). $CD169^+$ macrophages and TLS, previously linked to favourable outcomes, were analysed in primary breast tumours and lymph node metastases (LNM) from breast cancer patients. Interestingly, these markers were associated with opposing prognoses depending on if they were present in primary tumour (PT) or LNM. In LNM, $CD169^+$ macrophages and TLS correlated with better outcomes, while in PTs, they indicated worse prognosis. Further analysis revealed that presence of $CD169^+$ macrophages in PTs correlated with that of other immune cells known to suppress anti-tumour immune response, explaining the harmful prognosis.

In the second paper, to understand the opposing prognoses linked to $CD169^+$ macrophages in PT and LNM, their traits in the respective locations was studied. We showed that $CD169^+$ macrophages in primary breast tumours had a similar protein profile as $CD169^+$macrophages present in lymph nodes (LNs). In mice models, we showed that $CD169^+$ macrophages in primary breast tumours could be derived from monocytes, a type off immune cell that patrols the blood. Monocyte-derived $CD169^+$ macrophages with a similar profile cultured in the lab, displayed properties that suppressed immune cells like T-cells and natural killer (NK) cells, but the same cells promoted immune properties of B-cells. This explained their presence next to TLS, and their supporting role in antibody production. Furthermore, these findings emphasize the critical role of the tumour microenvironment in shaping macrophage function.

In the third paper, the protein profile of LN $CD169^+$ macrophages and lymphocytes that were spatially co-localised was analysed, to understand whether metastases affect their function. We showed that LNM significantly altered the protein profile of $CD169^+$ macrophages, B-cells, and certain T-cells, transforming the LN environment into an immunosuppressive state. This paper also showed that $CD169^+$ macrophages decline in LNs containing metastases. The suppressive tumour microenvironment in LNM likely hinders immune cell activation against tumours, worsening patient outcomes and promoting cancer progression.

In paper four, we investigated neutrophils, a major component of innate immunity. Our study focused on neutrophils present in LNs from cancer patients, a relatively unexplored population of cells. Neutrophils were found to decrease in numbers in

LNM compared to cancer-free LNs. This novel finding highlights a potential protective role of neutrophils against tumour progression. Additionally, neutrophils were observed interacting closely with B-cells in PTs, a relationship linked to worse prognosis. These results suggest that neutrophils play a critical yet underexplored role in breast cancer.

In conclusion, this thesis investigates the diverse roles of immune cells, particularly macrophages and neutrophils and their interplay with T- and B-cells, in breast cancer. It underscores the importance of the tumour microenvironment in shaping the behaviour of these cells and determining patient prognosis. By identifying key molecular pathways and immune cell interactions, this research provides insights for developing more personalised treatment approaches. Our findings contribute to a growing body of evidence that immune cells are not merely bystanders in cancer but active participants that can influence outcomes. These findings may contribute to the development of novel therapies targeting the tumour microenvironment, ultimately improving survival but also improving the quality of life for breast cancer patients.

Populärvetenskaplig sammanfattning

Bröstcancer är en global hälsoutmaning som drabbar miljontals kvinnor varje år. Det är den vanligaste cancerformen hos kvinnor och överträffar i vissa länder lungcancer som den vanligaste orsaken till cancerrelaterade dödsfall. I Sverige diagnostiseras mer än 200 kvinnor per 100 000 med bröstcancer varje år. Idag överlever 9 av 10 kvinnor som diagnostiseras med bröstcancer minst 10 år, en dramatisk förbättring jämfört med överlevnadsstatistiken för 40 år sedan. Denna framgång beror på betydande framsteg inom diagnostik och utveckling av behandlingsalternativ. Det slutgiltiga målet är dock fortfarande densamma: att bota och förbättra livet för alla patienter som drabbas av denna sjukdom.

Cancer uppstår när vissa celler i kroppen ackumulerar flera mutationer i sitt DNA. Dessa mutationer kan uppstå på grund av miljöfaktorer, såsom individens livsstil, men kan också vara ärftliga. Eftersom ackumuleringen av DNA-mutationer är en gradvis process blir cancer vanligare ju äldre vi blir. När en cell har ackumulerat tillräckligt många mutationer börjar den dela på sig okontrollerat, vilket leder till tumörer och slutligen spridning till andra organ. Denna process kallas metastasering och är den främsta orsaken till cancerrelaterade dödsfall.

Varje bröstcancerpatient har en unik uppsättning DNA-mutationer. Att identifiera gemensamma mönster i tumörer mellan patienter är därför avgörande för att utveckla förebyggande strategier och behandlingar. Till exempel behandlas hormonreceptorpositiva bröstcancerformer med terapier som riktar sig mot dessa receptorer. Vissa undergrupper, såsom trippelnegativ bröstcancer (TNBC), saknar hormonreceptorer och har därför sämre prognos på grund av begränsade behandlingsalternativ. För att utöka behandlingsalternativen så har ett forskningsområde uppstått som undersöker immunsystemets roll vid cancer. Denna avhandling fokuserar på detta forskningsområde, och är känt som tumörimmunologi. Syftet är att forska på hur kroppens immunceller interagerar med cancerceller och använda detta till att skapa nya immunterapier mot cancer.

Cancer kan ses som ett kroniskt inflammatoriskt tillstånd som utlöser ett immunsvar. Immunförsvaret kan delas in i två delar:

1. Det medfödda immunförsvaret, som utgör kroppens första försvarslinje.
2. Det adaptiva immunförsvaret, som tar längre tid att utveckla men är mycket mer specifikt och effektivt.

Dessa immunsvar kan modifieras med cancerimmunterapi, vilket är behandlingar som är utformade för att förstärka immunförsvarets förmåga att bekämpa tumörer. Hittills har dessa terapier främst fokuserat på att stärka T-cellerna, en nyckelkomponent i det adaptiva immunsvaret. Tumörer består dock av olika typer av immunceller, som alla spelar distinkta roller. Denna avhandling undersöker rollerna hos andra immunceller såsom makrofager, neutrofiler, T- och B-celler vid bröstcancerprogression.

I den första artikeln undersöktes en population av makrofager som identifieras av deras uttryck av proteinet CD169 ($CD169^+$ makrofager), samt deras samband med tertiära lymfoida strukturer (TLS), immuncellsaggregat som tidigare har kopplats till gynnsam prognos inom cancer. Dessa populationer analyserades i primära brösttumörer och lymfkörtelmetastaser (LNM) hos bröstcancerpatienter. Intressant nog var samma populationer associerade med motsatta prognoser beroende på om de återfanns i primärtumör (PT) eller LNM. I LNM korrelerade $CD169^+$ makrofager och TLS med en bättre prognos, medan de i PT indikerade en sämre prognos. Ytterligare analyser visade att $CD169^+$ makrofager i PT förekom samtidigt som andra immunceller som redan är kända för att dämpa immunsvaret, vilket förklarar den negativa prognosen.

I den andra artikeln, för att förstå de motsatta prognoserna kopplade till $CD169^+$ makrofager i PT och LNM, undersöktes deras funktion i PT jämfört med LNM. Vi kunde visa att $CD169^+$ makrofager i primärtumörer uppvisade samma proteinprofil som makrofager i LNM. I mös modeller kunde vi även visa att $CD169^+$ makrofager i primärtumör kunde bildas från monocyter, en typ av immuncell som patrullerar blodet. Monocyt-deriverade $CD169^+$ makrofager odlade i laboratoriet visade egenskaper som dämpar immunceller så som T-celler och naturliga mördarceller (NK-celler). Samtidigt främjade dessa makrofager B-cellers funktion, vilket förklarar deras närvaro nära TLS och deras stöd till antikroppsproduktion. Dessa fynd betonar vikten av tumörens mikromiljö för att forma makrofagers funktion.

I det tredje arbetet undersöktes proteinprofilen hos $CD169^+$ makrofager i lymfkörtlar, samt hos olika immunceller som var lokaliserade i närheten av dem. Detta analyserades för att förstå om metastaser påverkar immuncellers funktioner i lymfkörteln och därmed cancerns progression. Vi visade att metastaser signifikant förändrade proteinprofilen hos $CD169^+$ makrofager, B-celler och vissa T-celler, till ett immundämpande profil. Studien visade också att antalet $CD169^+$ makrofager minskade i lymfkörtlar med metastaser. Detta visar att LNM sannolikt skapar ett immundämpande tillstånd som kan försvåra aktiveringen av immunceller mot tumörer, vilket förvärrar patienternas prognos och gynnar tumörprogression.

I det fjärde arbetet undersöktes immunceller som kallas neutrofiler, vilka är en viktig del av det medfödda immunförsvaret. Vi undersökte specifikt neutrofiler i lymfkörtlar hos cancerpatienter, en relativt outforskad immuncellspopulation.

Neutrofilerna visade sig minska i antal i lymfkörtlar med metastaser jämfört med cancerfria lymfkörtlar. Detta nya fynd lyfter fram neutrofilernas potentiellt skyddande roll mot tumörer och tumörprogression. Dessutom observerades neutrofiler i nära kontakt med B-celler i primära tumörer, en relation som var kopplad till en sämre prognos. Dessa resultat tyder på att neutrofiler spelar en kritisk men outforskad roll vid bröstcancer.

För att sammanfatta, denna avhandling belyser de olika rollerna hos immunceller i bröstcancer, särskilt makrofager och neutrofiler, samt deras samspel med T- och B-celler. Avhandlingen tar upp vikten av tumörens mikromiljö för att forma immuncellernas beteende vilket påverkar patienters prognos. Genom att identifiera nyckelmekanismer och interaktioner mellan immunceller ger denna forskning insikter som förhoppningsvis kan användas för att utveckla mer personanpassade behandlingsmetoder i framtiden. Våra fynd bidrar till en växande kunskapsbas som visar att immunceller inte bara är medpassagerare i cancer, utan aktiva deltagare som kan påverka sjukdomens utgång. Dessa insikter kan visa vägen för nya terapier som riktar sig mot tumörens mikromiljö och därmed förbättra överlevnaden och livskvaliteten för bröstcancerpatienter.

Papers included in the thesis

Paper I: $CD169^+$ macrophages in primary breast tumors associate with tertiary lymphoid structures, T_{regs} and a worse prognosis for patients with advanced breast cancer.

Oscar Briem, Eva Källberg, Siker Kimbung, Srinivas Veerla, Jenny Stenström, Thomas Hatschek, Catharina Hagerling, Ingrid Hedenfalk and Karin Leandersson.

Cancers. 2023 February 16, 15(4):1262. DOI:10.3390/cancers15041262

Paper II: Breast cancer associated $CD169^+$ macrophages possess broad immunosuppressive functions but enhance antibody secretion by activated B cells.

Frida Björk Gunnarsdottir, **Oscar Briem**, Aida Yifter Lindgren, Eva Källberg, Cajsa Andersen, Robert Grenthe, Cassandra Rosenqvist, Camilla Rydberg Millrud, Mika Wallgren, Hannah Viklund, Daniel Bexell, Martin E. Johansson, Ingrid Hedenfalk, Catharina Hagerling and Karin Leandersson.

Front. Immunol. 2023 June 19, 14:1180209. DOI: 10.3389/fimmu.2023.1180209

Paper III: Altered immune signatures in breast cancer lymph nodes with metastases revealed by spatial proteome analyses.

Oscar Briem, Balázs Tahin, Asger Meldgaard Frank, Lina Olsson, Anna Sandström Gerdtsson, Eva Källberg and Karin Leandersson

Accepted, 2025 Mars 24, Journal of Translational Medicine

Paper IV: Neutrophils decline in breast cancer lymph nodes with metastasis.

Oscar Briem, Balázs Tahin, Asger Meldgaard Frank, Lina Olsson, Anna Sandström Gerdtsson, Karin Jirström, Eva Källberg and Karin Leandersson

Manuscript

Papers not included in thesis

I. G-MDSC induces immune exclusion via Ly6c downregulation in endothelial cells in breast cancer mouse models.

Oscar Briem, Eva Källberg, Vilma Persson, Meliha Mehmeti-Ajradini, Karin Leandersson

Manuscript

Introduction

Fundamental Concepts in Immunology

The immune system

The immune system is developed through evolution to primarily fence off pathogens and foreign substances such as bacteria, viruses, fungi and parasites. It's divided into three "lines of defence"[1]:

1. Anatomic and physiological barriers
2. Innate immunity
3. Adaptive immunity

The anatomic and physiological barriers represent organs, such as the skin, or the low pH environment found in the stomach, but this barrier will not be reviewed in this thesis. Instead, this thesis focuses on immune cells that are part of the innate and adaptive immune response in the context of the disease breast cancer. Innate immunity is the first line of defence against pathogens and comprises cells that respond first to infections. The innate immunity recognises foreign structures with low specificity and the innate immune system does not develop immunologic memory, it will therefore react identically should the same infection occur again. The adaptive immunity is, on the other hand, not only antigen-dependent but also antigen-specific. Antigens are structures that can be recognised by the immune system. If the antigens activate, stimulate or trigger the immune system, they are also called immunogens [2]. Antigens are divided into foreign antigens, that originates outside the body such as viruses, bacteria and food antigens. They can also originate from our own body. In this case they are called autoantigens or self-antigens and may give rise to autoimmune diseases [3]. It takes time to develop an antigen-specific response. Therefore, cells from the adaptive immunity are considered as the second line of defence against pathogens but they are more specifically educated to effectively eliminate them. Another capacity of the adaptive immune response is to develop memory against pathogens, which allows the host to respond quicker and stronger if a secondary infection of the same nature should occur [4,5]. The immune

system consists of a complex network of various immune cells that are collected under the umbrella term “leucocytes” which means white blood cells in Greek. Leucocytes develop from the hematopoietic stem cell progenitors in the bone marrow and differentiate into several branches of immune cell subgroups. The innate immune response derives from common myeloid progenitor cells and the adaptive immune response from the common lymphoid progenitor cells [4]. In this thesis the myeloid cells monocytes, macrophages and neutrophils will be discussed, as well as the T- and B-lymphocytes.

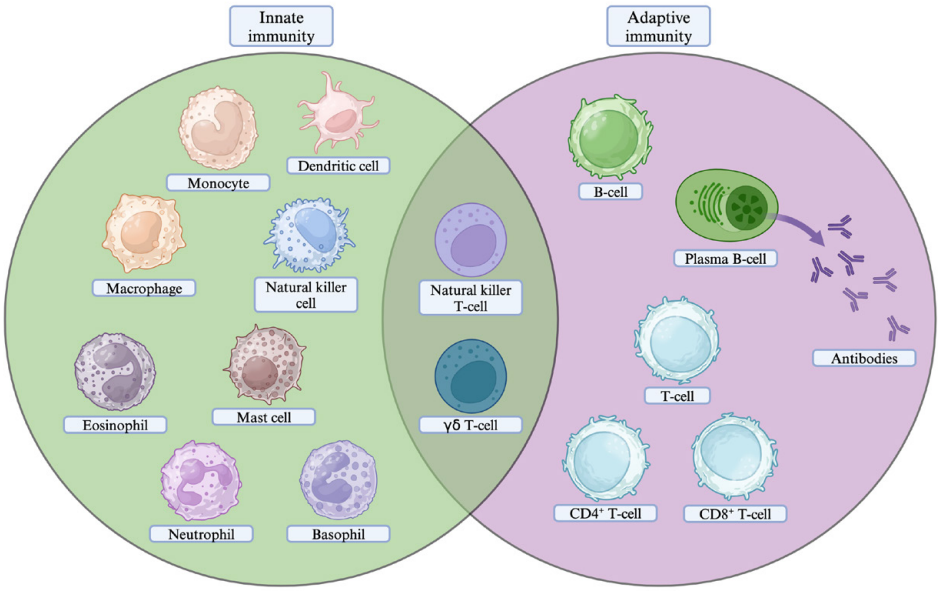

Figure 1. Overwiew of the immune system
Immune cells of the immune system are divided into the innate and adaptive immunity. The left circle illustrates cells from the innate immunity that recognises PAMPs and DAMPs. The right circle illustrates cells from the adaptive immunity that elicit a specific response against antigens. Cells in the middle of the circles represent cells with features from both the innate and adaptive immune response.

The innate immune system

The innate immune system is the first immune response triggered upon pathogen encounter. Cells from the innate immunity share specific receptors, pattern recognition receptors (PRRs), which recognise highly conserved structures expressed by microbes, pathogen-associated molecular patterns (PAMPs), or

immunological danger molecules released by endogenous dying cells or damaged tissues, damage-associated molecular patterns (DAMPs) [1,4]. DAMPs are therefore proteins, nucleic acids and metabolites released during infection or inflammation. The PRRs are divided into several families, some of them include Toll like receptors (TLR) which recognise extracellular microbial structures, or nucleotide oligomerization domain (NOD) like receptors that recognise intracellular microbial structures. The innate immunity also includes humoral elements such as complement proteins and anti-microbial peptides. Upon encountering pathogens, PRR activation leads to phagocytosis, a physiological process where innate immune cells engulf pathogens to clear and neutralise them [6]. PRR activation further leads to the release of inflammatory mediators called cytokines and chemokines. These are small soluble proteins which are important in cell signalling and trigger the recruitment of more leucocytes to the site of inflammation as well as activating them to fight the infection [7]. Innate immune cells recruited to the site of infection include several subtypes such as granulocytes (neutrophils, basophils, eosinophils, mast cells), monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells. Another important role of cells of the innate immune response is to initiate the adaptive immune response. This is done by antigen presenting cells (APC) which consist of macrophages and DCs. These cells engulf antigens by phagocytosis and present antigens to T-cells and B-cells via major histocompatibility complexes (MHC) [8].

Monocytes

Monocytes account for approximately 10% of circulating leucocytes in humans and are critical responding elements of the innate immune system [9]. They function as sentinel immune cells of the innate immunity that patrol the bloodstream, respond to inflammation or tissue damage, and have the capacity to differentiate into macrophages or DCs after tissue recruitment [10]. Monocytes circulate the blood in humans as three different subsets based on the expression of surface markers known as cluster of differentiation (CD), CD14 and CD16. These different types are called classical monocytes, intermediate monocytes and non-classical monocytes. They exert different roles in the innate immunity [11]:

- **Classical monocytes ($CD14^{+}/CD16^{-}$)**: Are also called migratory monocytes and represent the major population of monocytes. These cells respond to inflammatory signals and infiltrate tissues upon inflammation. They are primed for innate sensing and phagocytosis of pathogens. These are also most likely to differentiate into monocyte-derived DCs [12].

- **Intermediate monocytes ($CD14^{int}/CD16^{+}$)**: Represent a state between classical and non-classical monocytes. These monocytes have an increased cytokine secretion capacity.

- **Non-classical monocytes ($CD14^{low}/CD16^{+}$)**: Are also known as patrolling monocytes. These monocytes primarily patrol the blood and monitor endothelial cells (ECs) for damage or infection. They can also mediate phagocytosis of blood borne pathogens.

Recruitment of monocytes to inflamed tissues is orchestrated by chemokines. The classical monocytes express the chemokine receptor CCR2 while the intermediate and non-classical monocyte express CXCR1. CCL2, which is released by cells subjected to inflammatory microenvironments, will bind to CCR2 on monocytes and initiate their migration into the tissue [13]. The process of cell migration from blood to tissue is shared by immune cells and is called extravasation. Immune cells express chemokine receptors and adhesion molecules that interact with chemokine ligands and adhesion molecules on the endothelium cells lining the blood vessels. Extravasation can be summarised into four steps: tethering - when the leucocyte expands its membrane to the blood vessel wall; rolling – when the leucocyte binds to the endothelium layer and slows down; adhesion – when the leucocyte stops rolling and attaches completely to the endothelium wall; diapedesis – when the leucocyte migrates between ECs into the tissue [14,15]. Once they have migrated into the tissue, monocytes display significant plasticity, differentiating into various types of macrophages or DCs. By doing so, they become more efficient APCs or play pivotal roles in responding to inflammation, replenishing tissue macrophages, and coordinating immune responses based on environmental cues [9,10,14].

Macrophages

Overview and homeostatic functions

Macrophages are myeloid-derived cells central to the innate immune system, playing critical roles in maintaining homeostasis across all tissues [16]. Their origins are divided into:

- **Yolk-sac-derived macrophages**: These macrophages arise during embryonic development and persist in the human body as tissue-resident macrophages. They have self-renewal capacity and are primarily involved in maintaining homeostasis and mitigating inflammation.
- **Bone-marrow-derived macrophages**: These macrophages differentiate from circulating monocytes as they enter inflamed tissues. They are more inflammatory in nature and respond to infection and tissue damage by eating pathogens and producing pro-inflammatory mediators.

In steady-state conditions, macrophages are typically the primary immune cells present within tissues, acting as sensor cells that detect inflammatory triggers through the expression of PRRs capable of recognising PAMPs and DAMPs. Macrophages that are tissue-resident contribute to organ function by clearing dead cells and debris, and promoting tissue repair and regeneration[16]. During infections, tissue-resident macrophages are one of the first responders, while recruited monocyte-derived macrophages are one of the first innate waves of incoming immune cells. During infections, their main role is to engulf pathogens via phagocytosis and initiate an inflammatory response by secreting cytokines and chemokines that recruit and activate other immune cells [4].

Macrophage polarization states

Macrophages exhibit remarkable plasticity, with their behaviour and function being influenced by both their origin and signals from the local microenvironment [16,17]. Despite their origin, macrophages adapt to environmental cues that drive their diverse functions. They are often classified into the two extreme polarization states: M1 macrophages (classically activated) and M2 macrophages (alternatively activated). Though M1 and M2 macrophages have opposite functions, macrophage polarization is better described as a dynamic spectrum of polarization rather than these two discrete categories [18].

- **M1 macrophages (classically activated):** These macrophages are induced by stimuli such as bacterial lipopolysaccharides (LPS) and secrete pro-inflammatory cytokines like interferon gamma (IFN-γ). They are specialized in pathogen clearance by phagocytosis, and amplifying immune responses by recruiting additional immune cells to sites of infection or inflammation [16,18] and have an efficient antigen presentation capacity to activate the adaptive immune system [19].

- **M2 macrophages (alternatively activated):** These macrophages are induced by interleukins (ILs) e.g., IL-4 and IL-13 and associated with clearance of parasites or induced with IL-10 and transforming growth factor beta (TGF-β) in tumour microenvironments. In general they promote tissue repair, wound healing mechanisms, and release anti-inflammatory mediators [19]. Their anti-inflammatory role helps resolve inflammation and promote regeneration and angiogenesis after tissue injury [16,18].

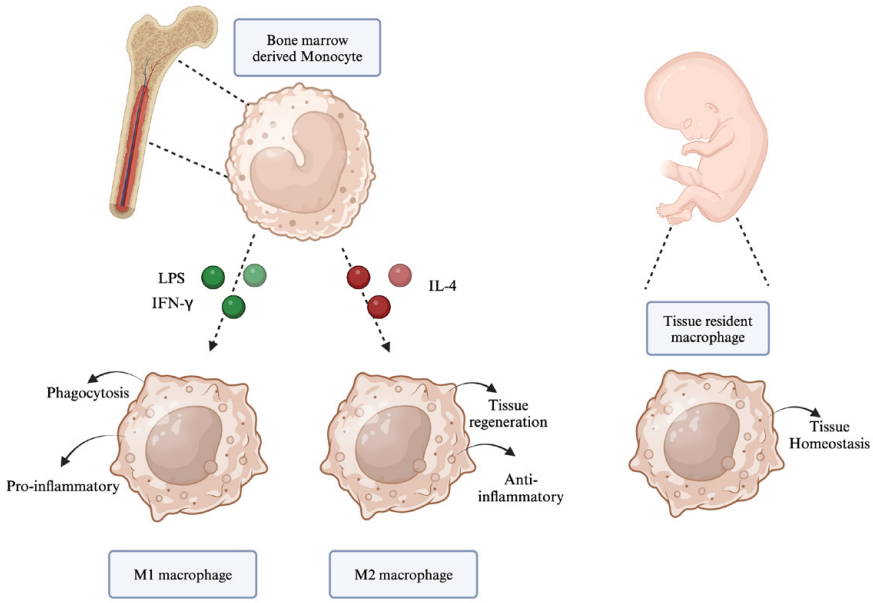

Figure 2. Macrophages polarization and origin

Schematic picture of macrophages origin and polarization. Tissue resident macrophages develop during the foetal stage, while recruited macrophages originate from bone marrow-derived monocytes. The tissue resident macrophages maintain tissue homeostasis while bone marrow-derived macrophages polarize into different phenotypes based on environmental cues. Although macrophages have a broad range of polarization phenotypes, they are commonly divided into the pro-inflammatory (M1) and anti-inflammatory (M2) macrophages.

Neutrophils

Development and function

Neutrophils are the most abundant leucocytes in the blood and account for 50-70% of circulating white blood cells [20]. These innate immune cells originate from the bone marrow and belong to the granulocyte family of myeloid cells. Granulocytes are also known as polymorphonuclear cells due to their lobed nuclei and granule-rich cytoplasm. The granules contain diverse antimicrobial peptides that are released into the extracellular matrix through degranulation following their activation. Neutrophils also display potent phagocytic activity, destroying pathogens in intracellular vesicles using degrading enzymes [4]. Beyond pathogen clearance, neutrophils perform additional roles, including the secretion of reactive oxygen species (ROS), the release of neutrophil extracellular traps (NETs), and the production of inflammatory mediators such as cytokines and chemokines to amplify

the immune response [21,22]. These mechanisms form the host's first major and initial line of defence against infections.

Neutrophil diversity and plasticity

Neutrophils are prevalent as different populations which exhibit significant diversity in terms of function. Neutrophil plasticity is influenced by environmental signals rather than developmental origins. This diversity is shaped by environmental cues such as chemokine gradients, cytokine environment, PRR activation, and circadian rhythms. Neutrophils follow a daily cycle, aging throughout the day and exhibiting functional adaptations during this process which affects inflammation and homeostasis. This heterogeneity in neutrophils divides them into the subtypes: mature neutrophils, immature neutrophils, aged neutrophils, and interferon-stimulated gene (ISG) neutrophils, each with distinct phenotype and function [20,22,23].

- **Mature neutrophils**: These neutrophils are released from the bone marrow primarily during the night and early morning once they have fully developed in the bone marrow. They are the main responders to infection and expresses the chemokine receptor CXCR2.

- **Aged neutrophils**: Are characterised by reduced CD62L and CXCR2 expression, alongside increased CXCR4 and CD11b expression. These neutrophils are characterised by their aged phenotype. They exhibit reduced migration potential, reduced potential of degranulation, and limited excessive inflammation due to reduced NET release capabilities.

- **Immature neutrophils**: These neutrophils are often observed during chronic inflammation and represents neutrophils released from the bone marrow prior to their complete maturation. They survive longer than mature neutrophils and exhibit anti-inflammatory roles in inflamed tissues.

- **ISG neutrophils**: These neutrophils are identified by their increased expression of inflammatory genes compared to mature neutrophils. They are associated with tumour microenvironments and chronic inflammatory conditions and are primed to fight infections and recruit inflammatory immune cells.

Similar to macrophages, neutrophils have different plasticity in cancer based on environmental cues. They can be classified into pro-inflammatory neutrophils (N1) and anti-inflammatory neutrophils (N2). These polarization states will be further discussed later in this thesis [24].

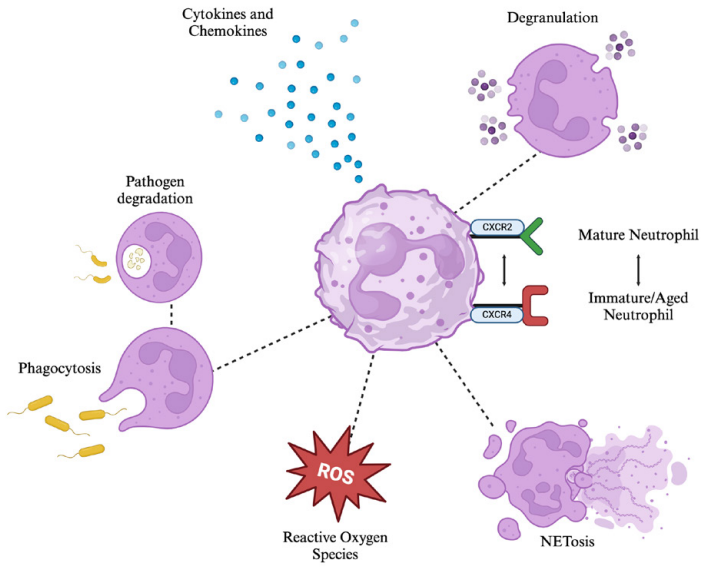

Figure 3. Neutrophil function and maturity
Neutrophils are the first immune cells to infiltrate inflamed tissue. Once they enter the inflamed tissue, their functions are to phagocytose and degrade pathogens, release cytokines and chemokines to attract other immune cells, release granules containing anti-microbial peptides, and release ROS and NETs. Neutrophils are regulated by a circadian rhythm which regulates certain receptors and indicates their maturity state. Neutrophils with different maturity exert different functions.

The adaptive immune system

The innate immune response has evolved to rapidly sense and eliminate pathogens. Nonetheless, the innate immune system does not recognise with high specificity and does not lead to immunological memory. This has driven the evolution of adaptive immune responses, which can be detected 72-96 hours after an infection has initiated. The adaptive immunity has developed diverse antigen specific receptors to recognise foreign pathogens with a high degree of specificity, leading to selective clonal expansion upon activation. By doing so, cells of the adaptive immunity are much more efficient at completely clearing infections. The key aspects of the adaptive immune response and how its cells differ from the innate immune cells is hence their specificity to non-self-antigens, their diversity, their clonal expansion capacity and, lastly, their immunological memory. With their specificity, they can recognise unique pathogen antigens and distinguish them from self-antigens. With their diversity, they have the ability to recognise an endless variety of epitopes. With their immunological memory, they can persist in the host for life and enable a rapid, strong response upon re-exposure to the same pathogen. The cells of the adaptive

immunity are T-cells and B-cells which are derived from the common lymphoid progenitor cells [25].

T-Cells

T-cells are a central component of the adaptive immune system and play diverse roles by either performing cytotoxic function themselves or interacting with other immune cells.

The T-cell receptor: Mechanisms of antigen recognition and activation

The T-cell receptor (TCR) is a fundamental structure of the specific immune response as it enables T-cells to recognise diverse antigens and mount an effective and specific immune response. The conventional TCR is composed of two chains: the alpha (α) and beta (β) chain and is expressed by the majority of T-cells in the peripheral blood (95%) [26]. Some T-cells TCR consist of the gamma (γ) and delta (δ) chain and represent 1-5% of T-cells in peripheral blood but have a higher proportion in the intestine (40 %) and the skin (10-30%) [27]. Each TCR is unique and recognises antigens presented as ligands by the MHC-I on all nucleated cells or MHC-II presented on APCs such as DCs or macrophages. Once the TCR on a T-cell binds to an antigen presented on MHC-I/II, they receive additional activation signals by co-stimulatory receptors and cytokines (see below). T-cells are divided into $CD4^+$ T-helper cells (Th) and $CD8^+$ cytotoxic T-lymphocytes (CTL) [25]. The $CD4^+$ T-cells support other immune cells in the inflamed tissue or tumour. They recognise antigens presented by MHC class II molecules on APCs and can further polarize into distinct subtypes depending on the cytokine stimulation. The cytokine environment triggers the activation of different transcription factors which induce the polarisation of $CD4^+$ Th into Th1, Th2, Th17, follicular helper T-cells (T_{FH}) and regulatory T-cells (T_{reg}) [28]. Once activated, Th cells either become memory T-cells for immunologic memory or effector T-cells that migrate to the site of inflammation. The $CD8^+$ CTLs destroy virus infected cells or tumour cells. They recognise antigens presented by MHC-I on all nucleated cells. Upon activation, CTLs become potent effector cells against intra-cellular infections and in anti-tumour immune responses. Their mechanism of function is to directly kill infected or malignant cells via cytotoxic mechanisms involving granzyme, perforin, or Fas ligand-mediated apoptosis [29].

T-cell subpopulations and their functional diversity

As mentioned, Th-cells can differentiate into various phenotypes upon activation, each with distinct roles in immunity. The Th1 cells secrete pro-inflammatory cytokines and are involved in recruiting and activating macrophages and stimulating CTLs by IFN- γ stimulation [28]. The Th2 cells are involved in macrophage activation towards the anti-inflammatory phenotype, recruitment of certain granulocytes, and

tissue repair [30,31]. Th17 cells are characterised by their secretion of IL-17 which is an important cytokine for neutrophil recruitment and the defence against extracellular bacterial and fungal infections. They are also involved in the pathogenesis of many autoimmune diseases [32]. The T_{FH} cells are located in secondary lymphoid organs (SLOs) such as lymph nodes (LNs) and are major helper cells for B-cell activation. They secrete IL-21 and IL-6 which are key cytokines for the affinity maturation and plasma cell differentiation of B-cells [33]. T_{regs} are comprised of natural Tregs (nT_{reg}) derived from the thymus and inducible Tregs (iT_{reg}) differentiated in the periphery. They express the immunosuppressive cytokines IL-10 and TGF-β that regulates the activity of other immune cells [33,34].

T-cell activation: A three-signal model

As mentioned above, T-cell activation requires a three-signal cascade [35]: Without all three signals, T-cells become anergic and functionally inactive [36].

1. **Antigen recognition**: The initial signal involves the interaction between the TCR and its specific antigen presented on MHC molecules. Th cells are activated by APCs that present antigens on MHC class II molecules. For malignant cells, antigens need to be displayed on MHC class I molecules to become detected by CTLs. For CTL activation, tumour antigens are therefore presented by APCs on MHC class I molecules, via a mechanism called cross-presentation [37].

2. **Co-stimulation**: The TCR signalling is amplified by co-stimulatory receptor interactions. Without this second signal, T-cells become anergic (unresponsive). The key co-stimulatory receptor is CD28 expressed on naïve T-cells, which binds to B7 (CD80/CD86) on APCs. Other co-stimulatory receptors include ICOS, 4-1BB, GITR, and OX40, each playing a role in enhancing T-cell activation and function. The co-stimulatory receptor can also be inhibitory (e.g. CTLA-4 and PD-1), as mentioned below.

3. **Cytokine signalling**: Fully activated T-cells require additional signals from cytokines in their near or direct (auto/paracrine) environment. IL-2 is the key cytokine that promotes T-cell expansion and survival. Optimal effector function, and the generation of specific Th population, is then generated by other cytokines (e.g., IL-12, IFN-γ, IL-6, IL-21, IL-17, IL-1β and TGF-β) which activates specific transcription factors determining Th lineage and transcription of effector cytokines (e.g. IFN-γ, IL-4, IL-17, IL-21, IL-22, IL-10) [38].

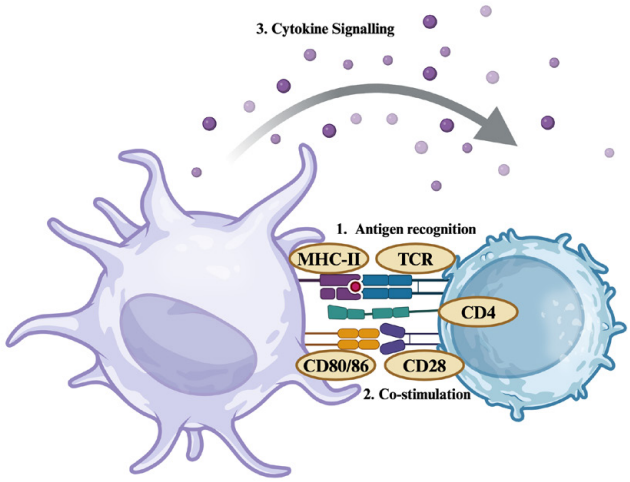

Figure 4. T-cell activation
T-cells require three signals for complete activation. Without all three signals, they become anergic. The first signal is antigen recognition with their TCR, either CD4 - MHC-II or CD8 - MHC-I. The second signal is a co-stimulatory signal between T-cell and the presenting cell, and the third signal is a cytokine environment which stimulates T-cells proliferation and differentiation.

T-cell tolerance

T-cell tolerance is essential for maintaining immune homeostasis by ensuring T-cells distinguish between self and non-self-antigens. It is divided into two main mechanisms: the central tolerance, which occurs in the thymus; and the peripheral tolerance, which operates in tissues outside the thymus [39]. By integrating central and peripheral tolerance mechanisms, the immune system effectively prevents autoimmunity while maintaining the capacity to respond to pathogens.

Central tolerance

T-cell central tolerance occurs in the thymus. Here T-cells undergo positive selection, where their TCRs first must recognise self-MHC molecules to continue their development. Negative selection occurs for self-reactive T-cells (those recognising self-antigens strongly) that are eliminated. Recognition of MHC class I or MHC class II will also determine their differentiation into $CD8^+$ CTLs or $CD4^+$ Th cells [39]. A subset of self-reactive thymocytes avoids deletion and instead differentiates into regulatory T_{regs} through agonist selection. These become nT_{regs} as compared to iT_{regs} that differentiate out in the periphery [40].

Peripheral tolerance

The peripheral tolerance mechanisms address self-reactive T-cells that manage to escape central tolerance, and T-cells activated by non-pathogen derived antigens encountered outside primary lymphoid organs, such as food-derived antigens. The key mechanisms of peripheral tolerance include clonal deletion, antigen sequestration, anergy, and immune regulation or suppression [41]. From these mechanisms, immune suppression is particularly interesting as it is frequently involved in tumour growth. Immune suppression is partly mediated by T_{regs} and inhibitory receptors or molecules like CTLA-4, IL-10 and TGF-β. These prevent self-reactive T-cell activation and effector functions [39].

B-Cells

B-cells are the source of the antibody-mediated immune response, which is also called the humoral immune response, and is an important component of the adaptive immune system. B-cells express and secrete immunoglobulins (Ig) that when membrane bound also function as their B-cell receptors (BCR; mIg). The BCR consist of two light chains and two heavy chains which, similar to the TCR, consist of variable and constant gene segments. The BCR is generated in the bone marrow through V(D)J recombination, which creates the diversity necessary for recognising the wide array of antigens [42]. B-cells undergo negative selection during this development to ensure self-tolerance. However, if a BCR is autoreactive, B-cells can undergo receptor editing, rearranging the light chain genes to produce a non-autoreactive BCR [43]. B-cell activation leads to the production of antibodies which are secreted and have many functions (discussed below).

Structure and function of antibodies

Ig are also called antibodies and contribute to immunity via several mechanisms including neutralisation, opsonization, complement activation, antibody dependent cellular cytotoxicity (ADCC) and antibody dependent cellular phagocytosis (ADCP). These mechanisms are managed by the five different Ig isotypes (IgM, IgD, IgG_{1-4}, IgA_{1-2} and IgE) each with distinct roles in humoral immunity [44].

- **Neutralisation (IgG, IgA, IgM)**: Antibodies bind to toxins or pathogens, thus preventing their cellular adhesion or toxin action.
- **Opsonization (IgG, IgM, IgA)**: After binding to an antigen, antibodies mark the cells for phagocyting cells such as neutrophils and macrophages. This mechanism is known as opsonization.
- **Complement activation (IgM, IgG)**: Antibodies binds to pathogens which triggers the classical complement protein pathway. The complement system can further kill pathogens via cell lysis.

- **ADCC/ADCP (IgG)**: After binding to the antigens, IgG_1 helps in the recruiting of effector cells, such as NK cells, to target antibody-coated cells and kill them via ADCC. Marked antigens can also enhance antigen-uptake by DCs leading to antigen presentation to T-cells.

B-cell activation

B-cell activation can occur via different pathways. These differ based on the localisation of the B-cells and the triggering signal. B-cell activation is divided into T-cell dependent (TD) and T-cell independent (TI) pathways. In general, B-cell activation and subsequent antibody production requires $CD4^+$ Th cells when the antigen is a protein. Yet, bacterial polysaccharide and repetitive epitopes can activate B-cells independent of T cells, via cross-linking their receptors. This is fundamental in early antibody responses for acute infections [45].

T-cell independent activation

TI B-cell activation is further divided into TI-1 and TI-2 B-cell activation depending on the triggering stimuli. TI-1 B-cell activation is initiated by TLR4 that recognises microbial ligands and induces a polyclonal, low-antigen-specific B-cell response [46]. TI-2 on the other hand is initiated by repetitive polysaccharides which extensively crosslink BCRs and deliver persistent signals to the B cells [47]. TI-2 B-cell activation leads to higher affinity antibodies and has some capability to enter germinal centres (GC) [48].

T-cell dependent activation

TD activation of B-cells occurs in the LNs with the help of $CD4^+$ Th cells and CD4 T_{FH}. When a naïve B-cell encounters an antigen that binds its BCR, it is internalized, processed and presented on their surface via MHC class II. The B-cells migrate to T-/B-cell borders in LNs where $CD4^+$ Th cells further activate them. If the $CD4^+$ Th cell TCR recognises the antigen expressed by the B-cell MHC class II, it establishes a co-stimulatory signal via the CD40/CD40L pathway. This leads B-cells into forming GC, where they eventually undergo complete activation, proliferation, class switch recombination (CSR) and affinity maturation. The interaction between a T-cell and B-cell is called linked recognition or cognate interaction. In the GCs, B-cells are divided into a dark zone and a light zone. They first enter the dark zone, where they proliferate and undergo somatic hypermutations (SHM) of their BCR. SHM introduces mutations into the variable regions of Igs, enhancing the affinity of the antibodies. Afterwards they migrate to the light zone which consists of follicular dendritic cells (FDCs) that recruit T_{FH} via the CXCL13/CXCR5 gradient. The B-cells test their BCR, that has undergone SHM, by acquiring antigens from FDCs which bind and store antigens in the form of immune complexes. Light zone CD4 T_{FH} then interact with B-cells that have gained improved affinity and provide

them with a survival signal via CD40/CD40L interaction, promoting B-cells to undergo further affinity maturation and CSR [49-51]. After maturation in the GCs, terminally differentiated B-cells exit the LNs as antibody secreting plasma cells or memory B-cells that persist as long lived cells [52].

While memory B-cells and plasma cells originate from GC, plasmablast differentiation is another B-cell maturation pathway that may occur. Plasmablasts reside outside primary and secondary follicles in so called extrafollicular foci. Plasmablasts are initially short-lived, antibody producing B-cells involved in early antibody production during infections and may be stimulated by TI and TD activation. Regardless they have the ability to differentiate into plasma cells and be isotype switched, but mostly they produce Igs with lower affinity [53].

Fundamental concepts in Cancer Immunology

The Tumour Microenvironment (TME)

Cancers can be broadly classified into haematological malignancies and solid tumours. For solid tumours, cancer is not merely a genetic disease but rather a complex ecosystem involving interactions between malignant cells and a variety of non-malignant cell types within the tumour microenvironment (TME). While genetic alterations in malignant cells are necessary for cancer initiation, the progression of the cancer depends on the characteristics of cells and signals in the TME. A microscopic examination of solid tumours reveals that the TME is comprised of multiple cellular components, including immune cells, cancer associated fibroblasts (CAFs), ECs, pericytes, neurons, adipocytes and extracellular matrix components. They collectively play critical roles in the pathogenesis of cancer [54]. This thesis focuses specifically on the myeloid immune cell populations within the TME and their contributions to cancer progression and prognosis, but also on their interaction with the adaptive immune response.

Endothelial cells and lymphatic endothelial cells

Immune cells can migrate into the TME via blood vessels or the lymphatic system. Blood vessels are composed of ECs, which line the blood vessels and are critical for supplying oxygen and nutrients to the TME. In the TME, ECs are often stimulated by vascular endothelial growth factor A (VEGFA), which is secreted by both tumour and immune cells, and leads to an abnormal vascularisation. The abnormal ECs cause altered properties in the TME such as reduced adhesion molecule expression, which limits immune cell infiltration. Moreover, ECs can produce immunosuppressive molecules that contribute to immune evasion [55]. Immune cells can also reach the TME via the lymphatic endothelial cells (LECs), which form the walls of lymphatic vessels and play a dual role in the TME. On one hand, lymphatic vessels provide a route for immune cells such as DCs to transport tumour antigens to LNs for adaptive immune activation. On the other hand, lymph angiogenesis facilitates metastatic dissemination, particularly to tumour-draining lymph nodes (TDLN). In general, the presence of LECs and the process of lymph angiogenesis in the TME are associated with poor prognosis due to their role in promoting metastasis and modulating the immune response [56].

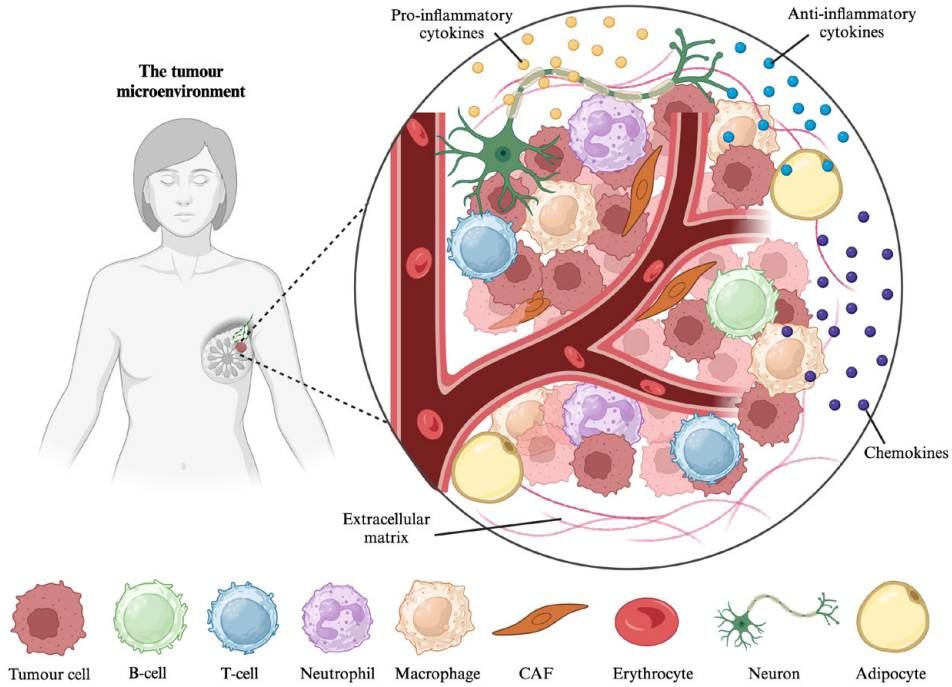

Figure 5. The Tumour Microenvironment
This illustration shows the complex heterogeneity of the tumour microenvironment (TME). Apart from tumour cells, the TME consists of several other types of cells including myeloid cells, lymphocytes, fibroblast, neurons and adipocytes. Signals and interactions between the cells of the TME shape the tumour and its progression.

Immune surveillance and immunoediting

Cancer is a multifaceted disease with intricate biological phenomena. For centuries, understanding the causes, growth mechanisms, and metastatic behaviour of cancer cells has been a central focus of cancer research. The functional transformations that normal human cells undergo to become cancerous have been extensively categorised in the landmark review "The Hallmarks of Cancer" and its sequel "Hallmarks of Cancer: The Next Generation" [57,58]. These frameworks have provided a unifying perspective on the shared traits among diverse cancer types, offering insights into the common cellular processes that drive tumourigenesis. The most recent update divides these hallmarks into fourteen distinct categories [59]. Importantly, not all cancers exhibit features from all hallmarks, and certain hallmarks can influence the activation of others. In this thesis, two hallmark are at focus in particular: Avoiding immune destruction and tumour promoting inflammation.

The notion that the immune system plays a pivotal role in cancer development dates back to 1909, when Paul Ehrlich first proposed that host defences might prevent neoplastic cells from progressing into tumours [60]. This initial hypothesis laid the foundation for what is now known as "Immune Surveillance", a concept conceived by Lewis Thomas and Frank Burnet. They hypothesised that the immune system recognises newly arising tumours through the expression of tumour specific neo-antigens that can induce an immunological reaction against the cancer. Thus, immune surveillance describes the ability of the immune system to recognise and eliminate cancer cells or, conversely, to shape their evolution. This dualistic role of the immune system, capable of both controlling and promoting cancer progression, later became the foundation of the cancer immunoediting hypothesis [61].

The cancer immunoediting hypothesis describes a dynamic interplay between the immune system and tumour cells that unfolds in three distinct phases: elimination, equilibrium, and escape [61]. These phases detail the progression from initial tumour suppression by immune cells to eventual immune evasion by tumour cells and occurs after the transformation of a healthy cell into a tumour cell. The details of immunoediting are described as the following:

1. **Elimination**: Describes the first phase of immunoediting, when the innate and adaptive immune systems collaborate to detect and destroy tumour cells. This stage is characterised by the restoration of tissue homeostasis as tumour cells are successfully eradicated – Immune surveillance.

2. **Equilibrium**: Describes the second phase of immunoediting. At this stage, the immune system can no longer eliminate tumour cells effectively. Instead, a prolonged state of balance ensues, during which tumour cells persist but are kept in check. At this stage, tumour growth remains limited, although progression may begin to develop.

3. **Escape**: Describes the last stage of immunoediting. At this phase, tumour cells have adapted, shaping an immunosuppressive and pro-tumour microenvironment. This enables them to evade immune detection and accelerate growth, ultimately leading to metastasis.

Understanding these phases is critical for elucidating the mechanisms by which cancer evades immune destruction, a hallmark that is central to the progression of many cancers. This thesis explores the intricate interplay between cancer cells and the immune system, with a specific focus on the mechanisms that allow tumours to avoid immune surveillance and promote disease progression.

Immune evasion

The development of a tumour is defined by a series of interactions between the tumour cells and their microenvironment. Consequently, progressing tumours become clinically manifested only when malignant cells manage to escape the immune system. The mechanisms underlying immune evasion evolve in response to selective pressures exerted by the immune system. These mechanisms were previously broadly categorised into two strategies: immune suppression and immune escape. Immune suppression involves the active inhibition of immune cell function through the release of immunosuppressive molecules, recruitment of regulatory immune cells, or remodelling of the tumour microenvironment into an immunologically inactive state [62]. In contrast, immune escape entails the direct avoidance of immune recognition by altering the expression of surface molecules, such as MHC proteins, by impairing antigen presentation pathways or having low tumour mutational burden [63].

A more recent framework categorises immune evasion into three distinct "C"s: camouflage, coercion, and cytoprotection [64]. This model, inspired by the three "E" phases of immunoediting, describes several mechanisms used by malignant cells to evade immune detection and destruction.

Camouflage

Camouflage is the primary mechanism through which malignant cells evade immune recognition. This mechanism of immune escape describes how malignant cells remain undetected by immune cells. This strategy involves defective antigen presentation and the creation of an immune-excluded tumour microenvironment. Following oncogenic mutations, malignant cells acquire a distinct tumour-antigenic landscape that CTLs typically recognise through the MHC-I machinery. However, mutations in genes encoding MHC molecules [65], loss of heterozygosity in chromosomes containing MHC loci [66], and epigenetic modifications [67], can impair antigen processing and presentation, leading to immune escape from T-cells. Tumours can also establish distinct microenvironmental phenotypes. Some exhibit an immune-inflamed phenotype, characterised by immune cell infiltration within the tumour parenchyma, while others develop an immune-desert phenotype, where immune cells are largely excluded from the tumour microenvironment [68]. This immune exclusion further supports the camouflage mechanism by preventing immune effector cells from engaging with malignant cells.

Coercion

In cases where malignant cells fail to camouflage themselves, they may still evade immune activity through coercion. This occurs when malignant cells are recognised by the immune system, but they interfere with immune effector cell function. The most prominent mechanisms of coercion include T-cell exhaustion and immune

suppression through metabolic and TME modulation [64]. The T-cell exhaustion is a state of T-cell dysfunction that arises during chronic immune stimulation, such as persistent infections or cancer. Exhausted T-cells exhibit reduced effector functions, including impaired IL-2 production, diminished proliferative capacity, and loss of cytotoxic activity [69]. This dysfunction results from both intrinsic and extrinsic negative regulatory pathways. Intrinsically, malignant cells upregulate inhibitory receptors, such as PD-L1, which interact with PD-1 expressed on activated T-cells, driving T-cell exhaustion [64]. Extrinsically, immunoregulatory cytokines such as IL-10 and TGF-β contribute to immune suppression, inhibiting the cytotoxic activity of T-cells and NK cells [70], and promoting the recruitment of T_{regs} and pro-tumour myeloid cells, such as M2 macrophages and N2 neutrophils [71]. Other cytokines, such as the colony stimulating factor 1 (CSF-1) and CCL2, further aggravate the immunosuppressive microenvironment by recruiting suppressive myeloid cell populations [72].

Cytoprotection

Even when recognised by immune cells, malignant cells can resist destruction through cytoprotective mechanisms. When T-cells or NK cells establish an immunological synapse with malignant cells, they release cytotoxic molecules such as granzyme B and perforin, express death receptor ligands like Fas ligand, or secrete tumour-targeting cytokines such as IFN-γ [73]. To counteract these cytotoxic pathways, malignant cells can alter the structure and function of the immunological synapse, thus disrupting cell death signalling pathways, and develop resistance to apoptosis [64].

The ability of cancer cells to evade immune detection and destruction represents a major barrier to effective immune-mediated tumour control. By controlling strategies of camouflage, coercion, and cytoprotection, tumours adapt to and evade immune responses, contributing to disease progression and resistance to therapy. Understanding these mechanisms is crucial for developing targeted immunotherapies aimed at overcoming immune evasion and restoring antitumour immunity.

Immune checkpoint inhibition and its mechanism

As discussed above, numerous mechanisms contribute to immune evasion by tumours. Among these, immune checkpoint inhibition (ICI) targets the "coercion" strategy of immune evasion, specifically addressing T-cell exhaustion, and remains the most effective immune therapy developed to counter immune evasion. The first ICI therapy was approved in 2011 and demonstrated remarkable success, particularly in solid tumours such as melanoma and non-small cell lung cancer (NSCLC), significantly improving survival rates [74]. To date, the U.S. Food and Drug

Administration (FDA) has approved nine immune checkpoint therapies, targeting four key checkpoint receptors: CTLA-4, PD-1, PD-L1, and LAG-3.

Immune checkpoints: The "off-switch" for T-cells

In parallel to the activation signals, T-cells can receive inhibitory signals through immune checkpoint receptors, which suppress their effector functions and maintain immune homeostasis. In the tumour microenvironment, these inhibitory signals are hijacked by malignant cells which promotes immune evasion. The primary checkpoint receptors targeted by current therapies include PD-L1/PD-1, CTLA-4, and LAG-3, with others like TIM-3 and TIGIT being actively studied [75].

Key immune checkpoints and their mechanisms

- **CTLA-4:** Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) competes with CD28 for binding to the co-stimulatory ligands B7 (CD80/CD86) on APCs. Unlike CD28, CTLA-4 suppresses T-cell activation by decreasing IL-2 production. CTLA-4 is also highly expressed on T_{regs}, where it facilitates immune suppression by reducing CD80/CD86 expression on APCs [76].
- **PD-1**: Programmed cell death protein 1 (PD-1) binds to its ligands PD-L1 and PD-L2. PD-1 activation recruits inhibitory motifs (ITIMs), which dephosphorylate the TCR signalling cascade, thereby attenuating T-cell activation [77,78].
- **PD-L1:** Ligand to PD-1 and expressed by tumour cells or APCs.
- **LAG-3**: Lymphocyte activation gene-3 (LAG-3) functions as a competitive receptor for CD4, binding to MHC class II molecules on APCs. The intracellular signalling pathways of LAG-3 remain under investigation, but its role in reducing T-cell proliferation and effector functions is well established.

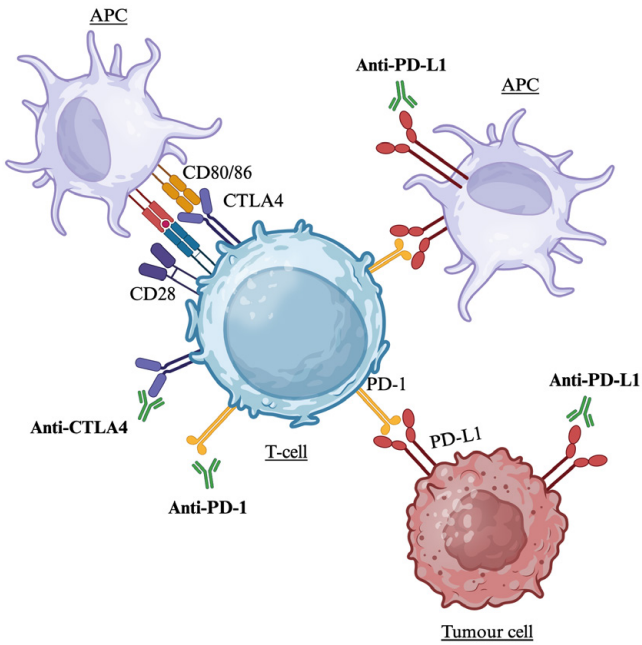

Figure 6. Immune checkpoint blockade
Current strategies employed by immune checkpoint blockades (ICB). The co-inhibitory signals necessary for T-cell inhibition are blocked by antibodies which target co-inhibitory receptors on either T-cells (CTLA4, PD-1) or on APCs/malignant cells (PD-L1).

Breast cancer

Breast cancer subtypes

Breast cancer is the most common cancer affecting women worldwide but may also affect men (<1% of cases) [79]. As the name suggests it affects the human breast which is a mammary gland composed of glandular tissue and supporting tissue. The supporting tissue consists of adipose tissue and connective tissue that provides structure for branching the ductal networks. The glandular tissue consists of lobules which are the functional units responsible for milk production. The milk is collected into collecting ducts which transport the milk to the nipple. The lobular and collecting ducts share a similar structure. They consist of a lumen, surrounded by luminal epithelial cells and an outer layer of myoepithelial cells, encapsuled by a basement membrane surrounding the whole structure. Upon diagnosis, breast cancer is classified into several categories, which are based on histological subtypes, histological grades, tumour grade and molecular subtypes [80]. Defining breast cancer within these different categories helps towards predicting prognosis and treatment alternatives for each patient.

Histological subtypes

The histological subtypes are classified into pre-invasive and invasive breast cancers and describe the aggressive nature of the cancer [81]. Cancer arising from epithelial tissues are called carcinomas. Breast carcinomas *in situ* are premalignant lesions from the mammary ducts, Ductal Carcinoma In Situ (DCIS); or mammary lobules, Lobular Carcinoma In Situ (LCIS) [82].These pre-invasive carcinomas are more common and are less aggressive cancer types because the carcinomas are growing *in situ*, meaning that they have not breached the basal membrane of the ducts and thus cannot spread to the vasculature in the surrounding stroma [83]. They are generally associated with better prognosis with 5 year-survival rates over 98%[84] Both DCIS and LCIS may progress into invasive carcinomas. Invasive breast cancers are more aggressive in nature as they invade surrounding tissues. They are divided into: Invasive Ductal Carcinoma (IDC), also termed "no special type" (NST) or Invasive Lobular Carcinoma (ILC) [80]. Invasive breast cancers are associated with tumour progression and a worse prognosis due to their ability to invade surrounding tissues.

Histological grades

The histological grade is a grading system that scores breast carcinomas based on their morphological features. The grading system delineates if the cancer cells have differentiated well (represented by a low score) or poorly (represented by a high score). Poorly differentiated carcinomas are associated with a worse prognosis and more aggressive tumour [80,82,85]. This histological grading system is called the

Nottingham system and is determined through immunohistochemistry (IHC). It evaluates three different histological morphologies which are:

- **Mitosis**: The rate of cell division.
- **Anisokaryosis**: The variations in nuclear size and shape of tumour cells.
- **Tubule formation**: The extent to which tumour cells form normal glandular structures.

Tumour staging

The tumour stage is determined using the TNM classification system [80], and provides information on the stage and progression of the cancer. It is based on three different parameters that are measured and summarised to evaluate treatment options and to evaluate the size and spread of the tumour. The three different parameters are:

- **T (Tumour size)**: The size of the primary tumour (PT).
- **N (Node status)**: Indicates the involvement and spread of the tumour to the nearby LNs called sentinel lymph nodes (SLN). In breast cancer, it is the axillary LNs that functions as SLN and are examined.
- **M (Metastasis)**: Indicates the presence of distant metastases in other organs of the body.

Hormone receptor status

The hormone receptors status plays the most important role prior to choosing an efficient treatment alternative in breast cancer. It classifies breast cancers into subtypes based on the expression of hormone- or growth factor receptors. It is a crucial predictive factor in breast cancer diagnosis. Because the breast is a mammary gland, it responds to hormones or growth factors produced by the body. These factors can affect the growth of the tumour. If the tumour cells express hormone receptors or growth factor receptors, they can be targeted with hormone therapy as targeted therapy. Hormone receptor status is routinely evaluated via IHC [80]. The molecular markers are:

- The oestrogen receptor (ER).
- The progesterone receptor (PR).
- The human epidermal growth factor receptor 2 (HER2).

Apart from these receptors, a proliferation marker is also evaluated via IHC:

- Ki-67: A marker of cellular proliferation.

Subtypes of breast cancer

Based on the hormone receptor and proliferation status, breast cancer patients are further classified into different molecular subtypes which predicts treatment option and prognosis from the disease. These molecular subtypes are based on the St. Gallen classification which is assessed by looking at histological features and IHC protein expression [86]. Breast cancer is divided into five subtypes [80,87]:

- **Luminal A (60–70% of cases)**: Represents tumours with strong ER and PR expression. On the other hand, they have a low HER2 expression and low Ki-67 index. These subtypes typically characterise low-grade tumours and are therefore associated with a favourable prognosis. They are treated with hormone therapy.

- **Luminal B/HER2$^-$ (10–20% of cases)**: Are also ER and PR positive, though lower than in Luminal A and have a low HER2 expression. In comparison to luminal A, these tumours have a higher Ki-67 index and represents higher-grade tumours. They are associated with an intermediate prognosis and are also treated with hormone therapy.

- **Luminal B/HER2$^+$ (13–15% of cases)**: These tumours are ER and PR positive but are also HER2 positive, with a higher histological tumour grade and higher Ki-67 index compared to luminal A subtypes. These tumours are associated with an intermediate prognosis and can be treated with HER2 targeted therapy and hormone therapies.

- **HER2 enriched (13–15% of cases)**: This subtype is included in the same prevalence range as the Luminal B/HER2$^+$ subtypes, as both of them are viewed as HER2$^+$ breast cancers. The HER2 enriched subtype represent non-luminal breast cancer tumours with low ER and PR expression but a high HER2 expression, a high histological grade and higher Ki-67 index. These patients have an intermediate prognosis and are treated with HER2 targeted therapies.

- **Triple-negative breast cancer (TNBC, 10–15% of cases)**: These tumours lack expression of ER, PR, and HER2 and have a high Ki-67 index. They are associated with the worst prognosis and offers limited treatment options as they lack the hormone receptors or growth factor receptor. These tumours can be treated by other therapies such as PARP inhibitors and chemotherapy[88].

Molecular subtypes of breast cancer based on a 50 gene expression signature is often used in research and personalised medicine. This system can be used to more precisely interpret the cutoff or ER, PR and HER2 expression and divide tumours into TNBC but also into normal-like breast cancer [87].

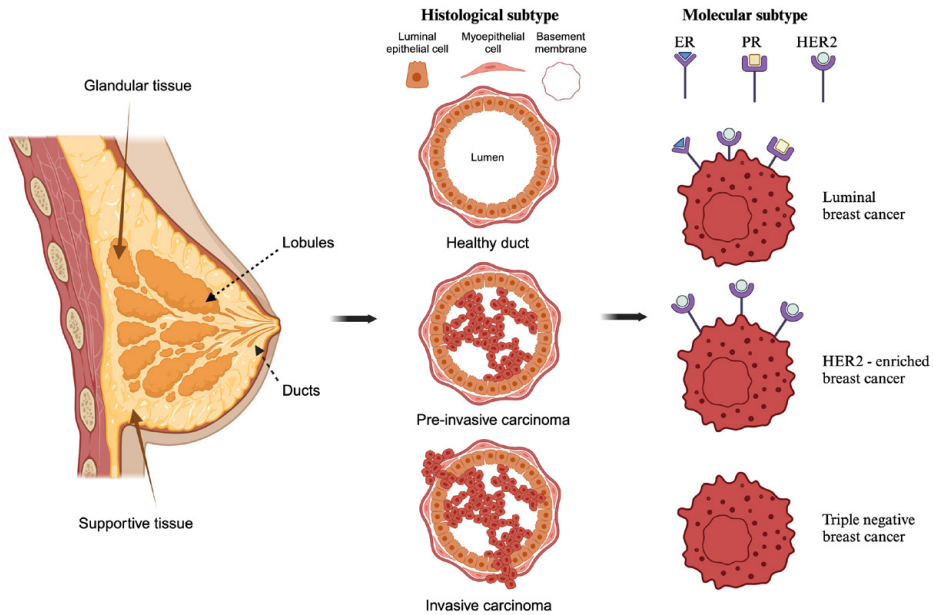

Figure 7. Breast cancer histological and molecular subtypes
The breast tissue consists of glandular and supportive tissues. The glandular tissue consists of lobular cells and collecting duct cells comprised of luminal and myoepithelial cells. In breast cancer, pre-invasive carcinomas invade the lumen, while invasive carcinomas invade the surrounding tissue. Breast cancer can further be categorised by expression of hormone- and growth factor receptors, indicating the aggressiveness of the cancer and treatment alternatives best suited.

Immune involvement in breast cancer

Breast cancer are solid tumours with a complex TME. The TME consists of non-malignant cell types such as lymphoid cells, myeloid cells and stromal cells. Due to the heterogeneity between subtypes of breast cancer, the TME and immune cell landscape also differ a lot. In general, immunogenicity and lymphocyte infiltration are higher for TNBC and HER2-enriched tumours and lower in the luminal subtypes[89,90]. A higher immunogenicity in these molecular subclasses leads to an

increased ability to generate an immune response against tumour specific antigens. Tumours with high tumour mutational burden are more immunogenic and therefore more likely to respond to current immunotherapies such as ICI. Due to this, tumour-infiltrating lymphocyte (TILs) are currently routinely used as a predictive and prognostic marker in TNBC and HER2 positive breast cancer. Nonetheless, current results from the clinic shows a poor benefit of ICI as a monotherapy in breast cancer. A clinical trial investigating this showed that in the overall population, ICI was associated with a median overall survival of 9,9 month while chemotherapy was associated with 10,8 months. However, patients with a high PD-L1 score showed 1 month improved survival with ICI compared to chemotherapy [91].

Nonetheless, better results have been achieved by combining ICI with chemotherapy. Yet, among multiple clinical trials, only two trials indicated beneficial prognosis in terms of longer progression-free survival (PFS) and event-free survival (EFS) using ICI in combination with chemotherapy. The first trial showed median PFS with pembrolizumab-chemotherapy of 9,7 months compared to 5,6 months with placebo-chemotherapy in TNBC [92]. The second trial showed EFS at 36 months in the pembrolizumab-chemotherapy group at 84.5%, compared with 76.8% in the placebo-chemotherapy group in TNBC [93]. This emphasises the need to develop better immunotherapies for breast cancer. In fact, many other immune components in tumours remain important to investigate in the TME. These cells are for instance macrophages, neutrophils and tertiary lymphoid structures (TLS).

Tumour-infiltrating lymphocytes (TILs)

TILs are important biomarkers for lymphocyte-mediated anti-tumour immunity. They primarily consist of $CD8^+$ T-cells, $CD4^+$ T-cells, and B-cells. T-cell TILs are often measured using the marker CD3 [94]. While normal breast tissue harbours minimal $CD4^+$ and $CD8^+$ T-cell populations, breast tumours and their surrounding stroma exhibit a significantly increased T-cell infiltration [95]. TIL infiltration is particularly pronounced in aggressive breast cancer subtypes, such as TNBC and HER2-enriched tumours [96,97]. TILs are further categorised based on their localisation within the TME. Stromal TILs (sTILs) represent the majority of TILs and reside in the stromal compartment of the tumour. Intratumoral TILs (iTILs), on the other hand, represent a smaller proportion of TILs that infiltrate and reside in the tumour mass itself [96]. The sTILs are physically excluded from direct contact with tumour cells, indicating an immune-excluded TME. Nonetheless, tumours with sTILs are correlated with better breast cancer treatment and survival compared with tumours lacking sTILs [98]. Tumours with a high iTILs, also have a better prognosis with improved pathological responses after chemotherapy. This is seen most notably in TNBC and HER2-enriched tumours [99,100]. In advanced breast cancers such as lymph-node-positive breast cancer, combined sTIL and iTIL prevalence ranges from 0-10% in luminal subtypes, 15% in $HER2^+$ cancers and 20% in TNBC. In the

previous study iTIL were determined as the percentage of mononuclear cells within the epithelium of the invasive tumour cell nests while sTILs were defined as the percentage of tumour stroma containing infiltrating lymphocytes [98]. However, these parameters can differ between studies.

As mentioned, TILs are most clinically relevant in the aggressive breast cancer subtypes TNBC and HER2-enriched. In TNBC, tumours harbour higher immunogenicity due to increased mutational burden and hence more neoepitopes[101]. Interestingly, a high $CD8^+$ TIL infiltration is associated with better response to chemotherapy and longer survival [102], although the underlying mechanism for better response rates is not known. $HER2^+$ amplified tumours have a lower mutational burden compared to TNBC, nonetheless high TIL levels are also associated with better responses to targeted therapy [103]. A feasible explanation indicates the involvement of ADCC by NK cells or unconventional T-cell populations such as natural killer T-cells (NKTs) and γδ T cells. In general, in TNBC and HER2-enriched subtypes, TIL levels correlate with favourable prognostic indicators, such as disease-free survival and overall survival. Luminal subtypes on the other hand, harbour lower levels of TILs that do not correlate with better prognosis [96,104,105]. TIL presence is often evaluated alongside PD-L1 expression to predict responses to immune checkpoint blockade (ICB) therapies [106]. However, despite having TIL-rich tumours, the proportion of breast cancer patients responding to ICB remains suboptimal. Hence, there is a need to identify additional immune biomarkers to improve predictive and prognostic precision in breast cancer.

B-cells have historically not been in focus when discussing tumour infiltrating immune cells, mainly because immunotherapy rather aims to target the T-cell compartment as they can exert cytotoxic effect on tumour cells. However, B-cells should not be disregarded as they modulate humoral immunity in cancer by producing antibodies that recognise tumour-associated antigens, mediating tumour destruction through ADCC and ADCP, but also have a function as APCs. Tumour-infiltrating B-cells exhibit diverse phenotypes, including naïve B-cells, memory B-cells, plasma cells, and regulatory B-cells (B_{regs}). The effector functions of B-cells in the TME include anti-tumour effects such as recruiting immune cells, priming T-cell responses, ADCC and ADCP; or pro-tumour effects, such as immunosuppression through B_{regs} [107]. These will be discussed below.

Tertiary lymphoid structures (TLS)

The presence of TILs can be further assessed through the formation of TLS. Initially described in autoimmune and infectious diseases as a hallmark of chronic inflammation, TLS are ectopic lymphoid aggregates resembling LN structures that facilitate local antigen presentation in non-lymphoid tissues [108]. In cancer, TLS were first identified in lung tumours, where a retrospective cohort study demonstrated

that TLS present within tumours, but not in adjacent non-malignant tissue, were associated with improved prognosis [109]. Since then, TLS and highly organised lymphoid aggregates have been observed across multiple cancer types, including breast cancer, where they have frequently been correlated with favourable clinical outcomes [110-114].

Composition and organization

As mentioned, TLS are complex structures that emerge in response to chronic inflammation, promoting adaptive immune activation in ectopic tissues [115]. Their cellular composition reflects their level of maturity, which in turn influences their prognostic impact. Structurally, TLS are organised lymphoid aggregates characterised by a central core consisting of B-cells which is surrounded by T-cells and antigen-presenting DCs. More specifically, they are composed of the following cell populations:

- **B-cells**: Naïve B-cells, GC B-cells, memory B-cells, plasmablasts, plasma cells, regulatory B-cells [116].
- **T-cells**: $CD8^+$ CTLs (tissue resident memory T-cells, exhausted T-cells), $CD4^+$ Th-cells (T_{FH}, T_{reg}, Th17) [116].
- **Myeloid cells**: cDC1, cDC2, pDCs and macrophages [117-119].
- **Stromal cells**: Fibroblastic reticular cells (FRCs) and FDCs.
- **Endothelial cells**: High endothelial venules (HEVs) and LECs.

TLS are functionally divided into two compartments, the T- and B-cell area, where tumour antigen specific immune responses are initiated and shaped. Both naïve T-cells and B-cells are recruited to TLS through HEVs, which are closely associated with TLS formation [115,120]. Additionally, lymphatic vessels are often found in proximity to TLS where memory T-cells may enter the TLS [121]. The two key compartments are:

- **The T-cell area**, where Th T-cells interact with mature DCs. This interaction leads to T-cell differentiation into T_{FH}, Th1, Th17. The T-cell area also consist of memory T-cells which are reactivated, thereby enhancing anti-tumour immune responses and improving survival [116,122].
- **The B-cell area**, which is further divided into the mantle zone, containing naïve B-cells and the GC with T_{FH} cells and FDCs which orchestrate B-cell differentiation [121].

Maturation and prognostic relevance

The degree of TLS organization plays a crucial role in their functional capacity and prognostic value. TLS are classified into three maturation stages, based on the composition and activation status of their lymphoid constituents [115,121,123]:

1. **Early TLS**: Unstructured lymphoid aggregates composed of T-cells and B-cells.
2. **Immature TLS (primary follicle)**: Lymphoid aggregates with distinct T-cell and B-cell zones and the presence of FDC.
3. **Mature TLS (secondary follicle)**: Fully developed TLS containing GCs and HEVs, representing active antigen presentation and immune activation.

In breast cancer, TLS presence has been associated with both favourable and unfavourable prognoses [110,124,125]. This variability likely reflects differences in the TME, which influences TLS functionality [126-128]. A deeper understanding of the TME factors that contribute to positive or negative outcomes is needed to pin-point the predictive value of TLS in breast cancer subtypes and optimising immunotherapeutic strategies.

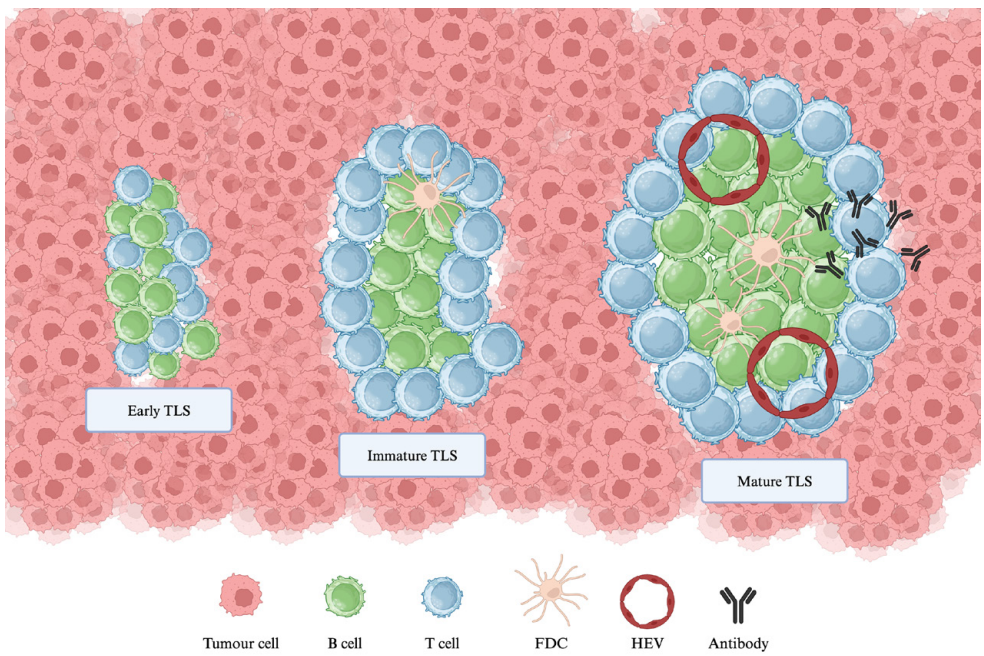

Figure 8. Tertiary lymphoid structures
Upon chronic inflammation, tertiary lymphoid structures (TLS) arise in the inflamed tissue to initiate the adaptive immunity. These structures also occur in cancer and can be categorised based on their maturity. Early TLS consist of lymphoid aggregates of B-cells and T-cells. Immature TLS consists of an organised structure of B-cells and T-cells with the presence of FDCs. Mature TLS consists of organised B-cell follicles with a GC and FDCs, adjacent to a T-cell zone, and the presence of HEVs that recruit T-cells and B-cells.

Macrophages in breast cancer

Although the presence of TILs in breast cancer is associated with improved responses to chemotherapy and enhanced survival outcomes in certain cases [129], they only represent a subset of tumour-infiltrating leucocytes. A substantial proportion of the tumour-infiltrating immune cell population is composed of tumour-associated macrophages (TAMs), which can constitute > 50% of all leucocytes in the TME[130,131]. Unlike TILs, TAM infiltration within the TME correlates with poorer prognosis. This negative prognostic association of tumour infiltrating immune cells is also observed with other immune cells such as neutrophils and T_{regs}. Nonetheless, among all tumour-infiltrating leucocytes, TAM infiltration is one of the most unfavourable prognostic indicators, affecting both ER-positive and ER-negative breast cancer patients [132].

The TME in breast cancer resembles a state of chronic inflammation, and its microenvironmental signalling cues play a pivotal role in TAM differentiation. TAMs, like other macrophages, exhibit significant plasticity which is influenced by various TME factors such as fibrosis, hypoxia, nutrient availability, lymphocyte-derived signals, and epigenetic modifications [133]. These environmental cues drive TAM polarization along a spectrum ranging from pro-inflammatory (anti-tumour) to anti-inflammatory (pro-tumour) phenotypes. As mentioned before, this polarization continuum is often referred to as the M1/M2 spectrum. The M1 macrophage exerts a pro-inflammatory response by mediating tumour cell death via ROS, phagocyting apoptotic cancer cells, and releasing inflammatory cytokines such as IL-6, IL-12 and TNF. The M2 macrophages promote angiogenesis, tissue reconstruction, and release anti-inflammatory cytokines such as IL-10, all leading to tumour progression [134]. However, it is essential to note that TAMs can share overlapping genetic and molecular characteristics, and their dichotomous states are not mutually exclusive [129]. The recruitment and differentiation of TAMs are dictated by the tumour landscape. Hypoxia within tumours induces stromal and tumour cells to secrete chemokines such as CCL2, CCL5, and CSF-1, which attract circulating monocytes [133,135]. Hypoxia also promotes the differentiation of monocytes into M2-immunosuppressive macrophages through the activation of hypoxia-inducible factor-2 alpha (HIF-2α). Overexpression of HIF-2α in tumours is associated with higher tumour grade and poor prognosis [136].

Anti-tumour TAMs exhibit tumour-suppressive functions through the release of inflammatory mediators and phagocytosis. They secrete cytokines such as TNF-α, iNOS, IL-6, and IL-12, which inhibit tumour cell growth and activates T-cells [130]. Through phagocytosis of cell debris, anti-tumour TAMs can present tumour antigens via MHC class II molecules, thereby activating T-cell responses by antigen presentation and expression of co-stimulatory molecules CD86 and CD80 [133,137]. Although anti-tumour TAMs have been associated with favourable prognosis in certain cancers, such as lung cancer, they remain relatively scarce in breast cancer and have not been established as beneficial prognostic indicators in this context [137].

Pro-tumour TAMs represent the predominant phenotype in breast cancer and are associated with tumour progression, metastasis, and immunosuppression. These macrophages are often identified by the marker CD163, and their high density in PTs has been correlated with poor patient prognosis. Pro-tumour TAMs are present both in the tumour stroma and tumour nests, contributing to similar prognostic outcomes [138,139]. The functional mechanisms of pro-tumour TAMs include angiogenesis, extracellular matrix remodelling, immunosuppression, and establishment of metastatic niches. Hypoxic regions within tumours release DAMPs due to necrosis, which perpetuates chronic immune stimulation and drives TAMs into M2 TAMs to suppress the inflammation [140]. Pro-tumour TAMs suppress the TME with effects on TIL activity. This is done through several pathways:

- **Immune checkpoint inhibitors**: Pro-tumour TAMs express immune checkpoint molecules, notably PD-L1, which inactivates T-cells. The presence of PD-L1 on macrophages is strongly associated with poor cancer prognosis [141].
- **Immunosuppressive cytokines**: These TAMs secrete IL-10 and TGF-β, which decrease $CD8^+$ T-cell antigen sensitivity, suppress pro-inflammatory cytokine production (e.g., IL-12), and promote T_{reg} differentiation [70,142,143].
- **Metabolic modulation**: TAMs express enzymes such as ARG1, which metabolize L-arginine, an essential substrate for T-cell proliferation [144].

Indirectly, TAMs also contribute to immune suppression by releasing cytokines that attract immunosuppressive cells. They can inhibit APCs, induce vascular dysfunction to prevent T-cell infiltration, and secret factors that lead to remodelling of the extracellular matrix, leading to denser tumours and immune exclusion [133].

$CD169^+$ macrophages

A distinct subset of macrophages expresses the membrane protein CD169 and have been associated with both beneficial and harmful prognosis in cancer. These macrophages can arise from circulating monocytes but are also found as tissue-

resident macrophages in SLOs, lungs, and bone marrow [145]. Depending on their anatomical location, CD169$^+$ macrophages perform diverse functions. This thesis investigates their roles within PTs and SLOs.

CD169 is a member of the SIGLEC (sialic acid-binding immunoglobulin-like lectin) family, which are cell membrane receptors. SIGLEC receptors are characterised by their ability to bind sialic acid and typically contain the intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs). ITIMs are further implicated in mediating intracellular signalling and endocytosis. The CD169 receptor is unique among SIGLECs, as it lacks ITIMs and possesses an unusually long extracellular domain comprising of 17 immunoglobulin domains [146]. This structural feature suggests that CD169 primarily facilitates cell-cell adhesion. Sialic acid is abundantly present on pathogens, apoptotic cells, and immune cells, underscoring the functional versatility of CD169$^+$ macrophages [145,147].

In LNs, CD169$^+$ macrophages are tissue-resident cells with significant roles in anti-tumour immunity. Their presence in TDLNs has been linked to favourable prognoses in breast cancer and other malignancies, including endometrial cancer, melanoma, colorectal cancer, and hepatocellular carcinoma [148-152]. Within SLOs, CD169$^+$ macrophages are further categorised into two distinct populations based on their anatomical location: subcapsular sinus (SCS) macrophages and medullary sinus (MS) macrophages [153].

The SCS CD169$^+$ macrophages are critical for lymphoid cell activation. They capture opsonized antigens or lymph-borne antigens, facilitate antigen exposure to underlying B-cell follicles and promote GC B-cell responses [154]. In contrast, MS CD169$^+$ macrophages specialize in phagocytosis, pathogen clearance, lipid sensing, and tissue remodelling [155]. In the context of cancer, the SCS CD169$^+$ macrophages can also contribute to anti-tumour immunity by taking antigens from cellular debris and presenting them to FDCs via extracellular vesicles [156]. Moreover, they are implicated in cross-presentation of antigens to T-cells, further enhancing adaptive immune responses [157,158].

CD169$^+$ macrophages are not restricted to LNs and may also arise within PTs. In breast cancer, the presence of these macrophages has been associated with unfavourable prognoses [159]. However, in other malignancies, such as glioblastoma, their presence correlates with beneficial prognosis by boosting T-cell responses [147]. It has further been shown that tumour infiltrating monocytes in a pro-inflammatory microenvironment aid towards re-activation of T-cells via cross-dressing [160], a mechanism potentially implemented by TAM CD169$^+$ macrophages since these cells have similar gene expression profiles[161]. Nonetheless, the role of TAM CD169$^+$ macrophages remain to be elucidated, especially how they influence the TME.

Neutrophils in breast cancer

The neutrophil-to-lymphocyte ratio (NLR) has emerged as a cost-effective and reliable prognostic marker for cancer that can be measured in blood samples [162]. Elevated NLR is associated with poor outcomes across various cancers and serves as an independent predictor for LNM for hormone-receptor positive breast cancer[163,164]. This association highlights the role of neutrophils in metastasis and tumour progression.

Neutrophils infiltrate the TME in response to chronic inflammation. Tumour cell derived cytokines and growth factors like IL-6, IL-1β, IL-17, CSF-1, and CSF-2 drive neutrophil recruitment [20]. In general, infiltration of neutrophils in the TME is associated with a worsened prognosis [165], with clinical evidence suggesting that neutrophils are important components for both tumour-promoting inflammation and immune suppression. But on the other hand, they sometimes also correlate with anti-tumour responses [20,166]. It is postulated that, once in the TME, neutrophils exhibit either pro-tumour or anti-tumour phenotypes depending on environmental signals. Similar to macrophage plasticity, neutrophils can hence, as mentioned before, be categorised as N1 (pro-inflammatory) or N2 (anti-inflammatory) neutrophils:

N1 neutrophils: Anti-tumour

- Can generate cytotoxic effects via TRAIL and nitric oxide (NO) which leads to apoptosis in tumour cells [167,168].
- Can promote immune responses by recruiting T-cells, presenting antigens via MHC-II, and secreting chemokines [169,170].

N2 neutrophils: Pro-tumour

- Can induce DNA damage by ROS release. This increases the mutational load and inflammation in cancer [171].
- Can release pro-growth cytokines (EGF, HGF, PDGF) and induce angiogenesis via MMP9 and VEGF [172].
- Can facilitate metastasis through NETosis, which traps circulating tumour cells and promotes adhesion to new sites [173]. Furthermore, they release mediators involved in extracellular matrix remodelling, leading to the escape of cancer cells from the tissue [174].
- Are associated with suppression of the adaptive immunity by releasing factors like ROS, iNOS, ARG1, LOX1, prostaglandin E2 (PGE2), or express PD-L1 [169].

Cancer-induced chronic inflammation further leads to the release of immature neutrophils from the bone marrow. These cells, often termed granulocytic myeloid-derived suppressor cells (G-MDSCs), infiltrate the TME and exhibit potent immunosuppressive properties. Analogous to N2 neutrophils, immature neutrophils support tumour progression by promoting ECM remodelling, immune suppression, and the creation of metastatic niches [175,176].

Lymph nodes

Lymph nodes (LNs) are SLOs of the immune system, serving as barriers against systemic pathogens and playing a central role in activating the adaptive immune responses. The human body contains approximately 500–600 LNs, strategically located to process lymph drained from adjacent organs and tissues [177,178] . In breast cancer, one of the most critical prognostic factors is the presence of lymph node metastases (LNM), with unfavourable prognosis increasing with the number of LNMs [179]. The first LNs to which tumour cells are likely to spread are the SLNs, located closest to the PT [180], consequently why they are also termed TDLN. Independent of breast cancer subtype, the presence of LNM is associated with a negative prognosis, as it defines a more advanced disease stage [181].

Lymph node structure and function

LNs are highly organised structures divided into lymphatic sinuses, specialized vasculature, and compartments of diverse immune cell populations. They are encapsulated by a fibrous outer layer (capsule) where afferent lymphatic vessels penetrate, allowing lymph to enter the SCS. From the SCS, lymph flows through trabecular sinuses, passing through the cortex and paracortex before reaching the medullary sinuses, where it exits via efferent lymphatic vessels [177,182]. Given their role as immunological hubs, LNs are critical for initiating adaptive immune responses. However, the mechanisms underlying the activation of LN-resident immune populations against tumour antigens remain incompletely understood. Most insights into LN-mediated immunity are derived from infectious disease models, where immune responses are elicited against viral or bacterial pathogens. Nonetheless, TDLNs can acquire tumour-derived antigens and contribute to shaping the immune response against malignant cells. Antigen presentation within TDLNs is mediated by multiple pathways, including the migration of DCs from the tumour, antigen capture by SCS $CD169^{+}$ macrophages, and the direct entry of soluble antigens into TDLNs [158,183,184]. Once in the LN, tumour antigens are presented to T-cells within the paracortex and B-cell at the border to, or within the cortex, leading to the activation of tumour-specific immunity [185].

T-Cell activation in the paracortex

T-cell activation and clonal expansion occur within the paracortex of LNs [178]. Naïve T-cells continuously circulate through the body and the majority of them (> 90%) enter LNs via HEVs, guided by the expression of CCR7 and the chemokine gradients CCL19 and CCL21, which retain them within the paracortex [186]. Nonetheless, a minority of T-cells may travel to LNs via the afferent lymph vessels,

consisting mostly of memory subtypes but also a few being of naïve subtypes [187]. Concurrently, activated DCs migrating from the TME also express CCR7, allowing them to home to the paracortex, where they present tumour antigens to naïve $CD4^+$ and $CD8^+$ T-cells [188]. In general, intracellular antigens are presented via MHC-I, while extracellular antigens are presented via MHC-II. However, to elicit a CTL response, DCs (cDC1) must efficiently present extracellular tumour antigens via MHC-I, a process known as cross-presentation [37,189]. Once activated, T-cells differentiate into stem-like progenitor effector T-cells in the LNs, and migrate to the TME, where they according to a leading theory are reactivated through co-stimulatory signals (cDC2) and execute their effector functions [190].

B-Cell activation in the cortex

Naïve B-cells are recruited to LNs by expressing CCR7 and CXCR5 which guides them to the cortex via CXCL13 gradients [191]. In the cortex, B-cells are located in extrafollicular compartments, primary and secondary follicles. The primary follicles consist of FDCs and naïve B-cells that search for a cognate antigen. The secondary follicles represent a later stage and consist of GCs with activated B-cells and FDCs[192]. Initial B-cell stimulation is mediated by FDCs and SCS $CD169^+$ macrophages, which capture soluble antigens from lymph and present them to underlying B-cells, leading to cross-linking of their surface receptors and endocytosis of the antigen [156,193,194]. Initially activated B-cells then migrate to the T/B-cell border where they are further activated by T-cells (linked recognition) [195]. Besides activating B-cells, the B/T-cell synapse is also crucial for the full maturation of T_{FH} and their location within GC [196]. Activated B-cells differentiate along extrafollicular compartments of GC follicles as previously mentioned [50,197]. After activation and maturation, memory B-cells or plasma cells, migrate to tumours and contribute to anti-tumour immunity by secreting antibodies against tumour antigens or function as APCs [198]. Nonetheless, plasma B-cells express CXCR4, which causes them to follow the CXCL12 gradient and, notably, most plasma cells reside in the bone marrow and in the medullary cords of LNs [52].

Macrophages in lymph nodes

LNs contain various macrophage populations, but two key subsets are characterised by CD169 expression and tissue residency [154]:

- SCS $CD169^+$ macrophages located at the interface between the subcapsular sinus and B-cell follicles.
- MS $CD169^+$ macrophages, situated around the medullary cords in the LN medulla.

Both subsets are positioned at critical sites where they interact with lymph, emphasizing their role in antigen processing and presentation. SCS macrophages function as initiators of adaptive immunity, capturing antigens from lymph and presenting them to FDCs or naïve B-cells [145,154]. Additionally, they contribute to anti-tumour immunity by mediating cross-presentation, leading to $CD8^+$ T-cell activation [158]. SCS $CD169^+$ macrophages are also involved in T-cell activation within cortical interfollicular regions (IFRs) of LNs [178,199,200]. Although the functional role of MS $CD169^+$ macrophages remains less well understood, they exhibit increased lysosomal activity, suggesting a role in antigen degradation and possibly supporting short-lived plasma cell survival [201,202].

Neutrophils in lymph nodes

Recent studies have highlighted the emerging role of neutrophils in modulating LN immune responses [203,204]. Although their precise function remains unclear, neutrophils have been observed interacting with various immune cell populations, including SCS $CD169^+$ macrophages, DCs, T-cells, and B-cells [203,205-207]. In cancer, the presence of neutrophils within B-cell follicles in TDLNs has been associated with improved prognosis, and they have been suggested to contribute to TI B-cell activation [205]. However, further investigation is required to elucidate their precise contributions to tumour immunity in LNs.

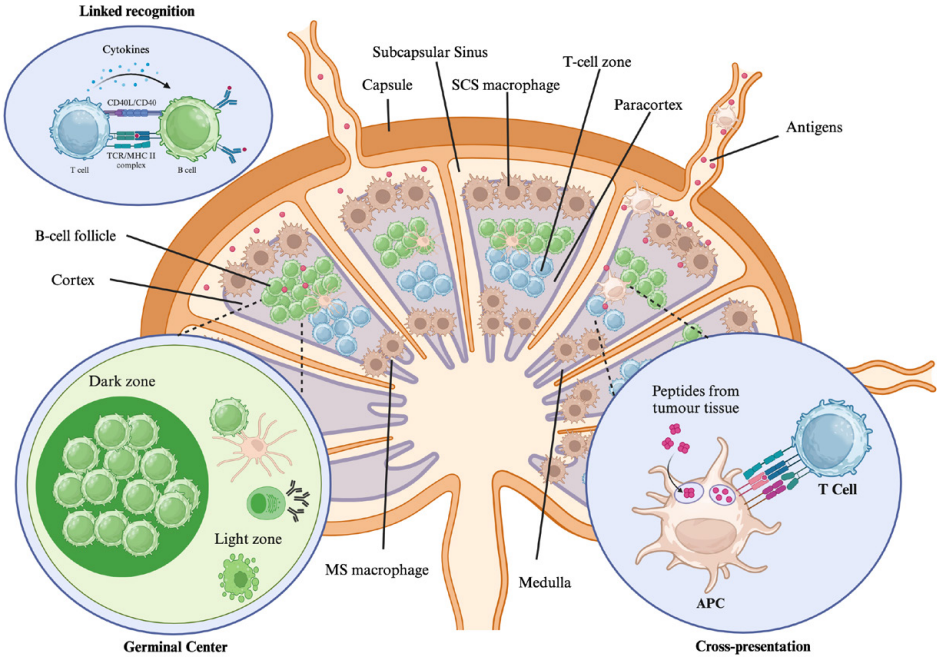

Figure 9. Structure of a lymph node

The LNs are secondary lymphoid organs that shape and activate the immune response against antigens. B-cell follicles in the cortex are presented with antigens by SCS macrophages or FDC or may acquire soluble antigens from the lymph. Once activated, the B-cells migrate to the T/B-cell border and become further activated by linked recognition with $CD4^+$ Th-cells. Once fully activated they enter GCs where they undergo affinity maturation before becoming plasma B-cells or memory B-cells. In contrast, T-cells are activated in the paracortex by DCs. Activated T-cells subsequently migrate to the tumour where they perform their effector function.

Emerging immunotherapeutic targets in breast cancer

Breast cancer can be classified into subtypes based on the molecular characteristics of the malignant cells. Among these, TNBC and HER2-enriched breast cancer are particularly suited for immunotherapy. These subtypes are often associated with a "hot" TME and are more aggressiveness compared to luminal A and luminal B breast cancers. Despite these distinctions, predictive biomarkers remain critical for assessing the likelihood of response to ICI. The most widely utilized biomarkers are PD-L1 expression and tumour mutational burden. As of 2022, more than 450 ICI therapies were in ongoing clinical trials for breast cancer. These trials predominantly investigate combinations of ICB with chemotherapy, with PD-1/PD-L1 blockade being the most studied approach, followed by CTLA-4 blockade [208]. Outcomes from these therapies can be categorised based on their use in metastatic versus early-stage breast cancer.

Metastatic breast cancer

Initially, clinical trials with ICI were conducted as a monotherapy in pre-treated metastatic TNBC patients. Response rates ranged from 5% to 20%, with PD-L1 blockade showing greater efficacy in tumours with low tumour mutational burdens[209]. However, ICI monotherapy failed to significantly improve overall survival (OS) compared to chemotherapy [91]. Consequently, subsequent clinical trials began to explore the combined effect of ICI with chemotherapy. In 2020, the combination of pembrolizumab, a PD-1 inhibitor, with paclitaxel, nab-paclitaxel, or gemcitabine plus carboplatin was shown to improve PFS in metastatic TNBC [92]. This combination is now FDA-approved for patients with PD-L1-positive TNBC tumours. For patients with metastatic HER2-positive breast cancer, combining anti-HER2 trastuzumab with pembrolizumab led to a 15% increase in response rates among patients with trastuzumab-resistant and PD-L1-positive tumours [210]. In contrast, metastatic luminal breast cancer patients have not demonstrated significant benefits from ICI therapies in initial clinical trials, likely due to the "colder" immune microenvironment (defined by absence of TILs and an anti-inflammatory signature)[68,211] in these subtypes.

Early breast cancer

Immunotherapy is also being evaluated in clinical trials for early-stage breast cancer. This setting could be more favourable for immunotherapy as the TME is less immunosuppressive and less influenced by prior treatments. Most clinical trials in early-stage breast cancer focus on neoadjuvant therapies rather than adjuvant therapies [208]. The majority of these trials are conducted in TNBC, where

neoadjuvant pembrolizumab combined with chemotherapy has been shown to improve EFS [93]. This combination therapy is now FDA-approved for the treatment of early-stage TNBC. For luminal and HER2-enriched breast cancers, data on ICI therapies are more limited. Combination therapies involving ICI and chemotherapy generally yield lower complete response rates in luminal subtypes [208,212], which aligns with their "colder" immune microenvironment based on low TIL presence.

Other immunotherapeutic strategies

In addition to ICI, other immunotherapeutic strategies, such as cancer vaccines and adoptive cell therapies (ACTs), are being investigated in clinical trials. Cancer vaccines aim to stimulate the immune system to recognise and attack cancer cells, rather than directly targeting specific receptors with monoclonal antibodies such as with ICI. The HER2 molecule, the first target for monoclonal antibody cancer therapy, has been a particular focus in cancer vaccine development. Currently, three HER2-targeting cancer vaccines are being studied [213]. These vaccines activate T-cells against distinct peptide domains of the HER2 protein. Early clinical results have not shown significant benefits in patients with HER2-enriched tumours but have demonstrated promises in patients with low general HER2 expression, regardless of oestrogen and progesterone expression [214].

ACT encompass several approaches, including TIL-based therapies, TCR gene therapies and Chimeric Antigen Receptor (CAR) T-cell therapies. These therapies involve isolating peripheral blood or tumour-resident T-cells, modifying and activating them *ex vivo*, before expanding them *in vitro*, and reintroducing them into the patient to target tumour cells [215]. Despite the potential of ACTs, early attempts have been inconclusive due to several challenges. These challenges are: i) the heterogeneous antigenic landscape of solid tumours, which complicates effective targeting; ii) the immunosuppressive nature of the TME which may hamper T-cell function; iii) limited infiltration of T-cells into the tumour nests in solid tumours. These challenges underscore the need for developing novel or modified immunotherapies that target the myeloid cell compartment and TME of breast cancer tumours.

Aims of the thesis

General Aim

The goal of tumour immunology is to elucidate the dynamics of the TME, with a particular focus on the interactions between diverse immune cell populations and tumour cells. A comprehensive understanding of these mechanisms would offer valuable insights into how immune cells contribute to either tumour suppression or progression. Such knowledge is pivotal for the development and refinement of immunotherapeutic strategies.

This thesis aims to investigate a specific aspect of tumour immunology: how specific immune cells of the myeloid immune compartment interact with the adaptive immunity in lymphoid structures of PTs and SLOs, and whether the local immune response in LNs is affected by LNMs.

Specific Aims

Paper I: To investigate the features and prognostic impact of $CD169^+$ macrophages in LNs (resident antigen-presenting macrophages) and their counterparts within breast TME and their relation to TLS.

Paper II: To delineate the phenotypic differences between LN-resident antigen-presenting $CD169^+$ macrophages and corresponding macrophages in breast tumour tissues. Additionally, this study seeks to explore the signalling pathways between $CD169^+$ macrophage populations and B-cells and T-cells, to elucidate their contributions to immune activation or suppression in breast cancer.

Paper III: To examine how LN breast cancer metastases influence LN architecture and immune signatures using spatial proteomics of paired UnLN and LNM. This study also aims to provide insights into the structural and molecular alterations in LNs cell populations upon metastasis by highlighting which proteins are up- or down-regulated during disease progression.

Paper IV: To elucidate the features of neutrophils in LNs and to investigate how their role in stimulating adaptive immune responses is modulated during tumour metastasis. This study aims to further identify neutrophil-driven mechanisms that influence the immune landscape in LNM and PTs. Lastly, this study aims to elucidate the prognostic impact of neutrophils in relation to B-cell and T-cells in PTs.

Main Experimental Methods

A combination of experimental methods, imaging analysis and bioinformatics approaches were utilized in this thesis. This chapter will highlight the key methodologies that contributed significantly to the main results in each paper. The strengths and limitation of each method are also discussed.

Patient cohorts and Study designs

Prospective and retrospective cohort designs

In this thesis, four different patient cohorts were investigated. A cohort refers to a group of individuals followed over a defined period of time, allowing researchers to predict disease outcomes and assess causal relationships. Cohort designs can be divided into prospective and retrospective cohorts, which strengths and weaknesses have been reviewed [216]. Three of the cohorts were retrospective studies, which analyse existing data to explore relationships between exposures and outcomes. Retrospective studies are advantageous due to their time efficiency, cost-effectiveness, and the typically larger sample sizes, which enhance statistical power. They are especially useful for generating hypotheses and identifying associations. However, retrospective studies are limited by potential biases in subject selection and data availability, which may not fully represent the general population. Furthermore, because these studies are observational, they cannot establish causality. In contrast, one cohort (SCAN-B) was part of a prospective study, which involves collecting new data over time by following participants and observing outcomes as they occur. The main advantage of prospective studies lies in their ability to explore specific research questions in detail and assess causality by linking exposure to outcomes. However, these studies are time-consuming, expensive, and often have smaller sample sizes, resulting in lower statistical power.

Spatial and high dimensional approaches

Spatial proteomics:

A pivotal technique in this thesis was spatial proteomics, which enables the detailed characterisation of the tissue architecture in healthy versus diseased states. Spatial proteomics combines proteomics data with IHC staining to profile specific cell populations within tissues while retaining their spatial context. In this thesis, the GeoMX digital spatial profiling (DSP) platform was used in Papers II, III, and IV. GeoMX employs two types of antibodies for tissue staining: morphological markers tagged with fluorophores to identify cells of interest and profiling antibodies tagged with UV-sensitive cleavable barcodes unique to each antibody to investigate the proteome. The morphological markers guide the selection of regions of interest (ROIs), which are then exposed to UV light to release the barcodes within the ROIs. The barcodes are then quantified to determine protein expression within the ROIs[217].

The main strength of spatial proteomics is that it enables high multiplex resolution of your samples while retaining the spatial context within the TME. In this thesis, this was necessary to be able to separate SCS macrophages from MS macrophages for instance. Proteomics has advantages over studying RNA transcripts, since it gives a clearer functional snap-shot analysis of possible mediators actually being expressed as proteins in real time. Furthermore, because it is a multiplex platform, simultaneous measurement of multiple proteins is possible, thus offering a comprehensive molecular profiling from regions of interest. This method is compatible with FFPE samples, which is a common way of preserving tissue in biobanks and the company also offers customizable panels for specific target of interest. However, there are some limitations with this method. Firstly, there is a limited proteome coverage as compared to transcriptomics analysis. The panel used assessed 47 different proteins, but with transcriptomics, hundreds of RNA transcripts can be investigated simultaneously depending on which kit you use. Another disadvantage is the cost of the method, meaning that large-scale cohort studies are more challenging to perform. There are also some resolution constraints, as single-cell protein expression is not applicable. Additionally, the method requires ROIs containing at least 20 cells, which may lead to contamination from adjacent cells.

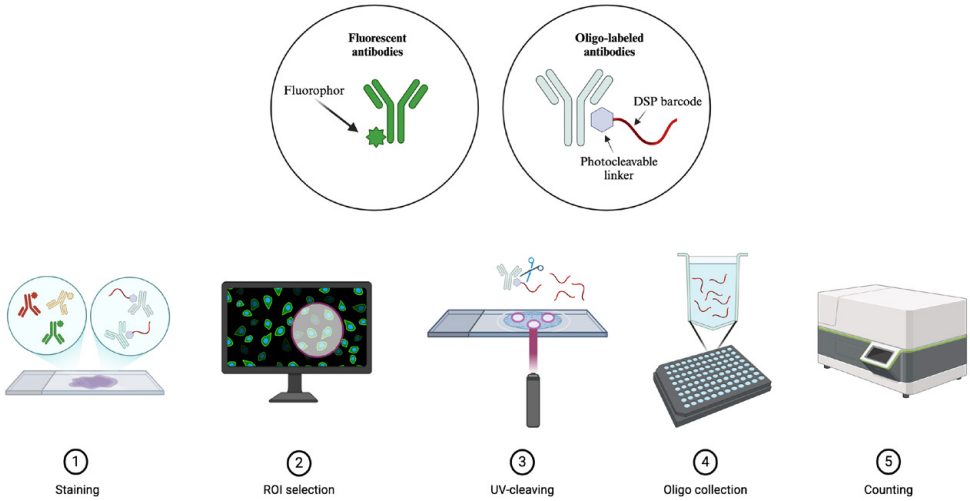

Figure 10. The GeoMx DSP platform
The GeoMx DSP platform can be described in five steps. First, the tissue sections are stained with morphological fluorescent markers and profiling markers tagged with a UV-cleavable barcode. The second step is to identify regions of interest (ROI) using your morphological markers. Thereafter, the tagged barcodes from the profiling markers are released by UV-light and collected into separates wells. Lastly, the bulk of barcodes from each ROI are counted using a nucleocounter.

Single-cell RNA-sequencing

Single-cell RNA sequencing (scRNA-seq) was another technique employed in this thesis. Unlike spatial proteomics, scRNA-seq provides higher resolution by characterising individual cells within a heterogeneous tissue. Tissues contain a complex heterogeneity of different cell types. The transcriptomic difference between neighbouring cells is often different and is best assessed with a single cell resolution. ScRNA-seq has revolutionised the field by providing vast databases which can be shared between researchers and used to answer different research questions. In this thesis, publicly available scRNA-seq datasets were utilized to explore macrophage and neutrophil phenotypes, as these cells exhibit high plasticity. The analysis workflow for scRNA-seq typically includes raw data processing, quality control, expression normalization, feature selection, cell population identification, and visualisation [218].

The strength with scRNA-seq is that it enables a broad applicability for discovery research. It enables detailed characterisation of transcriptomic heterogeneity between neighbouring cells, which is not possible with bulk RNA sequencing. Furthermore, publicly available datasets reduce costs and enable the exploration of diverse biological contexts. Lastly, it facilitates hypothesis generation and

identification of novel directions for experimental validation, as demonstrated in Paper IV. The main limitation with this method includes variability across datasets due to differences in sequencing platforms, protocols, batch effects, and normalization methods. There is also an inconsistency in cell type annotations across studies which affects data interpretation. Furthermore, the drawback when looking at single cell data is also that you lose the spatial context, and interpretation of cellular dynamics and/or interactions are harder to deduce. There are, however, new techniques that combine spatial transcriptomics with scRNA-seq interpretation.

Immunohistochemistry

Immunohistochemistry (IHC) was a pivotal method utilized in all the studies presented in this thesis. This technique is invaluable for visualizing the spatial localisation of cells of interest within tissue samples. Beyond its preclinical research applications, IHC is routinely employed in clinical practices and diagnostics. The method relies on the specificity of antibodies in a two-step procedure [219].

1. **Primary antibody binding**: The primary antibody binds to the target antigen, identifying the target cell.
2. **Secondary antibody binding**: The secondary antibody, conjugated with fluorophores or enzymes, binds to the primary antibody, enabling visualisation through immunofluorescence or enzyme-mediated colorimetric reactions.

The main strengths of IHC include its simplicity and affordability for investigating target localisation at the tissue or cellular levels. The enzyme-mediated colorimetric reactions are also persistent. Nonetheless, the technique has limitations, including variability in antibody specificity, potential loss of tissue information during processing, and its semi-quantitative nature, as it does not provide absolute quantification of the target abundance [220].

Tissue microarray - Triple IHC

A triple IHC staining was performed in all papers and was used to target several immune cell populations within the tissue sample of interest. Triple IHC was used on whole LN and tissue microarray (TMA) sections. The TMA consisted of tumour cores ranging from 0.6–1.0 mm and embedded into formalin-fixed paraffin-embedded (FFPE) blocks. The TMA sections were cut to a thickness of 4 µm and mounted onto slides. IHC was performed following a standardized protocol:

1. **Pre-treatment**: Sections underwent automated pre-treatment in the PT-Link system for antigen retrieval at pH 6.
2. **Staining**: Staining was conducted with two primary antibodies from different species at + 4 ºC with an overnight protocol.
3. **Detection**: Secondary antibodies (directed against the species from which the first antibodies were generated) tagged with horseradish peroxidase (HRP) respective alkaline phosphatase (AP) were added. HRP catalysed a substrate reaction to produce a DAB-brown-coloured signal while AP catalysed a substrate reaction to produce a red-coloured signal.

4. **Re-staining:** For subsequent stains, the HRP enzyme was inactivated through heat and low pH treatment, and non-specific binding sites were blocked before the next primary antibody application. New primary antibodies with a third species were added, followed by a new secondary antibody label with HRP. Emerald chromogen was used instead of DAB for the HPR enzyme to catalyse a blue-coloured signal.

FFPE samples were used in all staining protocols. The use of FFPE samples, rather than frozen sections, offered advantages such as prolonged preservation and enhanced stability. Additionally, enzyme-based detection methods provided durable signals that resist fading, allowing for sample re-analysis as compared to using fluorochromes based secondary antibodies.

Quantification of immune markers in tissue sections – QuPath

Given the extensive use of IHC in this thesis, the quantification of stained slides was thoroughly addressed. Two distinct approaches were employed to investigate immune cell infiltration in the tissue of interest:

1. **Manual Scoring**: Primarily used in paper I and II. Here, immune cell infiltration was assessed manually by authors, categorising infiltration as absent, low, moderate, or high. This method, while commonly used, is prone to inter-observer variability and human bias.

2. **Automated quantification with QuPath**: Primarily used in paper III and IV. Here, the QuPath software was used for image analysis to reduce human error and provide a detailed quantification of immune cell populations. For example, in paper III, QuPath quantified $CD169^+$ and $CD20^+$ cells in UnLN and LNM. Positive cell segments were classified using intensity thresholds for DAB (DAB OD max = 0.8), and the percentages of positive cells were calculated with the software to provide robust data on immune cell distribution within the tissue.

In vitro cell cultures

Cell cultures

The *in vitro* studies in this thesis were conducted in paper II, utilizing both primary cell cultures and continuous cancer cell lines. Primary immune cells were isolated directly from blood while continuous cancer cell lines were purchased. Primary immune cells were isolated from concentrated leucocytes, obtained from healthy blood donors. A Ficoll-Paque gradient was used to isolate peripheral blood mononuclear cells (PBMCs). The PBMC layer consists mostly of lymphocytes and monocytes [221]. These cells were further isolated for specific cultures of distinct immune cell populations. In this thesis, the main *in vitro* cultures were comprised of monocytes, B-cells and T-cells. All *in vitro* cultures were kept in humified atmosphere at + 37 ºC temperature and 5% CO_2.

Monocytes

Monocytes, accounting for 10–30% of PBMCs, were isolated using a monocyte isolation kit targeting $CD14^+/CD16^-$ cells. Isolation of monocytes from the PBMCs used magnetic anti-biotin microbeads, which binds biotin conjugated antibodies against T-cell, B-cells, NK-cell and DC markers, thus isolating monocytes through negative selection. Monocytes were cultured under serum-free conditions to reduce their spontaneous activation and were differentiated into specific macrophage subtypes. General condition for all monocyte/macrophages cultures comprised of either low/standard adherent plates, Opti-MEM media supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). Monocyte-derived macrophages were fully differentiated after 7 days and required the following conditions to stimulate specific macrophages polarization:

- M1 macrophages: Stimulated with CSF-2 day 0, 3 and 5 (10 ng/ml), LPS (100 ng/ml) day 5 and IFNγ (20 ng/ml) day 5.
- M2 macrophages: Stimulated with CSF-1 (10 ng/ml) day 0, 3 and 5 and IL-4 (20 ng/ml) day 5.
- $CD169^+$ macrophages: Stimulated with CSF-1 (10 ng/ml) day 0, 3 and 5, IL-4 (20 ng/ml) day 5 and IFNα/β (670 units/ml) day 5.

T-cells

T-cells represent the largest population of lymphocytes in the PBMC accounting for 70-85% of all cells. They are roughly divided in a 2:1 ratio between $CD4^+$ Th-cells and $CD8^+$ CTLs [222]. $CD4^+$ T-cells were mainly used and were isolated with negative selection to avoid contamination of microbeads in cell cultures. In certain co-cultures, these cells were activated with IL-2 and CD3/CD28 DynaBeads to provide them with co-stimulatory signals and induce their proliferation. General condition for all T-cell cultures comprised of low adherent plates, and growth in Opti-MEM media supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml).

B-cells

B-cells account for 2-10% of the cells from the PBMC layer [223]. B-cells come at different maturation stages and the B-cells compartment of the blood circulation are mature B-cells that express CD19 and CD20. The most abundant type of mature B-cells are naïve B-cells which express IgM and IgD and represent 70% of the PBMC B-cells [224]. B-cells were isolated using negative selection by labelling other cells in the PBMC layer. The unlabelled B-cells went through the magnetic column unhindered and were used for plasma B-cell or B_{regs} cultures. General conditions for B-cell cultures comprised of low adherent plates, and growth in Opti-MEM supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml). Specific culture conditions:

- Plasma B-cells: B-cells stimulated with Anti-IgM (4 h) and cultured with CpG (2.5 ug/ml), IL-21(50 ng/ml) and CD40L (1ug/ml) for 6 days.

Additional Material & Methods

Real-time qPCR:

Real-time quantitative polymerase chain reaction (RT-qPCR) was extensively used in paper II, primarily employing the Maxima SYBR Green/Rox kit. Briefly, SYBR Green binds to double-stranded DNA, emitting fluorescence proportional to the DNA quantity. The molecule SYBR green is a dsDNA-binding intercalating dye that binds non-specifically to dsDNA. SYBR emits a green fluorescence which increases as amplification proceeds. Thus, when the amount of DNA product increases, the number of SYBR green molecules incorporated into DNA also increases [225]. The advantage of this method is that it reduces the experimental cost because there is no need for the incorporation of a fluorescent reporter system in the primers design or the use of a fluorescent probe to a specific target sequence. However, the limitation of this method is that SYBR green can bind to non-DNA products such as primer dimers and can lead to an overestimation of the product. This must be accounted for by looking at melting curves to establish if there are significant levels of non-specific products.

The general protocol for RT-qPCR was the following:

- RNA extraction and purification.
- Reverse transcription to synthesize cDNA.
- Amplification with specific primers and SYBR Green detection.

Amplification results from the RT-qPCR were then illustrated with cycle threshold values, these values represent which amplification cycle where the produced cDNA components reached a detectable fluorescent value in a log scale. The earlier cycle threshold, the more expressed the transcripts was in the sample. The relative gene expression was then calculated by normalizing to housekeeping genes (e.g., GAPDH or SDHA) and analysed using the $2^{-\Delta\Delta Ct2}$ method [226].

Flow cytometry

Flow cytometry enables multiparametric analysis of immune cell populations and was also thoroughly used in paper II. The advantage with this method is that several parameters in the same sample can be analysed. Furthermore, information from hundreds of thousands of cells can be collected in a relatively short amount of time. Flow cytometers use a fluidic system, that aligns and moves cells through lasers one

by one. When they pass through the first laser, the light will scatter in different directions depending on the cells size and their cytoplasmic complexity. The forward scatter and size scatter are thus the first parameters investigated with flow cytometers. Flow cytometers also have a series of mirror that directs specific wavelengths emitted by fluorescent labelled cells to optical filters. These filters let specific wavelengths through and towards detectors called photomultiplier tubes (PMT). The light detected by PMTs are converted into a voltage pulse. Thus, when cells are in the centre of the lasers path, the maximum fluorescence emitted results in a peak in voltage pulse. When the cells leave the laser, this finishes the voltage pulse. Consequently, the more of your marker is present, the higher voltage signal is produced [227]. This enables categorisation of cells depending on cell surface receptors expression visualized by voltage pulses.

The advantage of flow cytometry is the ability to measure multiple cellular properties with the use of fluorochrome-conjugated antibodies at the single cell level. The disadvantage is that it is a complex method where technical issues can easily arise during the preparation of cells, and for every antibody used, calibration is necessary. Furthermore, different cell types have different autofluorescence properties, which can affect the interpretation of the results.

Cytometric Bead Array (CBA)

The Cytometric Bead Array (CBA) assay is a technique for capturing and quantifying soluble proteins such as chemokines and cytokines in liquid samples. Using capture beads of defined size and fluorescence, detection of the amount of chemokines and cytokines in cultures supernatants was assessed via flow cytometry. Each capture bead in the kit exhibits a distinct fluorescence and is conjugated to a specific antibody that recognises one specific analyte. After incubation, a detection reagent consisting of a mixture of phycoerythrin (PE)-conjugated antibodies was added, creating a sandwich complex consisting of capture bead/analyte/detection antibodies. These were used to reveal the fluorescent signal proportional to the amount of bound analyte, allowing identification based on the fluorescence signatures from both the bead and the detecting antibody. Quantification is then achieved by comparing fluorescence intensities to a standard curve generated from serially diluted standards. In this thesis, the specific analytes investigated were the chemokines CXCL8, CCL5, CXCL9, CCL2, CXCL10 and the cytokines CXCL8, IL-1β, IL-6, IL-10, TNF-α and IL-12. The main advantage with CBA is its multiplex function by investigating several analytes at once. However, CBA assays have some limitations such as cross-reactivity between antibodies, and a limited dynamic range compared to ELISA, especially for low abundance proteins.

Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying soluble substances such as peptides, proteins, antibodies and hormones. There are three ELISA formats, direct, indirect and sandwich ELISA. In this thesis, the sandwich ELISA method was used. Plates were coated with antibodies against the target protein of interest prior to adding supernatant samples. Once the protein binds to the antibody, detection antibodies are added, meaning that protein of interest is sandwiched between two primary antibodies that bind to different epitopes.

After binding, the sandwich ELISA uses the same principles as with the indirect ELISA by using labelled-secondary antibodies. However, the sandwich ELISA is more specific and sensitive compared to both the direct and indirect ELISA. This is because the method uses two primary antibodies. Nonetheless, a limitation with this method is the risk of cross-reaction with the secondary antibodies, leading to non-specific signalling. While several detection techniques such as chromogens, fluorescence and chemiluminescence are available, we used chromogens and the enzyme HRP. This detection method is direct and has a high reproducibility between experiments. Detection of the analyte is based on introducing the enzyme substrate to HRP which will convert the substrate to emit a specific colour. The amount of colour emitted reflects on the amount of protein captured. This reaction can be stopped by inactivating the enzyme. In this thesis, the sandwich ELISA method was implemented for evaluation of IL-1α, IL-1β, IL-6, IL-8, IL-15 and IgG.

AI implementation

The AI platform ChatGPT was utilized in this thesis to refine the scientific language and enhance the structural formatting of the introduction. Drafted sections of the thesis were copied into ChatGPT with the prompt: “Help me improve the scientific language of the following text.” The AI-generated text was subsequently reviewed and thoroughly proofread to ensure that the accuracy and integrity of the information remained unchanged. ChatGPT was not employed for any other purposes, such as generating original content for the introduction or references.

Ethical considerations

In this thesis, ethical approval was obtained by the Swedish Ethical Review Authority for all studies, spanning from Papers I to IV. A fundamental ethical principle in research involving human samples is obtaining informed consent from patients while ensuring data privacy and confidentiality. All studies were conducted in accordance with the Declaration of Helsinki. This declaration outlines ethical principles that safeguard the rights, safety, and well-being of participants, ensuring that research is conducted with integrity and respect for human dignity. This thesis involved ethical consideration implemented for four different patient cohorts, and for the use of primary human cells and continuous human cell lines in Paper II.

Cohort 1

This cohort was used in paper I and represents a retrospective study with tumour tissue from PTs and LNM of patients with advanced breast cancer. All patients were part of the randomized phase III TEX trial conducted between 2002 and 2007 [228]. Details of the clinical trial are available at ClinicalTrials.gov (NCT01433614). The trial included 304 women with advanced or inoperable metastatic breast cancer who received combination chemotherapy as first-line of treatment: epirubicin and paclitaxel alone or combined with capecitabine. Inclusion criteria required a life expectancy of at least three months and excluded patients with brain metastases or previous chemotherapy cycles. TMAs were generated with cores from PT FFPE blocks and if possible, paired LNM. From the original participants, 231 PT or LNM samples were possible to analyse. The other cases were excluded based on missing clinicopathological information or low-quality TMA cores. Ethical approvals were obtained from the regional committee in Sweden at Stockholm, (Dnr KI 02-206, KI 02-205), and Lund (Dnr 2009/658), Sweden.

Cohort 2

This cohort was also used in paper I. The cohort represented a large prospective and population-based cohort comprising 8164 breast cancer patients at the time of the analysis. These patients were enrolled by the multicentre, Sweden Cancerome Analysis Network-Breast (SCAN-B) which is managed by the southern healthcare region of Sweden [229] (ClinicalTrials.gov identifier NCT02306096). Patients enrolled in the cohort provided blood samples at the time of enrolment and tumour samples from the PT at the time of surgery. After routine assessment by a

pathologist, remaining tumour tissue was used for RNA sequencing to generate transcriptomic profiles for each patient. Ethical considerations and informed consent protocols were rigorously followed for all participants. Ethical permit for the cohort was approved by the regional ethical committee in Lund (Dnr 2007/155, 2009/658, 2009/659, 2014/8), the county governmental biobank centre, and the Swedish Data Inspection group (Dnr 364-2010).

Cohort 3

This retrospective cohort was used in paper III and paper IV and was not part of any clinical trial. Patient material included in the cohort provided whole sections of paired tissue biopsies from uninvolved lymph nodes (UnLN) and LNM. Historical FFPE material from five patients with invasive breast cancer and LNM were included in the cohort. Breast cancer subtypes were of luminal nature with PTs positive for ER and PR, and one patient also positive for HER2. Further clinicopathological information was not available. The ethical permit was obtained by the Swedish ethical review authority (Dnr 2021-04869). In this retrospective cohort, written consent was not required.

Cohort 4

In paper IV, another retrospective breast cancer cohort was used. The cohort provided TMAs with cores from PTs of 144 breast cancer patients. These patients were diagnosed with invasive breast cancer at the Malmö hospital between 2001 and 2002. Detailed descriptions of this cohort have previously been published [139]. Briefly, off the 144 patient samples, 109 tumours were of luminal A subtype, 8 were of luminal B subtype, 5 were of HER2-enriched subtype, and 15 were of TNBC subtype, 7 patients lacked information on their subtype. Ethical approval was obtained from the Ethics Committee at Lund University (Dnr 447-07). In this cohort, written consent was not required, but patients were given the option to opt out.

Primary human cells

Isolation of primary human immune cells was approved by the regional ethical committee at Lund University (Dnr 2021/04792). Concentrated leucocytes were obtained from healthy blood donors. The primary human cells were then isolated from the PBMC layer after Ficoll-Paque separation.

Continuous human cell lines

TNBC cell lines MDA-MB-231 (ATCC) and SUM-159 (BioIVT) were used. Authentication of the cell lines and transparency of our cell cultures conditions were published to facilitate reproducibility and data integrity. After working with them, these cell lines were safely disposed of, in biohazard containers to avoid environmental contamination or spreading of genetically modified cell lines.

Results and discussion

Paper I

CD169$^+$ Macrophages in Primary Breast Tumors Associate with Tertiary Lymphoid Structures, T_{regs} and a Worse Prognosis for Patients with Advanced Breast Cancer

Introduction and Results

Immunotherapy has revolutionized cancer treatment, particularly in malignant melanoma. However, in breast cancer, response rates to immune checkpoint therapies remains relatively low with complete clinical response only observed in combination with chemotherapy [230]. This underscores the need to develop novel immunotherapy modalities with improved efficacy. This also necessitates a deeper investigation into additional immune cell populations within the TME. In this study, we focused on a particular myeloid immune cell population with capacity to initiate specific immune responses: the SCS CD169$^+$ macrophages. SCS CD169$^+$ macrophages are tissue-resident macrophages localised between the cortex and SCS of LNs. The presence of CD169$^+$ macrophages in LNM has been associated with favourable prognosis in several cancers, including breast cancer[148,150-152,159]. These macrophages play a crucial role in capturing antigens from the lymphatic fluid and presenting them to underlying B-cell follicles or FDCs, thereby facilitating antigen-specific immune activation [231]. Interestingly, while the presence of CD169$^+$ macrophages in LNs correlates with improved prognosis, their infiltration into PTs has been associated with poor clinical outcomes [159]. Given their localisation in SLOs near B-cell follicles, we hypothesised that CD169$^+$ TAMs would accumulate near TLS in PTs. Furthermore, we hypothesised that if CD169$^+$ TAMs were co-localised with TLS, they might confer a favourable prognostic impact, as TLS have been shown to generate tumour-specific immune responses and are frequently associated with improved survival in various cancers [121].

CD169$^+$ TAMs associate with tertiary lymphoid-like structure (TLLS) and regulatory immune cells.

Our hypotheses were investigated by analysing clinicopathological features in a retrospective cohort of advanced breast cancer patients. Paired biopsies from PT and

LNM were subjected to triple IHC staining, allowing us to assess the presence of $CD169^+$ macrophages, T-cells and B-cells. Our findings revealed a strong association between $CD169^+$ TAMs and tertiary lymphoid-like structures (TLLS), characterised by lymphoid aggregates of T- and B-cells. This correlation was observed in both PTs (OR = 3.77; $P = 0.004$) and LNM (OR = 4.76; $P = 0.0001$). Since T_{regs} had previously been assessed in this cohort, we further examined their relationship with $CD169^+$ TAMs and TLLS. Our results demonstrated a positive correlation between $CD169^+$ TAMs and T_{regs} in PTs (OR = 2.06; $P = 0.057$), which was even more pronounced in LNM (OR = 2.87; $P = 0.046$).

The prognostic impact of $CD169^+$ macrophages with TLLS is beneficial in LNM but harmful in PT and is dependent on T_{reg} co-infiltration.

The prognostic significance of $CD169^+$ macrophage and TLLS co-infiltration was assessed using Kaplan-Meier survival analysis for two clinical outcomes: breast cancer-specific survival (BCSS) and recurrence-free interval (RFI). In PTs, $CD169^+$/TLLS infiltration was associated with a borderline negative prognostic impact, indicating a trend toward earlier mortality ($P = 0.059$) and earlier recurrence ($P = 0.123$). In contrast, in LNM, $CD169^+$/TLLS infiltration was significantly correlated with improved survival outcomes, showing a delayed mortality ($P = 0.016$) and a trend toward later recurrence ($P = 0.169$). In the same patient cohort, regulatory T_{regs} had previously been identified as independent prognostic markers in PTs but not in LNMs. Given their observed association with $CD169^+$ macrophages and TLLS, we further explored the potential confounding effect of T_{regs} on the prognostic impact of $CD169^+$/TLLS infiltration. Kaplan-Meier curves stratified by $FoxP3^+$ T_{reg} status in PTs revealed that co-infiltration of $CD169^+$ macrophages and TLLS was absent in tumours lacking T_{regs}. We then examined the prognostic role of $CD169^+$ TAMs based on T_{reg} presence. In T_{reg} negative tumours, $CD169^+$ TAM infiltration was associated with significantly worse prognosis, including an earlier recurrence ($P = 0.001$) and a trend toward earlier mortality ($P = 0.055$). Notably, the prognostic impact of $CD169^+$ macrophages was diminished in T_{reg} positive tumours. Multivariate Cox regression analysis confirmed that $CD169^+$ macrophage and TLLS co-infiltration was an independent prognostic factor for BCSS (HR = 2.88, $P = 0.007$) and RFI (HR = 2.15, $P = 0.035$).

$CD169^+$ TAMs associate with mature TLS signatures, T_{regs} and B_{regs} signatures.

A key limitation of IHC is its inability to determine the functional state of the identified structures. To address this, we analysed a prospective cohort with bulk mRNA sequencing of PT samples to investigate the gene expression correlations between $CD169^+$ macrophages, mature/active TLS, and immunoregulatory T_{regs} and B_{regs}. Our analysis revealed two distinct patient clusters in which $CD169^+$ TAMs in PTs were strongly associated with active TLS, as well as B_{reg} and T_{reg} gene signatures. These findings further validate our hypothesis that $CD169^+$ TAMs in

PTs are closely linked to mature TLS and contribute to an immunosuppressive tumour microenvironment characterised by regulatory lymphocyte infiltration.

Conclusion and Limitations

In Paper I, we demonstrate that $CD169^+$ TAMs in PTs are closely associated with TLS, similar to the SCS $CD169^+$ macrophages found near B-cell follicles in SLOs, but are instead associated with a worse prognosis. Furthermore, we establish that $CD169^+$ TAMs in PTs correlate with the presence of T_{regs} and B_{regs}, findings supported by both IHC and bulk RNA sequencing data. Our results suggest that TLS facilitate the recruitment and polarization of $CD169^+$ TAM, which in turn contribute to an immunosuppressive TME by promoting T_{reg} and B_{reg} infiltration. This immunosuppressive environment likely explains the adverse prognostic impact of $CD169^+$ macrophages in PTs. Notably, their prognostic role in LNMs was the opposite, indicating a context-dependent function.

Some limitations should be considered:

- Patient cohort composition: The IHC cohort primarily consisted of patients with luminal A and luminal B breast cancer subtypes. This imbalance in subtype distribution may introduce bias in the prognostic effects observed. Given that TNBC and HER2-enriched tumours are considered more immunogenic, investigating the role of $CD169^+$ macrophages and TLLS in these subtypes could provide a more comprehensive understanding of their prognostic significance and whether molecular subtype-specific factors influence macrophage accumulation or function.

- TLS characterisation: Defining TLS and determining their maturation state require multiple markers. In this study, we identified TLLS as lymphoid aggregates based on $CD20^+$ B-cells and $CD3^+$ T-cells. However, additional markers, such as FDC, HEVs, and proliferation markers, should be incorporated to confirm TLS identity and maturation status more robustly.

- Distinguishing TLS from B-cell follicles in LNMs: A major challenge in LNM analysis is differentiating TLS from B-cell follicles, as these structures are morphologically similar. The only reliable method to distinguish them is by assessing the surrounding cellular context. TLS are located within or in direct contact with metastatic tumour cells, whereas B-cell follicles are typically found within organised lymphoid tissue in the cortex. This distinction remains a limitation and warrants further investigation.

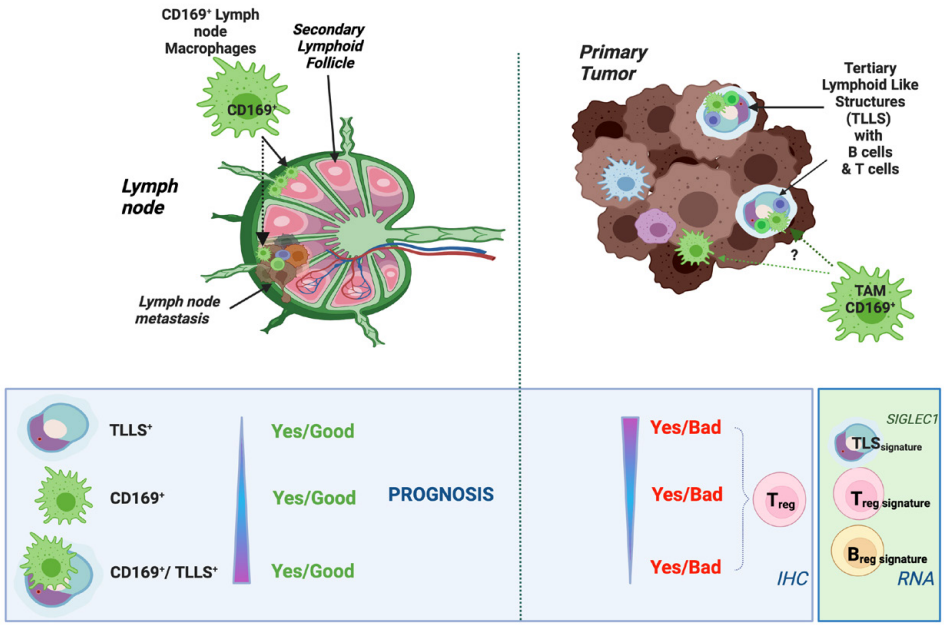

Figure 11. Graphical abstract Paper I

Graphical abstract summarising the results and conclusions from paper I [232]. Using IHC, $CD169^+$ macrophages were shown to correlate with TLLS and T_{regs} in PTs and LNMs. Their prognosis was harmful in PTs but beneficial in LNMs. Using bulk mRNA sequencing data, the gene for CD169 (*SIGLEC1)* was also shown to correlate with mature TLS, B_{reg} and T_{reg} gene signatures in PTs.

Paper II

Breast cancer associated CD169$^+$ macrophages possess broad immunosuppressive functions but enhances antibody secretion by activated B cells.

Introduction and Results

Macrophages are generally associated with poor prognosis in cancer patients [138], with a notable exception: the SCS CD169$^+$ macrophages in LNMs [159,233]. The precise role of these macrophages remains to be fully elucidated. In Paper I, we demonstrated that CD169$^+$ macrophages exert distinct functions in tumour progression depending on their localisation, either within LNM or the PT [232]. In both settings, CD169$^+$ macrophages were associated with regulatory T_{regs} and TLS, suggesting a close relationship with adaptive immune cells. These findings prompted further investigation into the role of CD169$^+$ TAMs within the TME, whether they are derived from tissue-resident macrophages or bone marrow-derived monocytes, their phenotypic characteristics, and their functional interactions with other infiltrating immune cells.

CD169$^+$ TAM in breast cancer originates from bone marrow derived monocytes.

To determine the origin of CD169$^+$ TAMs in humans, we utilized a xenograft mouse model. Immunodeficient NSG mice were engrafted with TNBC cell lines (MDA-MB-231 or SUM-159) alone or in combination with primary human monocytes. After 21 days, tumours were harvested and preserved in FFPE blocks. IHC staining for CD169 revealed that the engrafted human monocytes differentiated into macrophages in the SUM-159 tumour model, indicating that CD169$^+$ TAMs can be monocyte-derived. However, in the MDA-MB-231 model, CD169$^+$ TAM differentiation did not occur, suggesting that their development is dependent on TME-specific factors. To identify factors driving CD169$^+$ TAM differentiation, we examined inflammatory and tumour-derived mediators. Given that type I IFNs have been previously implicated in CD169 upregulation on circulating monocytes and are produced by SCS CD169$^+$ macrophages, we hypothesised that type I IFNs could promote CD169$^+$ TAM differentiation. Indeed, stimulation of M2 macrophages *in vitro* with type I IFNs led to upregulation of CD169, as confirmed by flow cytometry. Additionally, polyinosinic-polycytidylic acid (Poly I:C), a TLR3 agonist that induces type I IFN production, similarly enhanced CD169 expression. Further analysis of TNBC cell lines revealed that SUM-159 expressed higher levels of type I IFN mRNA compared to MDA-MB-231, potentially explaining the differential induction of CD169$^+$ TAMs in the xenograft models.

CD169$^+$ TAM have a unique phenotype which resemble the phenotype of tissue resident CD169$^+$ macrophages.

To characterise the phenotypic similarities between LN resident CD169$^+$ macrophages and CD169$^+$ TAMs, we performed spatial proteomics analysis and analysed publicly available scRNA-seq data. In LNs, CD169$^+$ macrophages are spatially clustered, allowing for spatial proteomics analysis. In contrast, within the TME of PTs, CD169$^+$ TAMs are more diffusely distributed, requiring scRNA-seq-based analysis. Unexpectedly, both LN CD169$^+$ macrophages and CD169$^+$ TAMs shared expression of multiple macrophage markers, including CD68, CD163, and HLA-DR. They also exhibited immunostimulatory features, expressing STING, CD80, 4-1BB, and OX40L, as well as immunoregulatory markers such as PD-L1, VISTA, IDO1, LAG-3, TIM-3, and Arg1. To further validate these findings, we assessed the expression of surface markers in *in vitro* differentiated CD169$^+$ macrophages using flow cytometry and RT-qPCR. The *in vitro* generated CD169$^+$ macrophages exhibited a similar surface marker profile to CD169$^+$ TAMs, including elevated expression of CD163, PD-L1, STING, VISTA, and OX40L, in contrast to M2-polarized macrophages. These results indicate that CD169$^+$ TAMs closely resemble LN resident CD169$^+$ macrophages and that monocytes, when exposed to an M2-polarizing TME, can differentiate into CD169$^+$ TAMs with a molecular signature similar to their *in vivo* counterparts.

CD169$^+$ TAM mimics M2 macrophages in relation to T-cells but enhance IgG and IL-6 production from activated B-cells.

To investigate the functional properties of CD169$^+$ macrophages, we analysed their cytokine and chemokine secretion profiles and their interactions with adaptive immune cells. Using V-PLEX protein assays, ELISA, and RT-qPCR, we determined that CD169$^+$ macrophages predominantly secrete the cytokines IL-6 and IL-15, while their chemokine secretome was CXCL10, CCL2, and CCL17. To assess their impact on T- and B-cell function, autologous and mixed lymphocyte reaction (MLR) assays were performed. These assays demonstrated that CD169$^+$ macrophages did not enhance T- or B-cell proliferation. However, suppression assays revealed that CD169$^+$ macrophages inhibited T-cell proliferation, similar to M2 macrophages. This immunosuppressive effect was likely mediated by PGE2 and IL-10, both of which were upregulated in CD169$^+$ macrophages at the mRNA level, as well as by ROS, as ROS inhibition reversed the suppressive effects in MLR assays. Given that CD169$^+$ macrophages are associated with TLS and secrete IL-6, we further examined their influence on activated B-cells. Co-culture experiments revealed that CD169$^+$ macrophages significantly increased IL-6 and IgG production from activated B-cells, potentially explaining their co-localisation with TLS in PTs.

Conclusion and Limitations

In Paper II, we investigated the role of CD169$^+$ TAMs in breast cancer and demonstrated that these macrophages originate from monocyte-derived precursors under the influence of a specific TME, particularly type I IFNs. We showed that CD169$^+$ TAMs share phenotypic characteristics with LN resident CD169$^+$ macrophages and that *in vitro* generated CD169$^+$ macrophages exhibit a similar phenotypic profile. Notably, CD169$^+$ macrophages were found to exert immunosuppressive effects on T-cells via ROS, PGE2, and IL-10, while simultaneously enhancing IgG and IL-6 production in B-cells, supporting their association with TLS in PTs.

Some limitations should be considered:

- Comparative analysis differences: The methodologies used to analyse LN resident CD169$^+$ macrophages and CD169$^+$ TAMs is a limitation. In spatial proteomics (GeoMx) analysis, protein expression from LN resident CD169$^+$ macrophages were normalized to total protein expression of CD45$^+$ immune cells. These were primarily B-cells present in the cortex of LNs. B-cells also express HLA-DR and CD40 and may lead to the underrepresentation of these markers by LN CD169$^+$ macrophages. Additionally, scRNA-seq data for CD169$^+$ TAMs may not fully correspond to protein expression due to post-transcriptional regulation and mRNA degradation.

- Limitations of *in vitro* models: Macrophages in the TME are influenced by a complex array of signals that vary across tumour contexts. *In vitro* systems cannot fully replicate these conditions, thus limiting their ability to capture the complete functional repertoire of CD169$^+$ macrophages.

- Flow cytometry limitations: flow cytometry analysis requires cell detachment. This method is particularly difficult on macrophages which tend to adhere strongly to culture plates. The cell detachment procedure can result in cell death and loss of certain macrophage subpopulations. Consequently, some phenotypic data may be lost during sample processing.

Despite these limitations, our findings provide novel insights into the role of CD169$^+$ TAMs in breast cancer and their interactions within the TME. Future studies should explore their prognostic and therapeutic potential across different breast cancer subtypes.

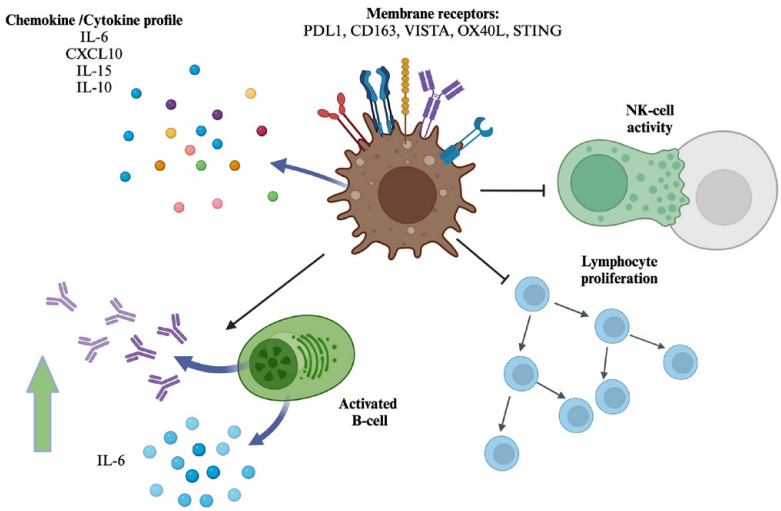

Figure 12. Graphical abstract Paper II
Graphical abstract summarising the results and conclusion from paper II. CD169^{+} macrophages cultured *in vitro* show a distinct phenotype associated with immunostimulatory and immunoinhibitory markers. They inhibit T-cell proliferation but on the other hand, they improve IgG and IL-6 secretion of activated B-cells.

Paper III

Altered immune signatures in breast cancer lymph nodes with metastases revealed by spatial proteome analyses.

Introduction and Results

SLNs/TDLNs are often the first site of metastasis in breast cancer. Patients with LNM generally have a poorer prognosis [181]. It remains unclear whether this is due to disseminated disease or alterations in the adaptive immune response. LNs contain two populations of $CD169^+$ macrophages: SCS macrophages and MS macrophages. Both are strategically positioned to interact with lymphatic flow, facilitating antigen capture and presentation [155]. Given the prognostic relevance of SCS $CD169^+$ macrophages in breast cancer, it is essential to elucidate their precise role in local anti-tumour immune responses. In Papers I and II, we demonstrated that these macrophages are associated with B-cell activation and enhanced immune responses. Based on these findings, we hypothesised that the proteome of SCS $CD169^+$ and MS $CD169^+$ macrophages is altered following the establishment of metastases in LNs. To investigate this, we employed spatial proteomics to analyse distinct cortical regions composed of macrophages, T-cells, or B-cells in paired LNs with (LNM) or without (UnLN) metastases from breast cancer patients. Our aim was to gain a deeper understanding of the immune mechanisms occurring in key immune cell populations during breast cancer progression.

SCS $CD169^+$ macrophages are reduced in UnLN compared to LNM.

LN sections from each patient were stained and analysed using IHC and GeoMx DSP analysis. IHC staining for CD169 distinguished SCS and MS subsets from cortical regions and medulla of LNs, while CD20 staining outlined B-cell follicles in the cortex. Using QuPath image analysis, the number of $CD169^+$ macrophages was quantified. Our results revealed a significant reduction in the overall number of $CD169^+$ macrophages in LNM compared to UnLN. Notably, this decrease was primarily observed in SCS macrophages, while MS macrophages remained unchanged in number. To determine whether the remaining SCS $CD169^+$ macrophages underwent proteomic alterations, we analysed their protein expression profiles. Two proteins, Bcl-xL and FAP-alpha, were differentially expressed in LNM. Given the role of Bcl-xL in apoptosis regulation, its downregulation suggests that SCS $CD169^+$ macrophages are depleted in LNM through apoptotic cell death.

MS $CD169^+$ macrophages exhibit an altered proteome in LNM compared to UnLN.

We further characterised the proteomic changes in MS $CD169^+$ macrophages. Five proteins showed differential expression in LNM compared to UnLN. Notably, Granzyme A and Arginase 1 were upregulated in LNM. These findings suggest that

MS CD169$^+$ macrophages are actively interacting with T-cells within the LN. Upon metastasis, MS macrophages appear to acquire an immunosuppressive phenotype, characterised by increased Arg1 expression, a potent inhibitor of T-cell responses. Interestingly, although Granzyme A is not produced by macrophages, it can be internalized and subsequently contributing to pro-inflammatory signalling and extracellular matrix degradation. Consistent with this, fibronectin, a key extracellular matrix component, was reduced in LNM.

Cortical LN regions exhibit distinct immune cell populations with altered proteome in LNM.

Spatial proteomic analysis of CD45$^+$ cortical regions revealed three distinct immune cell populations:

1. **B-cell follicles with active GCs**: Characterised by CD20, Ki-67, and Bcl-6 expression.
2. **B-cell follicles without active GCs**: Characterised by CD20 and CD40 but lacking Ki-67 and Bcl-6.
3. **Interfollicular T-cell-rich regions (IFR T-cells)**: Characterised by CD3, CD4, and CD8 expression and the absence of CD20 and CD40.

In B-cell follicles with active GCs, several proteins were upregulated in LNM, indicating an active B-cell response. This was evidenced by increased expression of Bcl-6 and ICOS, suggesting an attempt to mount an immune response even in the presence of metastatic cells. However, proteins associated with GC contraction, including PD-L1, FoxP3, and CD25, were also upregulated, suggesting premature GC shutdown and possibly impaired anti-tumour immunity. In contrast, the IFR T-cell-rich regions exhibited a distinct proteomic shift in LNM, with widespread downregulation of multiple proteins. Several T-cell activation markers, including Ki-67, were decreased, indicating reduced T-cell proliferation and activation in the presence of metastases. Given that IFR T-cells interact with SCS CD169$^+$ macrophages, their diminished activation is likely a consequence of the loss of SCS CD169$^+$ macrophages in LNM and the emergence of an immunosuppressive environment driven by MS CD169$^+$ macrophages.

Conclusion and Limitations

In Paper III, we demonstrate that LNs harbouring breast cancer metastases exhibit distinct proteomic alterations compared to uninvolved LNs. These changes affect multiple immune cell populations:

- SCS $CD169^+$ macrophages undergo apoptosis, likely driven by Bcl-xL downregulation, leading to their depletion in LNM.
- MS $CD169^+$ macrophages do not decline in number but instead acquire an immunosuppressive phenotype, characterised by increased Arg1 expression.
- While B-cell numbers ($CD20^+$ cells) remain unchanged, B-cell follicles with active GCs exhibited a premature contraction signature, as indicated by increased expression of immunoregulatory proteins such as PD-L1, FoxP3, and CD25.
- IFR T-cells show reduced expression of activation and proliferation markers, likely due to both the loss of SCS $CD169^+$ macrophages and the immunosuppressive milieu induced by metastases.

Some limitations should be considered:

- Spatial proteomics: One primary limitation of spatial proteomics is its resolution; each ROI contained multiple cells, leading to potential contamination from neighbouring cell populations. This was particularly evident in MS macrophage regions, where T-cell markers such as ICOS and CTLA-4 were detected in UnLN. Another limitation is the inability to distinctly separate lymphocyte subsets within the cortex due to the limited number of morphological markers available in the GeoMx platform (restricted to four markers). In this study, CD169 was used to identify macrophages, CD45 and follicle structures to define cortical lymphocytes, Pan-CK to identify metastatic cells, and DAPI for nuclear staining. CD45 was the only marker used for defining cortical regions. This constraint led to an uneven representation between B-cell follicle and IFR T-cell-rich regions and further prevented direct comparison of B-cell follicles lacking GCs between UnLN and LNM.
- Cohort size: The study cohort was limited to five patients. Small sample sizes increase the risk of selection bias and reduce statistical power, making it more challenging to detect significant differences and increasing the likelihood of Type II errors. Consequently, findings from this cohort may not be generalizable to larger populations.

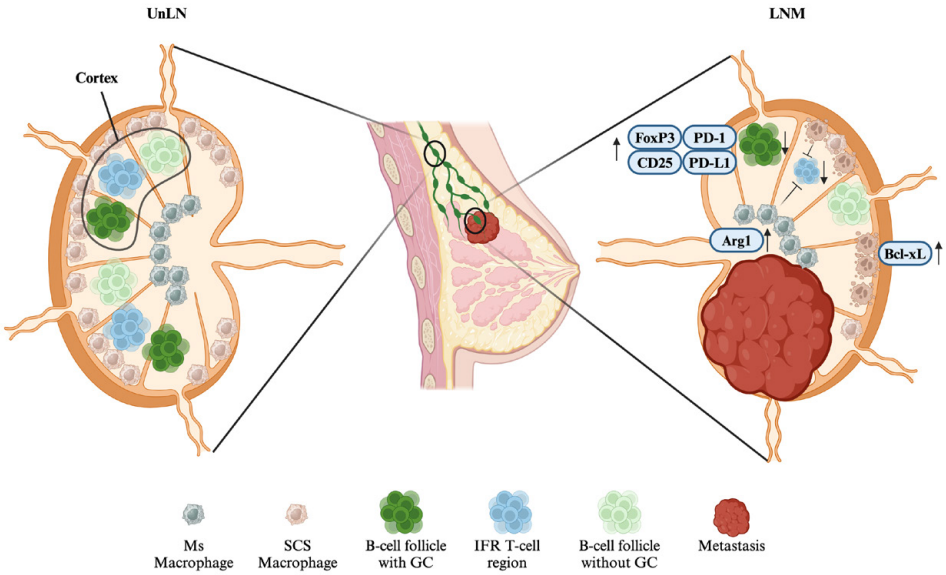

Figure 13. Graphical abstract Paper III

Graphical abstract summarising the results and conclusion from paper III. In LNM, the proteome of several immune cell population is altered compared to UnLN. In LNM, SCS Macrophages downregulate Bcl-xL; MS macrophages upregulate Arg1; B-cell follicles with GC upregulate FoxP3, CD25, PD-L1, PD-1; IFT T-cell regions have a reduced expression of Ki67.

Paper IV

Neutrophils decline in breast cancer lymph nodes with metastasis

Introduction and Results

Neutrophils play a critical role in breast cancer progression, yet their contribution to the adaptive immune response and their role in SLNs/TDLNs remain underexplored. As demonstrated in Paper III, the adaptive immune response is altered in the presence of LNM. Recent studies have identified neutrophils as an immune cell population capable of contributing to antigen presentation within LNs in infectious models [234-236]. However, their relationship with the B-cell compartment within LNs remains poorly understood in cancer. Given this gap in knowledge, we hypothesised that neutrophils represent a crucial immune cell population in LNs, capable of localising to B-cell follicles and potentially facilitating TI B-cell activation. Thus, this study aims to identify neutrophil-driven mechanisms influencing the immune landscape in LNM.

Neutrophils declines from LN compartments in LNM compared to UnLN.

Neutrophil presence in LNs was investigated using IHC and GeoMx DSP in the same patient cohort investigated in Paper III. Neutrophils were identified using the myeloperoxidase (MPO) and CD66b marker. In healthy LNs, neutrophils were observed infiltrating multiple regions, including B-cell follicles and T-cell zones. Next, we investigated neutrophil infiltration in matched LNs with or without metastases from five breast cancer patients. Automated quantification using QuPath revealed a significant reduction in neutrophil numbers in LNM, both in absolute numbers and relative to the LN area. Further analysis of specific LN compartments showed a consistent trend of reduced neutrophil presence across all regions, with a significant decrease in the medulla and trabeculae tracts in every patient. Using GeoMx DSP, we analysed cortical regions from LNM and UnLN. Across all ROIs, CD66b expression was downregulated in LNM compared to UnLN. Further compartmentalised analysis revealed that CD66b downregulation was most pronounced in IFR T-cell regions, indicating a more pronounced loss of neutrophils in this area.

Neutrophils exhibits tissue specific phenotypes.

To investigate the phenotypic characteristics of neutrophils within LNs, we compared their gene expression profiles to those in blood and breast tissue using publicly available scRNA-seq databases. In LNs tissue, neutrophils expressed several markers associated with T-cell or B-cell activity. Most notably, they expressed NAMPT, BAFF (*TNFSF13B*), APRIL (*TNFSF13*), HMGB1 and LL-37.

Interestingly, compared to UnLN, neutrophils in LNM had increased expression of BAFF and APRIL, both proteins involved in TI B-cell activation. Further analysis of polarization markers revealed distinct neutrophil phenotypic differences based on tissue location: while neutrophils in blood and breast tissue exhibited an N2-like phenotype, those in LNs demonstrated a shift toward an N1-like polarization. This suggests that neutrophils in LNs may play a role in initiating adaptive immune responses, unlike their counterparts in other tissues.

Neutrophils in PTs associate with B-cells and T-cells.

Given their expression of TNFSF13B, a marker associated with TI B-cell activation, we further examined the potential role of neutrophils in B-cell activation within a breast cancer cohort. Neutrophils, identified by CD15 staining, were correlated with B-cell infiltration ($P = 0.015$). Clinicopathological analysis revealed that tumours with neutrophil and B-cell co-infiltration exhibited increased T-cell infiltration (CD3 OR = 3.38) and enrichment of M2 macrophages (CD68 OR = 13.63, CD163 OR = 4.44). Additionally, there was a trend toward a correlation with FoxP3 expression (FoxP3 OR = 6.19, $P = 0.075$). Notably, co-infiltration of neutrophils and B-cells was associated with reduced overall survival in ER^+ patients. However, multivariable Cox regression analysis demonstrated that this prognostic impact was dependent on confounding factors, including age, tumour size, histological grade, nodal status, and Ki67 expression.

Conclusion and Limitations

In Paper IV, we demonstrate that neutrophils represent another specific immune cell population present in healthy LNs, LNM and UnLN. During metastatic progression, neutrophil numbers are significantly reduced in LNM compared to in UnLN, as evidenced by IHC and spatial proteomics using the GeoMx DSP platform. We further characterised the phenotype of LN-resident neutrophils using scRNA-seq, revealing an N1 polarization that implicates their role in initiating adaptive immune responses. Specifically, markers such as NAMPT, BAFF (*TNFSF13B*), LL-37, HMGB1 and APRIL (*TNFSF13*) suggest a potential role in modulating B-cells while markers such as HLA-DR, IL-1β and ICAM-1 suggest a potential role in modulating T-cells. Finally, we investigated neutrophil interactions within primary breast tumours and found that neutrophils infiltrate tumours and associate with both B-cell and T-cell infiltration. Importantly, co-infiltration of neutrophils and B-cells was associated with poorer prognosis in ER-positive breast cancer patients.

Some limitations should be considered:

- Gene expression analysis: Neutrophils are a challenging immune population to study due to their inherently low RNA content per cell and endogenous RNA degradation upon maturation and isolation. As a result,

publicly available scRNA-seq datasets contain relatively few well-characterised neutrophils, potentially leading to reduced accuracy of transcriptional analyses.

- Cohort size: Additionally, this study was conducted on a small cohort when assessing neutrophil presence in LNs. Similar to Paper III, findings from small cohorts may not be representative to larger patient populations and may introduce selection bias, reducing the statistical power of our results. However, this limitation applies primarily to our findings on neutrophil depletion in LNs and not to our observations from PTs of the larger breast cancer cohort.

Despite these limitations, our findings highlight the dynamic role of neutrophils in LNs and tumours, underscoring their potential involvement in modulating adaptive immune responses during breast cancer progression.

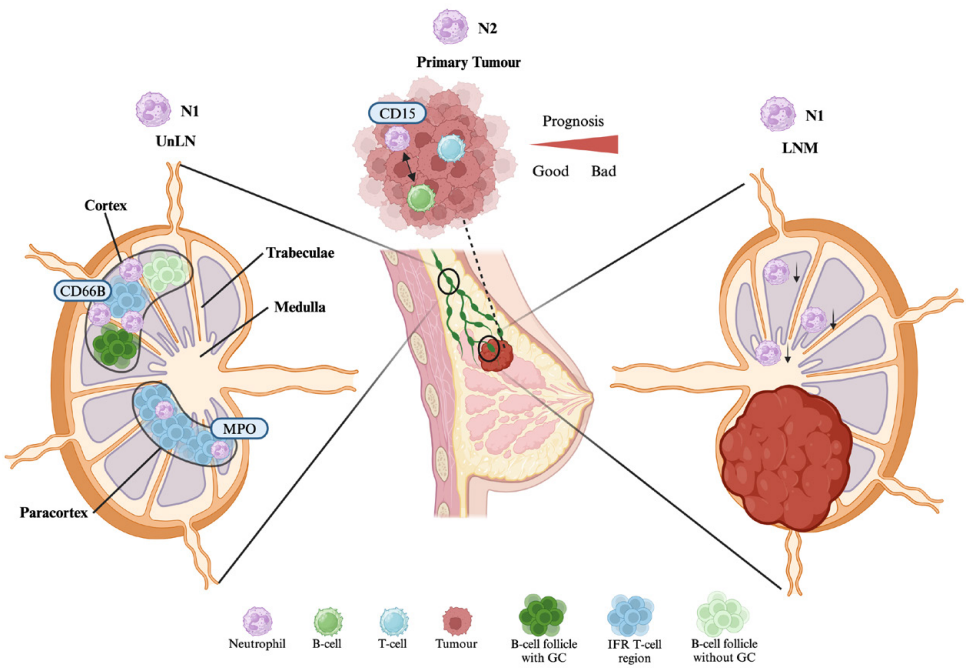

Figure 14. Graphical abstract Paper IV
Graphical abstract summarising the results and conclusion from paper IV. Neutrophils in LNs express markers involved in TI B-cell activation and T-cell response. In LNM, neutrophil numbers decline compared to UnLN. In PTs, neutrophils correlate with B-cells, T-cells and a worse prognosis in ER^+ patients.

Discussion and concluding remarks

Breast cancer prognosis is determined by multiple factors, one of the most critical being the presence of metastases in the SLNs/TDLNs [80,179], as these nodes typically represent the first site of tumour dissemination. LNs are essential immune structures that orchestrate the adaptive immune response against specific antigens [237]. In cancer, this includes the recognition of tumour-specific antigens, which are presented by APCs to activate antigen-specific B-cells and T-cells. A fundamental question addressed in this thesis is how immune cells of the myeloid immune compartment interact with lymphoid structures and adaptive immunity. Furthermore, this thesis investigates if the local immune response in LNs is compromised by the metastasis or if LNMs are primarily markers of the disease progression.

In **Paper I**, we investigated the role of $CD169^+$ TAMs and their association with TLS in PTs and paired LNM. Our key finding was that these macrophages were associated with TLSs in both LNMs and PTs. While their presence in LNMs correlated with a favourable prognosis, the opposite was observed in PTs. This finding is in contradiction to previous literature, where TLSs generally have been linked to improved outcomes in breast cancer patients [238]. However, this discrepancy may be explained by differences based in breast cancer subtypes. Previous studies reporting favourable prognosis were primarily focused on patients with immune "hot" TMEs, such as those with TNBC or HER2-enriched tumours [238,239]. In contrast, our study primarily included patients with Luminal A and Luminal B breast tumours, which are considered to have "cold" TMEs. Additionally, our cohort consisted of patients with advanced-stage breast cancer, suggesting that once tumours begin metastasising, TLSs alone may not be sufficient to counteract disease progression. We also found that TLSs in PTs were associated with B_{reg} and T_{reg} infiltration, suggesting a potential immunosuppressive feedback mechanism that could inhibit anti-tumour responses within TLSs, as has been reported in other cancer models [240]. The co-occurrence of $CD169^+$ macrophages and T_{regs} is likely mediated by the CCL22/CCR4 chemokine axis, given that $CD169^+$ macrophages can secrete CCL22 upon encountering apoptotic cells [241]. Another finding in this study was that the prognostic impact of $CD169^+$ macrophages and TLSs was only significant in patients with immune infiltration in either the PT or LNMs, but not in both. This suggests that the detrimental prognostic impact of immune cell infiltration in PTs may counteract the beneficial prognostic role of immune

infiltration in LNMs. Alternatively, it is possible that infiltration of CD169$^+$ TAMs into PTs occurs simultaneously to their depletion from LNMs in breast cancer patients.

Building on the findings from Paper I, **Paper II** aimed to elucidate why CD169$^+$ macrophages are associated with favourable prognosis when present in LNMs but a poor prognosis when present in PTs. Specifically, we sought to better understand their functional role in PTs. Our results suggest that CD169$^+$ TAMs may originate from bone marrow-derived monocytes and can differentiate under the influence of a type I IFN TME. Interestingly, a type I IFN signature is also associated with T_{reg} activation and LNM development [242], further explaining the relationship between CD169$^+$ macrophages and T_{regs}. Furthermore, the *in vitro* generated CD169$^+$ TAMs exhibited characteristics of both SCS CD169$^+$ macrophages, including type I IFN production, and MS CD169$^+$ macrophages, as evidenced by high CD163 expression. These macrophages also secreted CXCL10, a potent chemoattractant for T-cells and T_{regs} in breast carcinomas [243], as well as IL-6, which is critical for B-cell activation and GC formation in autoimmune diseases [244]. This may explain why CD169$^+$ macrophages were localised to TLSs in both Paper I and Paper II, suggesting that they play a crucial role in TLS initiation. Phenotypically, these macrophages expressed both immunogenic and immunosuppressive markers in LNs and tumours. While their expression of immunosuppressive markers may explain their adverse prognostic impact in PTs, their dual expression of activation and inhibitory receptors likely underpins their distinct prognostic roles in different tissue contexts. Interestingly, monocyte-derived TAMs with similar signatures as the CD169$^+$ TAMs analysed in Paper II (CXCL10, IL-15, IFN-I) were recently shown to cross-dress tumour antigens and promote restimulation of primed T-cells [160]. This mechanism was inhibited by PGE2 and raises the question whereas CD169$^+$ TAMs may also be able to cross-dress or if they are implicated in the suppression of this phenomenon via PGE2.

Given the findings in Paper II, further investigation was warranted to determine whether LN-resident CD169$^+$ macrophages exert anti-tumourigenic effects or simply disappear in late-stage LNMs. Additionally, further research to differentiate between SCS and MS CD169$^+$ macrophages was necessary. In **Paper III**, we addressed these questions using spatial proteomics to analyse specific immune cell populations within LNs. We confirmed that SCS CD169$^+$ macrophages were depleted in LNMs, consistent with previous reports in breast cancer and head and neck cancer [245,246]. Our study further provided a clear distinction between SCS and MS CD169$^+$ macrophages, demonstrating that the regression was specific to the SCS population. We identified Bcl-xL as a potential mediator of SCS CD169$^+$ macrophage depletion. Conversely, MS CD169$^+$ macrophages remained present in LNMs but exhibited a shift in their proteomic profile, with increased expression of the immunosuppressive marker Arg1. The loss of SCS CD169$^+$ macrophages in LNMs likely reduces the acquisition and presentation of tumour-specific antigens

to B-cells, and IFR T-cells as shown by reduced expression of markers of proliferation and activation in the IFR T-cell regions [199,200] . Proteomic analysis further indicated that monocyte-derived $CD169^+$ macrophages from Paper II shared greater phenotypic similarity with MS $CD169^+$ macrophages based on CD163 expression. Additionally, we observed contraction and shutdown of GCs within activated B-cell follicles, as evidenced by upregulation of FoxP3, CD25, and PD-L1. This is consistent with prior studies demonstrating that T_{regs} and T_{FH} can suppress anti-tumour immune responses by inducing GC shutdown [247]. Taken together, these findings suggest that both T-cell and B-cell responses are impaired in LNMs, indicating that LNMs not only serve as markers of systemic disease dissemination but also actively contribute to disease progression by suppressing adaptive anti-tumour immunity.

Our findings in Paper III further indicated that additional immune cell populations are affected in LNMs. In **Paper IV**, we specifically examined the role of neutrophils in LNs, a topic that had been relatively unexplored. Neutrophils have been shown to interact with SCS $CD169^+$ macrophages, DCs, B-cells, and T-cells in the context of infections and inflammatory diseases [203,204,206]. Here, we investigated their role in cancer metastasis. We demonstrated that neutrophils were depleted in LNMs, particularly in IFR T-cell regions of the cortex, and in the medullary and trabecular compartments of LNs. Using scRNA-seq, we found that LN-resident neutrophils expressed BAFF, APRIL, NAMPT, LL-37, OX40 and HMGB1, suggesting a potential role in TI B-cell activation. We further analysed whether these neutrophils exhibited an N1 or N2 polarization. While they expressed markers of both phenotypes, the predominant population expressed N1 markers, including CXCR2, IL-1β, and ICAM1. In contrast, neutrophils in breast tumours displayed greater expression of N2-associated markers, such as CXCL8 and CXCR4 [175,248]. We further assessed neutrophil and B-cell interactions in PTs. In our study, neutrophil infiltration in breast tumours correlated with both B-cell and T-cell infiltration, and co-infiltration of neutrophils and B-cells was associated with poor prognosis. However, this prognostic effect was dependent of other clinicopathological variables. Recent studies suggest that neutrophils promote metastasis, which may explain their association with poor prognosis in our cohort. Collectively, our findings in Paper IV underscore the need for further investigation into the role of neutrophils in immune regulation within LNs and the TME.

Clinical implications

The research presented in this thesis can be categorised as preclinical translational research, which aims to bridge the gap between basic science and clinical applications by translating laboratory findings into potential clinical practices. The insights gained from this work can contribute to the development of novel immunotherapies for targeted treatments and the identification of new biomarkers, thereby enhancing diagnostic tools for breast cancer.

The identification of molecular markers is essential for designing tailored therapies in cancer [249]. In **Paper I**, we demonstrate that TLS or lymphoid aggregates, traditionally associated with a favourable prognosis in cancer, are instead correlated with poor prognosis in patients with advanced Luminal A and Luminal B breast cancer. These findings align with observations from ongoing clinical trials, where immunotherapy has yet to significantly improve survival or treatment response in luminal breast cancer [208]. A plausible explanation for this outcome is the immunosuppressive TME associated with $CD169^+$ macrophages in PTs. To address this, targeting the development of $CD169^+$ macrophages within tumours present a potential strategy to enhance immunotherapy efficacy and improve treatment responses in breast cancer patients. Current immunotherapies against TAMs mainly target CSF-1 and the CCL2/CCR2 chemokine axis to reduces TAM infiltration in tumours [250]. Findings from **Paper I and II** further supports the approach of targeting TAMs, as $CD169^+$ TAMs are associated with a worse prognosis. Because $CD169^+$ macrophage differentiation is driven by type I IFN, inhibiting this signalling pathway, or blocking the mediators released by $CD169^+$ TAMs (e.g., PGE2 and ROS), may prove beneficial as a macrophage-based immunotherapy, particularly in the context of Luminal A/B breast cancer.

Beyond the adverse prognostic role of TLS in PTs, this thesis also identifies additional biomarkers in **Papers III and IV**, specifically within the LN microenvironment. The immunosuppression observed in LNM offers new opportunities for immunotherapeutic intervention. Currently, ICI such as Ipilimumab (anti-CTLA4) and Nivolumab (anti-PD-L1) are administered intravenously to induce systemic immune activation [251]. However, localised administration of these agents directly to TDLNs may enhance treatment efficacy by reactivating adaptive immune responses against tumour-specific antigens. This strategy could be explored as a neoadjuvant therapy prior to surgery, as SLNs are typically excised to reduce metastatic risk.

Additionally, the depletion of neutrophils from LNM presents another potential therapeutic target. Incorporating neutrophil markers into LN biopsy diagnostics could detect neutrophil depletion, enabling the implementation of personalised treatment strategies aimed at restoring LN neutrophil homeostasis. However, the precise role of neutrophils in LN immunity remains incompletely understood and warrants further investigation before targeted neutrophil-based therapies can be developed for breast cancer.

Overall, this thesis underscores the significance of personalised medicine in breast cancer treatment. Effective translational research not only optimises treatment strategies but also accelerates the transition from discovery to clinical application, ultimately contributing to evidence-based, patient-centred care. We propose that the biomarkers identified in this thesis have the potential to serve as predictive markers for immunotherapy response in luminal breast cancers. Moreover, the integration of advanced imaging techniques could enhance the accuracy and sensitivity of current diagnostic methods. However, these advancements must be carefully weighed against their economic feasibility, as they would significantly increase healthcare costs.

Future perspective and Summary

Breast cancer remains a major global health challenge, affecting millions of women annually. Despite significant advancements in diagnostic techniques and treatment options, it remains the second leading cause of cancer-related mortality worldwide. In several countries, it has even surpassed lung cancer as the deadliest malignancy. This underscores the urgent need for continued research to enhance patient outcomes and ultimately achieve curative therapies. This thesis focused on the immunological landscape of breast cancer, aiming to identify novel molecular and cellular targets for future therapies, and provide clinical insights into disease progression. The findings presented contribute to our understanding of tumour immunity while also raising new research questions that warrant further investigation.

TLS and immune suppression in breast cancer progression

In **Paper I**, tumour-associated TLLS were found to correlate with a favourable prognosis in LNM. However, a major challenge in studying TLS within LNM is the complex architecture of SLOs, which makes it difficult to distinguish TLS from secondary lymphoid follicles in histological sections. Addressing this distinction remains a critical goal in the field of TLS immuno-oncology. Other research questions arose from this paper within the SCAN-B cohort. The analysis of the SCAN-B breast cancer patient cohort identified several patient clusters with TLS presence. Notably, not all subset of patients exhibited TLS in association with $CD169^+$ TAMs, B_{reg} and T_{reg} gene signatures. Conversely, other patients displayed TLS and $CD169^+$ TAMs with lower B_{reg} signatures. Investigating the prognostic significance of these subgroups could help determine whether the presence of $TLS/B_{reg}/T_{reg}/CD169^+$ TAMs is linked to worse outcomes, whereas $TLS/CD169^+$ TAMs alone may be beneficial. The association to worse prognosis of $TLS/CD169^+$ TAMs was observed in Luminal patients. Stratifying these findings based on breast cancer molecular subtypes, particularly in the large SCAN-B cohort exceeding 8,000 patients, would allow for a more robust statistical analysis and should also be investigated. Furthermore, if TLS-mediated immune suppression contributes to poor prognosis in advanced luminal breast cancer, immune checkpoint blockade (e.g., anti-PD-1/PD-L1 or CTLA-4 inhibitors) could serve as a promising therapeutic approach to reactivate TLS-driven immune responses. Clinical trials targeting luminal breast cancer patients with TLS-positive tumours could help validate this hypothesis.

$CD169^+$ macrophages and immune regulation in breast cancer

In **Paper II**, we demonstrated that monocyte-derived $CD169^+$ macrophages secrete CXCL10 and CCL17, chemokines known to recruit T-cells subsets, among them, regulatory T_{regs} as shown in breast and lung cancer [120,243]. However, T_{reg} migration assays did not confirm directed migration toward conditioned media from *in vitro* generated $CD169^+$ macrophages. Despite this, our findings from **Paper I** indicated a strong association between $CD169^+$ macrophages and T_{reg} infiltration in both PTs and LNs, suggesting an indirect or alternative mechanism of recruitment. Further investigation into the role of CXCL10 and CCL17 in $CD169^+$ TAMs is necessary to clarify their contribution to T_{reg} accumulation. If these macrophages are implicated in T_{reg} recruitment, targeting $CD169^+$ macrophage differentiation may offer therapeutic benefits by reducing $FoxP3^+$ T_{reg} infiltration and mitigating immune suppression. Potential strategies to inhibit $CD169^+$ macrophage differentiation would include targeting type I IFN, lymphotoxin-α/β, and CSF-1/2 and could be investigated *in vitro*. Based on recent findings [160], the role of $CD169^+$ TAMs should also be investigated in relation to promoting or preventing trogocytosis. Blocking ROS or PGE2 by COX2 inhibitors *in vitro* or *in vivo* warrants further investigation in relation to priming CTLs reactivation.

Immune suppression in LNM

Findings from **Papers I, III, and IV** highlight the need for further research into immune suppression occurring within LNM and its impact on treatment responses. A deeper understanding of these mechanisms could enhance the prediction of immunotherapy efficacy and inform surgical decisions regarding LN dissection. In **Paper III**, we observed the decline of SCS $CD169^+$ macrophages from LNM, with evidence suggesting that the anti-apoptotic protein Bcl-xL plays a role in this process. While targeting Bcl-xL may represent a potential therapeutic approach, the development of a macrophage-specific delivery system is crucial to avoid unintended effects on metastatic tumour cells. Moreover, **Paper III** revealed that GC contraction occurs in LNM, a phenomenon that remains poorly understood. At present, no therapeutic interventions specifically target GC dysfunction in LNM. However, ICI (e.g., anti-PD-1/PD-L1 or CTLA-4 blockade) may not only restore TLS activity in PTs but also reverse GC shutdown within LNs. If proven effective, such approach could reduce the need for SLN dissection, which is often performed to mitigate metastatic spread but can lead to long-term complications such as lymphedema [252]. The possibility of replacing surgical intervention with immunotherapy to preserve LN function while enhancing anti-tumour immunity warrants further investigation.

Neutrophil dynamics in LNs and breast cancer progression

In **Paper IV**, we characterised the role of neutrophils in LNs, though the prognostic implications of neutrophil depletion remain an open question for future studies.

Furthermore, the proteomic profiles of TANs compared to LN-resident neutrophils in breast cancer remain poorly defined. Further research is needed to determine whether neutrophils contribute to tumour progression via immunosuppressive mechanisms or if they play a more complex, dual role in regulating adaptive immune responses. Ongoing work involving co-culture experiments with neutrophils from fresh LNs and B-cells aims to elucidate the precise role of neutrophils in modulating TDLN function. Lastly, to further investigate the relationship between PT neutrophils and LNM neutrophils, a prognostic study from the TEX cohort with paired PT and LNM samples is being planned. With these results, the prognostic implication of neutrophils and their role in TI B-cell activation can further be elucidated.

Conclusion

This thesis underscores the importance of personalised medicine in breast cancer treatment. By identifying immunological markers that stratify patients into distinct prognostic groups, such as luminal breast cancer versus TNBC or HER2-enriched subtypes, our findings may contribute to the development of tailored therapeutic strategies. Translational research bridges the gap between fundamental discoveries and clinical applications, ensuring that novel treatment modalities reach patients more efficiently. We propose that the biomarkers and cell populations identified in this work hold potential as predictive markers for immunotherapy response in breast cancer. Continued research in these areas will be essential for refining breast cancer treatment strategies and improving patient outcomes.

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Paper I

cancers

MDPI

Article

CD169$^+$ Macrophages in Primary Breast Tumors Associate with Tertiary Lymphoid Structures, T_{regs} and a Worse Prognosis for Patients with Advanced Breast Cancer

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Simple Summary: We here show that CD169$^+$ TAMs in primary breast tumors are associated with tertiary lymphoid-like structures (TLLSs), T_{reg} and B_{reg} signatures, and a worse prognosis for the patient. In contrast, CD169$^+$ TAMs and TLLSs present in lymph node metastases were associated with better prognosis. We propose that the negative prognostic value related to CD169$^+$ TAMs and TLLSs in primary breast tumors is a unique consequence of an immunosuppressive tumor environment in advanced breast cancers. This knowledge is important for understanding the immune landscape in breast cancer and for future targeted therapies.

Abstract: The presence of CD169$^+$ macrophages in the draining lymph nodes of cancer patients is, for unknown reasons, associated with a beneficial prognosis. We here investigated the prognostic impact of tumor-infiltrating CD169$^+$ macrophages in primary tumors (PTs) and their spatial relation to tumor-infiltrating B and T cells. Using two breast cancer patient cohorts, we show that CD169$^+$ macrophages were spatially associated with the presence of B and T cell tertiary lymphoid-like structures (TLLSs) in both PTs and lymph node metastases (LNMs). While co-infiltration of CD169$^+$/TLLS in PTs correlated with a worse prognosis, the opposite was found when present in LNMs. RNA sequencing of breast tumors further confirmed that *SIGLEC1* (CD169) expression was associated with mature tertiary lymphoid structure (TLS), and T_{reg} and B_{reg} signatures. We propose that the negative prognostic value related to CD169$^+$ macrophages in PTs is a consequence of an immunosuppressive tumor environment rich in TLSs, T_{regs} and B_{regs}.

Keywords: breast cancer; lymph node; macrophage; CD169; T_{reg}; B_{reg}; TLS

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1. Introduction

Breast cancer is a high-impact disease in our society. With a high mortality rate, due to metastasis, breast cancer is the fifth deadliest cancer type worldwide and even passed lung cancer in incidence rate in 2020 [1]. The need for novel therapies and improvement in current treatment regimens is urgent.

In general, breast cancers are divided into various subtypes depending on hormone receptor expression status (estrogen receptor, ER, and progesterone receptor, PR) and human epidermal growth factor receptor 2 (HER2) status. Expression of these receptors has a large impact on choice of current treatment protocols and on breast cancer prognosis. While receptor positive breast cancers are more common (ER$^{+/-}$PR$^{+/-}$HER2$^{+/-}$), triple negative breast cancers (ER$^-$PR$^-$HER2$^-$; TNBC) are less common and have the worst prognosis with few treatment options [2,3].

In breast cancer, the response rate to immune checkpoint blockade is still relatively low [4,5]. While immune checkpoint inhibitors focus on promoting cytotoxic T-cell activation, other immune cell populations infiltrating the tumor microenvironment (TME) are being further investigated in order to increase our understanding and the efficacy of current treatments [6,7]. Among the most important immune populations in the TME are macrophages and the myeloid immune cell compartment [8].

Macrophages are innate myeloid immune cells with a wide plasticity. They are broadly divided into either tissue-resident macrophages or recruited monocyte-derived macrophages [9]. Apart from this division, macrophage subsets are further characterized by their polarization state. There are two extreme macrophage polarization states, often being referred to as M1- and M2-like subsets, with a plethora of subpopulations ranging in between them, depending on localization, microenvironment and the type of disease in which they are active [10,11].

Lately, a tissue-resident macrophage subpopulation with expression of the surface marker $CD169^+$ has been attracting attention, due to its highly prognostic impact in cancer and autoimmune disease [12]. $CD169^+$ is expressed and upregulated predominantly on macrophages found in organs such as lungs, bone marrow and secondary lymphoid organs (SLOs) [13]. In the SLOs, the $CD169^+$ macrophages are either subcapsular sinus (SCS) $CD169^+$ macrophages or medullary $CD169^+$ macrophages, with slightly different origin and function [14,15]. Their main function there is associated with lymphoid cell activation and regulation [16,17]. While the SCS $CD169^+$ macrophages capture opsonized antigens or lymph-born antigens, allowing antigen encounters with underlying B-cell follicles and thus inducing a germinal center B-cell response, [18] the medullary sinus $CD169^+$ macrophages are efficient at phagocytosis, pathogen clearance, sensing lipids and inducing tissue destruction [18,19]. In a tumor context, $CD169^+$ macrophages can originate from activated monocytes [20] that infiltrate tumors, hence becoming tumor-associated macrophages (TAMs) [21].

In 2012, it was reported that $CD169^+$ macrophages located in the paracortical region of lymph nodes were able to catch tumor antigens and use cross-presentation to activate CD8 T-cells [16]. It was also shown that SCS $CD169^+$ macrophages could recognize sialic acid decorated apoptotic bodies from tumor cells, facilitating B cell anti-tumor immunity [22]. These initial findings were followed by several cohort studies presenting evidence that high presence of $CD169^+$ macrophages in lymph nodes of cancer patients was associated with a beneficial prognosis [23–26]. We recently confirmed this phenomenon in breast cancer and showed that the presence of $CD169^+$ macrophages in breast cancer lymph node metastasis (LNM) was associated with a better prognosis, while surprisingly the presence of $CD169^+$ tumor-associated macrophages ($CD169^+$ TAMs) in the primary tumor (PT) was not [27].

The functional localization of $CD169^+$ macrophages surrounding lymphocyte follicles in SLOs led us to speculate whether infiltrating $CD169^+$ macrophages in PTs ($CD169^+$ TAMs) would localize with tumor-infiltrating lymphocytes (TILs). We specifically investigated whether the $CD169^+$ TAMs in PTs would localize to tertiary lymphoid structures (TLS) or tertiary lymphoid-like structures (TLLSs), similar to the spatial positions they have in secondary lymphoid follicle structures, and the prognostic effect thereof. We here provide evidence that $CD169^+$ TAMs associate with TLLS, T_{reg} and B_{reg} signatures in breast cancers, leading to an adverse clinical outcome when present in PTs, while the opposite effect was observed in LNMs.

2. Materials and Methods

2.1. Ethical Declarations

Written informed consent was received from the patients included in the clinical trials presented in this study, and ethical approvals for the clinical trials were obtained from the regional ethics committees in Sweden: Stockholm (Dnr KI 02-206 and KI 02-205) and Lund (Dnr 2009/658) [28,29].

2.2. Patient Cohorts and Study Design

Two patient cohorts were used in this study; the first cohort was a retrospective cohort study based on primary tumors and lymph node metastases from patients with locally advanced and metastatic breast cancer from the randomized phase III TEX trial performed between 2002–2007 [28]. Detailed information about the clinical trial is found at clinicaltrials.gov with identification number NCT01433614. Briefly, the clinical trial comprised 304 women with advanced or inoperable metastatic breast cancer. Participants received two types of combination chemotherapy as the first line of treatment: Epirubicin and Paclitaxel alone or combined with Capecitabine. Among several criteria that have been described in detail previously [28], the enrolled participants had to have a life expectancy of at least 3 months, no brain metastases and they were not permitted to join the study if they had performed previous chemotherapy treatment cycles. From the 304 participants, formalin-fixed, paraffin-embedded blocks from primary tumors and synchronous lymph node metastases were collected wherever possible for tissue microarray (TMA) construction, as described previously [29], enabling further analysis of the tissue with immunohistochemistry (IHC). A simplified study design of the cohort is illustrated in Figure 1A.

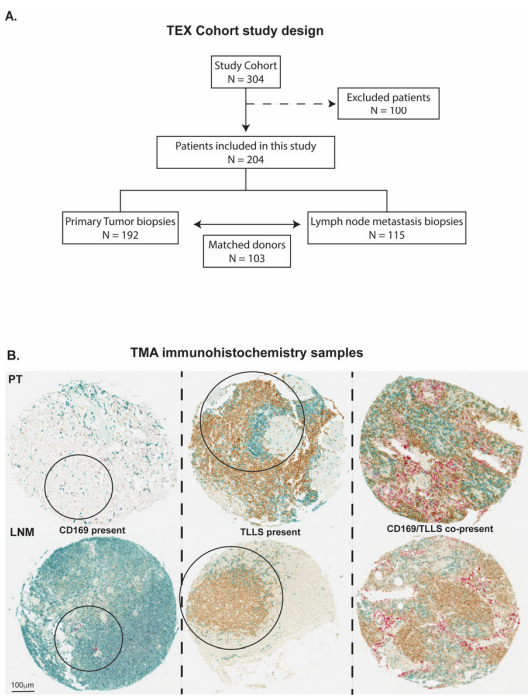

Figure 1. The TEX cohort study design flow chart and immunohistochemical staining examples from the cohort. (**A**) 204 patients were included from the retrospective TEX cohort, giving altogether 192 primary tumor and 115 lymph node metastasis biopsy samples; excluded patients lacked or had missing biopsy cores. (**B**) Tissue microarray (TMA) sections and immunohistochemistry using the three markers CD169 (red), CD20 (brown) and CD3 (blue). The staining panel allowed for identification of three types of cell infiltration/presence patterns, which are highlighted in circles, in primary tumors (PTs) and lymph node metastases (LNMs); $CD169^+$ macrophages only ($CD169^+$), tertiary lymphoid-like structures only (TLLS), and $CD169^+$ macrophages together with TLLS ($CD169^+$/TLLS).

The second cohort was a broad prospective, population-based cohort used in order to validate our findings from the smaller cohort using RNA sequencing data. This cohort was compromised of 8164 patients enrolled in the Sweden Cancerome Analysis Network—Breast (SCAN-B) initiative [30], and was approved by the regional ethical review board in Lund, Sweden. Detailed information from the cohort is found at ClinicalTrials.gov with identification number NCT02306096. Fresh biopsy samples were taken from each patient during the primary surgery by pathologists performing their routine clinical diagnostics. All analyses were performed in accordance with patient consent and ethical regulations, and the biopsies were used to gather RNA sequencing data.

2.3. Immunohistochemistry and Scoring

IHC was performed on the TMA cohort where all primary tumor and lymph node metastases were scored for the different immune cell surface markers, CD169, CD20 and CD3, using the protocol previously described [29,31]. FoxP3 had been annotated previously [31]. In brief, TMA blocks were sectioned to a thickness of 4 mm prior to mounting. The sections contained cores with diameters of 800 µm and were pre-treated with the PT-link system before staining with an Autostainer Plus (DAKO, Santa Clara, CA, USA) at pH6 with an overnight staining protocol. The following antibodies and dilutions were used for staining: anti-CD169$^+$ macrophages (1:100, Invitrogen, Clone SP216, Waltham, MA, USA), anti-CD20$^+$ B-cells (1:100, Abcam, Clone L-26, Cambridge, UK), anti-CD3$^+$ T-cells (1:100, Abcam, Clone 11084, Cambridge, UK) and developed with a triple staining IHC kit from Abcam. A previous staining performed by authors (J.S. and C.H.) used mouse monoclonal anti-FoxP3 (ab20034, clone 236A/E7, Abcam, 1:400, Cambridge, UK) to annotate T_{regs} as previously published [31].

CD3$^+$ T-cells, CD20$^+$ B-cells and CD169$^+$ macrophages were annotated individually by authors O.B., E.K. and K.L. The following scores were used: for CD169, CD169$^+$ expression present = 1, and CD169$^+$ expression absent = 0; for CD20, CD20$^+$ clusters in spatial contact with CD3$^+$ T-cells present = 1, and CD20$^+$ absent or present as dispersed single cells (not in clusters) or without spatial contact with CD3$^+$ T-cells = 0. Since we did not include a follicular dendritic cell marker, the CD20$^+$/CD3$^+$ B/T cell clusters will be referred to as TLLS, and not TLS. The purpose of this scoring was to classify immune cell infiltration into three different categories: (1) CD169$^+$ macrophages positive tumors/metastases; (2) TLLS positive tumors/metastases (CD20$^+$/CD3$^+$); (3) tumors/metastases with presence of CD169$^+$ and TLLS (CD169$^+$/CD20$^+$/CD3$^+$), as represented in Figure 1B.

T_{reg} (FoxP3$^+$) annotations were published previously and performed by authors J.S. and C.H. [31]. The T_{regs} scoring strategy ranged from 0–3 and furthermore also categorized absence–presence (0–1). In the present study, the T_{regs} (FoxP3$^+$) (0–1) score was used, solely exploring correlation between presence or absence of T_{regs} and its effect within the three different immune cell infiltration categories (CD169$^+$, TLLS, CD169$^+$/TLLS).

2.4. Statistical Analyses

Statistical analyses were performed with IBM SPSS statistics (version 27), with all statistical tests being two-sided with $p \leq 0.05$ considered as significant results. In the TMA cohort, age at diagnosis ranged from 27 to 71 years old with an overall median age at diagnosis of 51 years. A total of 21% of included patients were alive at the time of data collection (July 2013) and the median follow-up time for patients alive was 10.5 years. Age, tumor size, lymph node status, metastatic stage, PT receptor status, lymph node receptor status and adjuvant therapy given are presented in Table 1.

Correlations between clinicopathological factors and immune cell infiltration in PTs and LNMs were assessed using cross tabulation tables. Odds ratios with a 95% confidence interval were correlated to 5-year recurrence-free interval (RFI), 5-year breast-cancer-specific survival (BCSS), tumor sizes above 20 mm, expression of the receptors (ER, PR, HER2), high Ki67 levels (>15%) and presence of T_{regs}, TLLSs or CD169$^+$ macrophages. All clinicopathological factors were set as binary values; thus, significant correlations with immune

cell infiltration were analyzed with the chi-square test or with Fisher's exact test when fewer observations than 20 were seen.

Table 1. Patient characteristics and clinicopathological features of patients included in the TEX study [28].

Patient Characteristics		No. of Patients	Percent (%)
Age	>50	92	45.1
	<50	112	54.9
Tumor size (T)	T1 (0–20 mm)	83	40.7
	T2 (20–50 mm)	95	46.6
	T3 (>50 mm)	16	7.8
	T4 (Invasion)	9	4.4
	Missing	1	0.5
Regional lymph nodes (N)	N0	65	31.9
	N1	124	60.8
	N2	9	4.4
	N3	2	1.0
	Missing	4	2.0
Metastasis (M)	M0	185	90.7
	M1	18	8.8
	Missing	1	0.5
PT receptor status			
ER	Neg	36	17.6
	Pos	152	92.2
	Missing	16	7.8
PR	Neg	80	39.2
	Pos	107	52.5
	Missing	17	8.3
HER2	Neg	172	84.3
	Pos	17	8.3
	Missing	15	7.4
LNM receptor status			
ER	Neg	28	13.7
	Pos	74	36.3
	Missing	102	50.0
PR	Neg	64	31.4
	Pos	38	18.6
	Missing	102	50.0
HER2	Neg	77	37.7
	Pos	13	6.4
	Missing	114	55.9
Adjuvant therapy given			
Chemotherapy	No	106	52.0
	Yes	98	48.0
Endocrine	No	92	45.1
	Yes	112	54.9
Radiotherapy	No	55	27.0
	Yes	149	73.0

Abbreviations: ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2.

The prognostic outcome of immune cell infiltration was analyzed with Kaplan–Meier plots and log-rank tests to exclude the null hypothesis of equal prognostic effect for BCSS or RFI based on specific immune cell infiltration in tumor tissues. Effects on BCSS and RFI were calculated based on infiltration of $CD169^+$ macrophages alone, TLLSs alone, or

dual infiltration of $CD169^+$ macrophages and TLLSs ($CD169^+$/TLLS). The follow-up data from the TMA cohort enabled a long time to event scale for BCSS and RFI, since all patients had presented with locally advanced or metastatic disease at the time of inclusion into the clinical trial. The time to event scale for BCSS and RFI in the current analysis was censored at 25 years after primary tumor diagnosis. For LNM biopsies, the time to event scale for BCSS and RFI was set to 10 and 25 years after diagnosis in order to focus on both early and long-term prognostic effects, since lymph node metastases were present at primary diagnosis in the majority of patients in the TEX cohort.

Univariable followed by multivariable Cox regression analyses were also performed to estimate hazard ratios (HRs) for recurrence or death from breast cancer according to $CD169^+$, TLLS and $CD169^+$/TLLS infiltration in PTs and LNMs. The same time to event scale as for the Kaplan–Meier analyses was maintained and the multivariable models accounted for hormone receptor/growth factor expression status (ER, PR, Her2), T_{regs} presence, Ki67 levels, tumor size and age at primary diagnosis. Results were illustrated using forest plots showing HRs with a 95% confidence interval.

2.5. Gene Expression Analyses

Gene expression analyses of TLS gene signature [32], B_{reg} signature [33], T_{reg} (*FoxP3*) signature [34] and $CD169^+$ TAMs (*SIGLEC1*) were performed using RNA sequencing data from the SCAN-B cohort, following the same procedure as previously described [30,35]. Expression data were extracted as fragments per kilobase per million reads for each case and transformed into a logarithmic scale. Five gene classifiers representing different subtype predictors were used to classify samples into the intrinsic breast cancer subtypes according to the PAM50 gene signature [36]. Prior to analysis, a batch correction was performed via ComBat in order to remove potential bias associated with technical variations. After correction, the data were uploaded unto The Institute for Genomic Research MultiExperiment Viewer (TIGR MeV) version 3.1, and differences in gene expression were determined through hierarchical clustering using median-centered gene correlations where status 1 or above represented upregulated expression and -1 or below represented downregulated expression. RNA sequencing results are presented with heat maps, showing Pearson correlation distance and complete hierarchal clustering linkages.

3. Results

3.1. $CD169^+$ TAMs Associate with TLLSs in PTs

Since SCS $CD169^+$ macrophages in lymph nodes have a functional localization surrounding B cell follicles and are associated with a beneficial prognosis in cancer patients, we first set out to investigate the localization pattern of PT-infiltrating $CD169^+$ macrophages ($CD169^+$ TAMs) in relation to $CD20^+$/$CD3^+$ B/T cell clusters (referred to as TLLSs) in primary breast cancer tumors (Figure 1B).

Localization patterns were investigated with odds ratios (ORs), as shown in Tables 2 and S1. Firstly, $CD169^+$ TAMs in PTs were indeed correlated with the presence of TLLSs (OR = 3.77, p = 0.004) and furthermore showed a trend for T_{reg} infiltration (OR = 2.06, p = 0.057). $CD169^+$ TAMs also correlated with B cells as only marker, unrelated to TLLSs (OR = 5.26, p = 0.017). In LNMs, $CD169^+$ presence ($CD169^+$ LNM) was found to also correlate with TLLS presence (OR = 4.76, p = 0.0001) and T_{reg} infiltration (OR = 2.87, p = 0.046).

$CD169^+$ LNMs further correlated with decreased odds of tumor size above 20 mm (OR = 0.42, p = 0.041) and increased odds of surviving beyond 5 years (OR = 2.20, p = 0.045), while CD169 in PTs ($CD169^+$ TAMs) correlated with high Ki67 levels (OR = 2.33, p = 0.021) (Table 2). This was in line with our previously published data using another breast cancer cohort regarding $CD169^+$ infiltration in PTs ($CD169^+$ TAM) and LNMs ($CD169^+$ LNM) [27]. $CD169^+$ TAMs also correlated with decreased odds of tumor size above 20 mm (OR = 0.47, p = 0.019) and decreased odds for expression of ER (OR = 0.28, p = 0.0001).

Table 2. Odds ratio table comparing CD169$^+$ macrophage infiltration in PTs and LNMs with tumor and metastasis clinicopathological features as well as other immune cell infiltration.

Clinicopathological Features		CD169$^+$ PT				CD169$^+$ LNM			
		OR	95% CI	*p*-Value [a]	*n*	OR	95% CI	*p*-Value [a]	*n*
Age	>50				85	1			48
	<50	0.96	0.51–1.81	0.90	106	0.89	0.43–1.88	0.77	67
Overall Survival	>5 years	1			67	1			42
	<5 years	1.21	0.61–2.36	0.59	124	2.20	1.01–4.79	0.045	73
Relapse free interval	>5 years	1			108	1			77
	<5 years	0.61	0.31–1.22	0.16	73	1.37	0.61–3.06	0.44	35
Tumor size	T1	1			80	1			34
	>T1	0.47	0.24–0.89	0.019	110	0.42	0.19–0.97	0.041	80
Ki67$^+$ PT	Neg	1			24	1			37
	Pos	2.33	1.183–4.600	0.021	24	1.26	0.535–2.989	0.67 [b]	16
Ki67$^+$ LNM	Neg	1			14	1			31
	Pos	1.47	0.550–3.923	0.45[b]	9	0.69	0.285–1.662	0.51[b]	12
ER PT	Neg	1			36	1			21
	Pos	0.28	0.13–0.60	0.001	147	1.43	0.55–3.75	0.63	85
ER LNM	Neg	1			24	1			28
	Pos	0.76	0.26–2.28	0.77	69	0.53	0.22–1.29	0.18	70
PR PT	Neg	1			79	1			42
	Pos	0.59	0.31–1.12	0.11	103	1.18	0.54–2.59	0.68	61
PR LNM	Neg	1			57	1			60
	Pos	0.34	0.11–1.01	0.053	37	0.68	0.30–1.56	0.36	37
HER2 PT	Neg	1			166	1			95
	Pos	1.77	0.61–5.17	0.37[b]	16	0.65	0.17–2.46	0.74 [b]	10
HER2 LNM	Neg	1			69	1			77
	Pos	0.99	0.24–4.06	1[b]	13	1.41	0.41–4.76	0.76 [b]	12
Cell infiltration association									
FoxP3 PT	Neg	1			68	1			40
	Pos	2.06	0.99–4.26	0.057	107	0.76	0.34–1.72	0.51	56
FoxP3 LNM	Neg	1			24	1			26
	Pos	0.70	0.23–2.13	0.57	49	2.87	0.99–8.27	0.046	54
TLLS PT	Neg	1			165	1			91
	Pos	3.77	1.61–8.82	0.004	26	2.04	0.58–7.27	0.36 [b]	12
TLLS LNM	Neg	1			39	1			46
	Pos	1.02	0.40–2.62	0.97	64	4.76	2.12–10.71	0.0001	69

Abbreviations: PT = primary tumor; LNM = lymph node metastases; OR = odds ratio; 95% CI = 95% confidence interval; *n* = number of patients; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; [a] = Fisher exact test unless otherwise stated, two-tailed; [b] = Pearson's chi square test, two-tailed.

Additionally, in PTs, the presence of CD169$^+$/TLLS structures showed a trend towards association with decreased odds of surviving the first 5 years (OR = 0.29, p = 0.053), decreased expression of ER in both PTs and LNMs ($OR_{PT\text{-}ER}$ = 0.26, p = 0.042, $OR_{LNM\text{-}ER}$ = 0.10, p = 0.051), and high Ki67 levels in PTs (OR = 5.43, p = 0.018). In sharp contrast, CD169$^+$ /TLLS in LNMs was significantly correlated with increased odds of surviving breast cancer the first 5 years (OR = 3.51, p = 0.005; Table S1).

To explore the potential univariable role of TLLS infiltration (CD20$^+$/CD3$^+$; "TLLS") without CD169$^+$ co-localization, further OR analysis was performed between TLLSs and other patient and tumor characteristics (Table S1). Results indicated that TLLSs in PTs per se (TLLS$^+$ PT) was significantly correlated with reduced odds of breast-cancer-related

death (OR = 0.41, p = 0.045) and lower odds for recurrence (OR = 0.31, p = 0.018) within 5 years after diagnosis. Furthermore, TLLS was significantly correlated with greater odds for infiltration of T_{regs} (FoxP3) (OR = 8.54, p = 0.001) into PTs. Again, and in contrast, TLLSs in the LMNs per se (TLLS$^+$ LNM) was significantly correlated with increased odds of surviving beyond 5 years (OR = 2.63, p = 0.018), and decreased odds of PR expression in LNM (OR = 0.43, p = 0.044).

In summary, CD169$^+$ macrophages were associated with TLLSs both in PTs and in LNMs, but when present in PTs this was associated to a worse prognosis for the patients, the opposite of what was seen in LNMs.

3.2. CD169$^+$ TAMs and TLLSs as Prognostic Markers for Breast Cancer Patients

Investigating prognostic impact, Kaplan–Meier plots for each variable (CD169$^+$/TLLS; CD169$^+$; TLLS) showed unique survival patterns. In general, a worse prognosis was seen with CD169$^+$ and TLLS infiltration in PTs, while infiltration in LNMs showed a better prognosis. In PTs, CD169$^+$/TLLS co-infiltration was a borderline prognostic marker associated with worse BCSS (p = 0.059) (Figure 2A). To estimate if the observed effect was caused by the dual infiltration pattern (CD169$^+$/TLLS), or was solely from one type of cell infiltration (CD169$^+$ or TLLS), individual Kaplan–Meier plots with corresponding log-rank tests were performed. Both CD169$^+$ infiltration (p = 0.047) (Figure 2B) and TLLS infiltration (p = 0.001) (Figure 2C) in PTs showed evidence for an adverse BCSS. A similar significant inferior outcome regarding RFI was seen only for TLLS infiltration (p = *0.006*) (Figure 2D–F). Infiltrating B cells as only variable (CD20) however, did not have an impact on survival (BCSS p = 0.38; RFI p = 0.82).

Conversely, CD169$^+$/TLLS presence in LNMs showed significant evidence of improved BCSS in the first 10 years (p = 0.016) (Figure 2G). Individually, CD169$^+$ LNM presence remained significant (p = 0.023), while TLLSs showed weaker evidence (p = 0.083) for a beneficial prognostic effect on BCSS (Figure 2H,I). For RFI, no statistically significant correlations were observed for any type of immune cell infiltration; the survival curves, however, did trend towards longer RFI upon TLLS infiltration alone or CD169$^+$/TLLS co-presence, suggesting a potential beneficial prognostic effect (Figure 2J–L). The beneficial prognostic effects for CD169$^+$ and TLLS in LNMs, however, were lost in the long-term 25-year follow-up (Figure S1).

Because the prognostic effect was opposite that based on tumor localization (PT vs. LNM), we further investigated the prognostic impact for patient matched biopsies. Interestingly, the results suggested that CD169$^+$ TAM infiltration in PTs was relevant as a prognostic factor only if CD169$^+$ macrophages in LNMs were absent, and vice versa. The same finding was true for TLLSs (Figure S2).

Hence, the observed opposite prognostic effects in PTs and LNMs were seen both for individual (CD169$^+$ or TLLS) and dual infiltration patterns (CD169$^+$/TLLS).

3.3. T_{reg} Infiltration Impacts the Prognostic Effect of CD169$^+$ TAMs

In previous research using the TMA cohort, T_{reg} infiltration in PTs was found to be an independent prognostic factor for decreased BCSS, but the prognostic effect was lost in LNMs [31]. In line with this, in the present study we show that CD169$^+$ TAMs trended towards an association with infiltration of T_{regs} (OR = 2.06, p = 0.057), and that TLLSs correlated with T_{regs} (OR = 8.54, p = 0.001) (Tables 2 and S1). Interestingly, CD169$^+$/TLLS dual infiltration in PTs (CD169$^+$/TLLS PT) was significantly associated with the opposite, meaning a decreased presence of T_{regs} (FoxP3$^+$) in PTs (OR = 0.59, p = 0.007) (Table S1). To interpret this, we further investigated the impact of CD169$^+$ TAMs and TLLS infiltration based on a FoxP3$^+$ (T_{reg}) strata in PT biopsies only. To our surprise, we found that PTs lacking T_{reg} infiltration also always lacked CD169$^+$/TLLS dual infiltration, implying that co-infiltration of CD169$^+$ TAMs and TLLSs is necessary for the presence of T_{regs}, and vice versa. Therefore, Kaplan–Meier plots with corresponding log-rank tests could not be performed with a FoxP3$^+$ strata for CD169$^+$/TLLS and TLLs. Impor-

tantly, however, individual analysis of CD169$^+$ infiltration alone (CD169$^+$ TAM) associated with shortened RFI (P_{RFI} = 0.001) and a trend towards association with shortened BCSS (P_{BCSS} = 0.055) only in the absence of FoxP3$^+$ T_{regs} in PTs (Figure 3A,B). Hence, the prognostic effects of CD169$^+$ TAMs alone for BCSS and RFI were completely lost in the FoxP3$^+$ T_{regs} positive patient strata (Figure 3C,D).

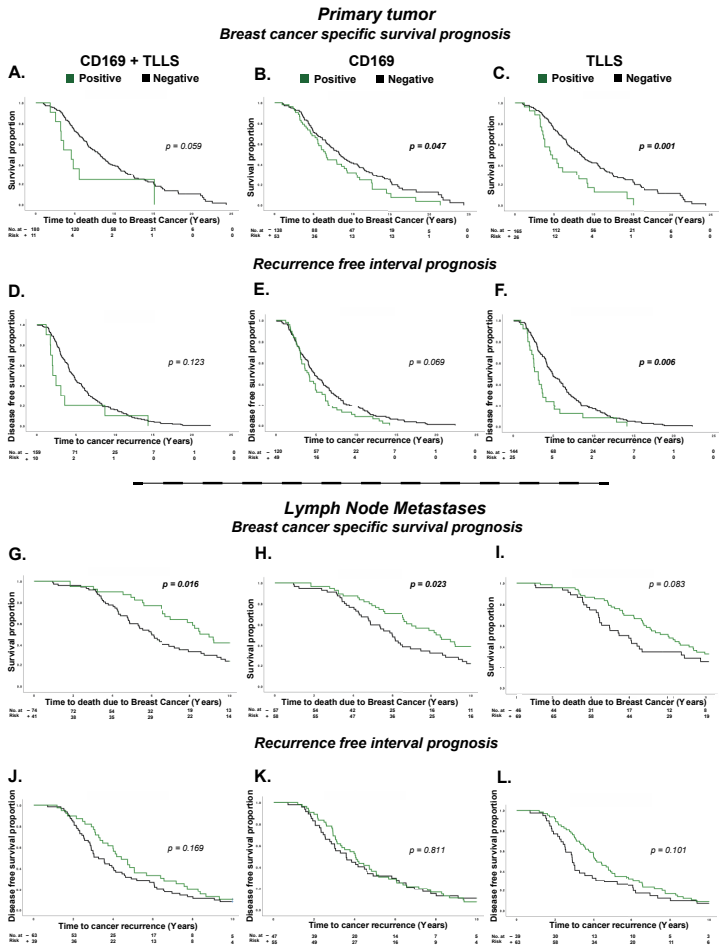

Figure 2. Kaplan–Meier survival plots investigating differences in 25-year breast cancer specific survival (BCSS) and recurrence free interval (RFI) for specific immune cell populations infiltrating tumors. P values by the log-rank test are highlighted in bold when significant. In panels (**A**–**F**), the impact of immune cell infiltration for CD169$^+$ TAMs, TLLSs and CD169$^+$ TAMS/TLLS was investigated as prognostic markers for BCSS and RFI in primary tumors (PTs). In panels (**G**–**L**), the impact of CD169$^+$ TAMs, TLLSs and CD169$^+$ TAMS/TLLS on BCSS and RFI was investigated in lymph node metastases (LNMs). Green lines indicate PTs and LNMs with CD169$^+$ TAMs, TLLSs or CD169$^+$ TAMS/TLLS infiltration, and black lines indicate the absence of CD169$^+$ TAMs, TLLSs or CD169$^+$ TAMS/TLLS.

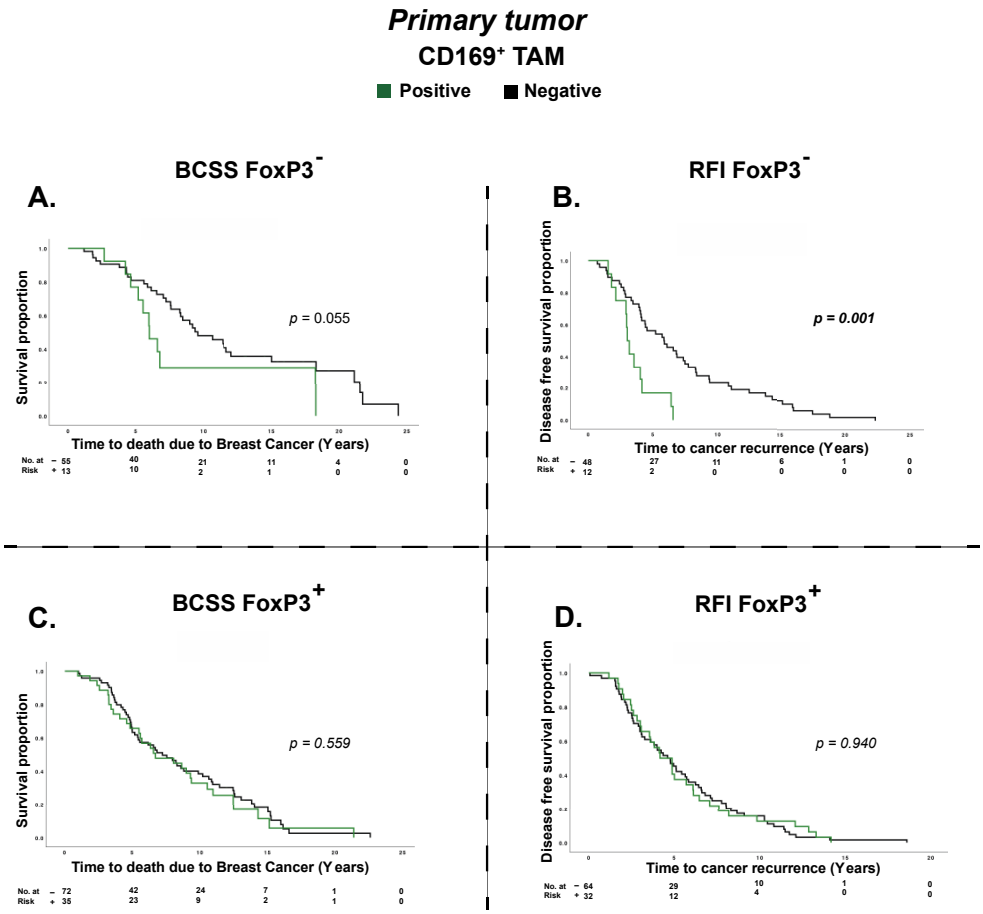

Figure 3. Kaplan–Meier survival plots with a FoxP3 strata on BCSS and RFI in PTs only. *p* values by the log-rank test and highlighted in bold when significant. Panels (**A**,**B**) show correlations for BCCS and RFI with CD169$^+$ TAMs in FoxP3 negative cases, while panels (**C**,**D**) show correlations for BCCS and RFI with CD169+ TAMs in FoxP3 positive tumors. For all panels, green lines indicate PTs with CD169$^+$ TAMs and black lines indicate patients with the absence of CD169$^+$ TAMs.

Altogether this suggests that co-infiltration of CD169$^+$/TLLS may be necessary for the presence of T_{regs}, and that the presence of CD169$^+$ TAMs alone may only have a prognostic impact in breast tumors lacking T_{regs}.

3.4. CD169$^+$ TAMs and TLLSs Show Unique Independent Prognostic Effects

Multivariable Cox regression analyses were done to compare the effects from each type of cell infiltration biomarker adjusted for several potential confounders. Included confounders taken into account were: age, nodal status, tumor size, Ki67, receptor status (ER, PR, HER2), T_{regs} presence, TLLS (CD20$^+$/CD3$^+$) presence and CD169$^+$ macrophage presence (Figures S3 and S4). The prognostic impact was calculated with HR with a 25-year

timeline in PT samples and a 10-year timeline in LNM samples for BCSS and RFI. For BCSS, after multivariable adjustments, dual infiltration of $CD169^+$/TLLS in PTs showed an independent HR value correlating to a worse prognosis (*HR* = 2.88, 95%CI: (1.33–6.2), $p = 0.007$), hence even stronger than the univariable effect (*HR* = 1.90, 95%CI: (0.97–3.75), $p = 0.063$) (Figure S3A). In contrast, in LNMs, $CD169^+$/TLLS dual infiltration was correlated with improved survival in univariable analysis only (*HR* = 0.54, 95%CI: (0.33–0.90), $p = 0.017$). In the multivariable analysis, $CD169^+$/TLLS dual infiltration in LNMs showed a similar trend but with weaker statistical evidence (*HR* = 0.45, 95%CI: (0.20–1.02), $p = 0.057$) (Figure S3B). $CD169^+$ and TLLS infiltration were next analyzed separately. Multivariable adjustments further strengthened TLLSs as an independent prognostic marker in PTs ($HR_{TLLS} = 1.73$, 95%CI (1.03–2.93), $p = 0.040$), while evidence for $CD169^+$ TAMs as a prognostic factor in PTs decreased ($HR_{CD169} = 1.07$, 95%CI: (0.67–1.71), $p = 0.77$) compared to their univariable effects ($HR_{TLLS} = 2.14$, 95%CI: (1.38–3.35), $p = 0.001$; $HR_{CD169} = 1.43$, 95%CI (1.002–2.04), $p = 0.049$) (Figure S3C,E). In contrast to PTs, multivariable analyses of $CD169^+$ LNMs showed both stronger HRs and stronger correlation to decreased risk of death from breast cancer, while evidence for TLLS infiltration being an independent prognostic factor decreased ($HR_{CD169} = 0.48$, 95%CI: (0.23–0.99), $p = 0.046$; $HR_{TLLS} = 0.72$, 95%CI: (0.40–1.31), $p = 0.28$) compared to their univariable effects ($HR_{CD169} = 0.59$, 95%CI: (0.37–0.93), $p = 0.025$; $HR_{TLLS} = 0.66$, 95%CI: (0.41–1.06), $p = 0.085$) (Figure S3D,F).

Regarding the independent prognostic impact on RFI, it was clear that the prognostic effect was not as important as for BCSS. Multivariable analysis for $CD169^+$ TAMs and TLLS infiltration, respectively, showed non-significant HR correlations in both PTs and LNMs. However, $CD169^+$/TLLS dual infiltration in PTs was significantly correlated with higher HRs of recurrence (*HR* = 2.15, 95%CI: (1.06–4.38), $p = 0.035$) (Figure S4).

Our data thus showed that $CD169^+$/TLLS dual infiltration in PTs of advanced breast cancer patients was an independent prognostic marker with regards to both BCSS and RFI.

3.5. $CD169^+$ TAMs Associate with Both Mature TLS and B_{reg} Gene Signatures

To investigate whether the TLLSs associating with $CD169^+$ TAMs in PTs were functional tertiary lymphoid follicles, and to confirm the association between $CD169^+$ TAMs and TLLSs in breast cancer, gene signatures of mature tertiary lymphoid structure (TLS) [32] from bulk RNAseq from 8164 patients of the SCAN-B cohort were investigated, allowing analysis in a larger, contemporary and representative cohort. Initial results (Figure 4A), showed that $CD169^+$ expression (gene name, *SIGLEC1*) indeed correlated with a mature TLS gene signature for a specific cluster of patients, indicating functional TLS formations. This specific cluster also had upregulated levels of the gene *MS4A1*, encoding CD20. For all clusters, the molecular subtype, although being dominant for more aggressive subtypes (luminal B, HER2-enriched, basal), was not restricted to one subtype cluster. This implies that TLS formation can occur in all subtypes of breast cancer.

To further investigate the possible immunological role of the $CD169^+$ TAMs in the PT, we analyzed other immunoregulatory gene signatures [33,34]. $CD169^+$ TAM upregulated clusters correlated with the B_{regs} gene signature (Figure 4B). Notably, a cluster of patients with $CD169^+$ TAMs further associated with TLS, B_{reg} and T_{reg} gene signatures (Figure 4C), indicating an immunosuppressive function for these TLSs. More importantly, gene signatures for TLS formation were not seen in patient clusters that lacked $CD169^+$ TAMs, and the same observations were true for T_{reg} and B_{reg} gene signatures. Lastly, $CD169^+$ TAM positive clusters also associated with genes important for $CD169^+$ macrophage biology, such as *CD163*, *CSF-1*, *LTA* and *LTB* (Figure 4D). Complete heatmaps with the highlighted clusters are presented in Figure S5.

In summary, this implies that $CD169^+$ TAMs are closely connected to mature TLS formation, and T_{reg} and B_{reg} infiltration, in PTs of breast cancer patients.

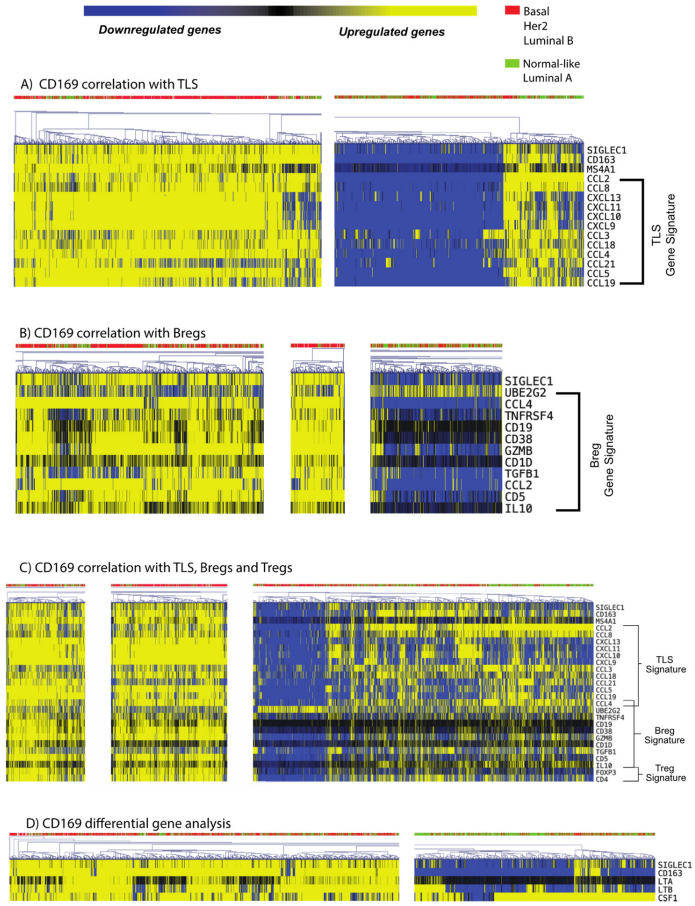

Figure 4. Heat map associations between SIGLEC1 (CD169) and gene signatures for (**A**) tertiary lymphoid structures (TLSs), (**B**) Bregs and (**C**) Tregs. Patients are characterized based on their molecular subtype of breast cancer, aggressive subtypes in red (basal-like, HER2$^+$ and luminal B), and luminal A or normal-like subtypes in green. Upregulated genes are shown in yellow while downregulated genes are shown in blue. The threshold for upregulation/downregulation was set at 1/-1 based on median-centered genes relations. The highlighted areas represent clusters with positive cell infiltration, taken from full-scale analysis shown in Supplementary Figure S4. (**D**) SIGLEC1 (CD169) was correlated with the CD169$^+$ macrophage differentiation markers CD163, LTA, LTB and CSF1.

4. Discussion

In secondary lymphoid organs (SLOs), the CD169$^+$ SCS lymph node macrophages surround B cell follicles to aid in antigen delivery and to regulate immune responses [19]. Our initial hypothesis was therefore that CD169$^+$ TAMs would associate with TLLSs in PTs, just like CD169$^+$ SCS macrophages do in SLOs, hence aiding or regulating immune activity. CD169 is primarily expressed on activated monocytes and macrophages, with occasional

expression on T cells and mature dendritic cells (DCs) [37,38]. Here, co-stainings with CD3 ruled out T cells expressing CD169; however, we cannot exclude that follicular DCs could potentially express microvesicles containing CD169 derived from macrophages [39]. Our original published data, which were supported by this present study, showed that $CD169^+$ TAMs infiltrating PTs were indeed associated with a worse prognosis for breast cancer patients [27]. We here show that $CD169^+$ TAMs infiltrating PTs actually do associate with TLLSs in PTs, similar to the $CD169^+$ SCS resident lymph node macrophages and B-cell follicles in SLOs. We show that $CD169^+$ macrophages are in close spatial association with TLLSs in PTs, and surprisingly also with presence of T_{regs}. In spleen, $CD169^+$ macrophages are dependent on lymphotoxin α1ß1 generated from B-cell follicles [40,41]. An explanation for the co-localization of $CD169^+$ TAMs and TLLSs in breast tumors could therefore be a local secretion of lymphotoxin α1ß1 in tumors with TLSs. Indeed, our bulk RNA-sequencing data from the SCAN-B cohort showed that upregulation of CD169/*SIGLEC1* also correlates with *LTA1* and *LTB1* upregulation.

Both $CD169^+$ TAMs and TLLSs in PTs were clearly associated with a worse prognosis, in contrast to $CD169^+$ macrophages present in LNMs, which had a beneficial effect on prognosis. This finding is in disagreement with previous literature where TLS presence in primary breast tumors was a positive prognostic factor [42,43], as a meta-analysis has shown TLS-presence to generally be associated with a beneficial prognosis in breast cancer [43]. However, in previous studies, the beneficial effect of TLSs as a prognostic marker in PTs was highly dependent on breast cancer molecular subtypes (HER2 amplified [44] or TNBC [45]). These data should be put into relation with the TMA cohort used in the present study, which was comprised mostly of luminal tumors (n = 143) and very few $HER2^+$ (n = 9) and TNBC (n = 24) tumors. All the patients used in the TMA cohort, furthermore, had developed metastatic disease and therefore had a poor prognosis. This could indicate differential impact of TLS depending on the molecular subtype or due to the advanced stage. One observation supporting this was made by Figenschau et al. [46], who verified that tumors with a higher level of tumor infiltrating immune cells correlated with intra-tumoral TLS formation, higher tumor grade and a higher degree of inflammation, thus leading to worse prognosis. We also found that presence of $CD169^+$ LNM and $CD169^+$ PT showed lower odds of having a large tumor size. This is surprising given the worse prognosis seen for $CD169^+$ TAMs in PTs, although when adjusting for multiple variables the prognostic effect of $CD169^+$ PT was lost. Nonetheless, these findings could indicate a more aggressive behavior and hence microenvironment of primary tumors with $CD169^+$ TAMs already at a low tumor size in this cohort with advanced breast cancer patients. Lastly, a general difficulty when investigating TLSs in lymph node metastases is the lobular structure of the SLO, since secondary lymphoid follicles may be difficult to separate from TLSs in the sectioned lymph node metastases. How to discriminate TLS from secondary lymphoid follicles will be an important issue to solve for the TLS immune oncology field in the future.

Using RNA sequencing data from the large SCAN-B breast cancer cohort, we showed that *SIGLEC1* (CD169) expression in primary breast tumors clustered with the expression of functional TLS signatures, indicating that $CD169^+$ TAMs actually do associate with mature tertiary lymphoid follicles also in primary tumors. A fraction of these were enriched for B_{reg} and T_{reg} signatures, thus possibly inducing immunosuppression and adverse prognostic effects in breast cancer patients. This would be supported by a recent study showing that the presence of TLSs with B_{reg} and T_{reg} infiltration was associated with a worse prognosis in primary breast tumors from invasive ductal carcinoma (IDC) and ductal carcinoma in situ (DCIS) [47]. In the present study, we evaluated this hypothesis using RNA sequencing data. In fact, there was a clear subcluster of patients with high transcript levels for $CD169^+$, TLS gene signatures, and B_{reg} and T_{reg} signatures, possibly leading to immunosuppression and hence a worse prognosis. Furthermore, TLS and B_{reg} gene signature transcripts were only present for subsets of patients with a higher infiltration of $CD169^+$ TAMs, implying that $CD169^+$ TAMs, TLS and B_{regs} may interact. On the other hand, patients with both

CD169 and TLS signatures, presenting with lower expression of the B_{regs} signature, were numerous in comparison. The significance of these findings in relation to outcome will be interesting to evaluate and will be a future goal.

Another important result from our study was the correlation between TLS, $CD169^+$ TAMs and $FoxP3^+$ T_{regs}. We found a strong correlation between $FoxP3^+/CD4^+$ T_{reg} signatures and $CD169^+$ expression (*SIGLEC1*) in the SCAN-B cohort, and the T_{reg} gene signature also correlated with TLS and B_{reg} gene signatures. Indeed, based on our IHC results using the TMA cohort, TLS infiltration was almost exclusively present when T_{regs} were also present in PTs. In fact, several papers have shown that the presence of T_{regs} is associated with a worse prognosis in breast cancer [31,48,49]. As the main purpose of T_{regs} is to suppress lymphocytes, it is likely that $FoxP3^+$ T_{reg} infiltration is a natural feedback response following high levels of TLLS formation, suppressing these lymphocytes. A previous in vivo study investigating the correlation between T_{regs} and TLS in lung cancer showed that T_{regs} actively suppress the anti-tumor response from TLSs. In the same model, T_{regs} depletion reversed this effect and led to T-cell expansion starting at the TLS sites and promoting tumor destruction [50]. It was also recently shown that tumors affect local lymph node immune tolerance epigenetically via type I IFNs, eventually promoting distant metastasis facilitated by tumor-antigen specific T_{regs} in a malignant melanoma model [51]. As lymph node $CD169^+$ macrophages are known type I IFN producers [12], the correlation seen here between $CD169^+$ macrophages and T_{regs} could possibly be involved in this epigenetic reprograming [51]. Even more interesting is that T_{regs} infiltration is associated with a higher risk of death and relapse, especially for ER positive breast cancer patients [49], which is in line with our TMA cohort that was predominantly of luminal subtype. The presence of T_{regs} likely inhibits reactivation of T-cell responses; thus, despite the presence of TLLSs, the immune system is unable to counteract tumor growth. A final interesting finding regarding T_{regs} from the present study was that co-infiltration of $CD169^+$ TAMs/TLLS is necessary for the presence of T_{regs}, and that $CD169^+$ TAMs alone only had a prognostic impact in tumors lacking T_{regs}. This most probably means that T_{regs} have a dominant functional role over $CD169^+$ TAMs. $CD169^+$ TAMs may also play another important role here in relation to T_{regs} infiltration, as it has been shown that $CD169^+$ macrophages can upregulate CCL22 upon interaction with apoptotic cells, leading to $FoxP3^+$ T_{reg} recruitment via a CCL22/CCR4-mediated chemotaxis gradient [52]. The same mechanism might apply within breast cancer patients with high tumor grade, with an abundance of apoptotic cells or necrotic cells, thus recruiting $CD169^+$ TAMs which release CCL22 and recruit T_{regs}.

Lastly, the results we obtained, where the presence of $CD169^+$ TAMs and TLLSs were correlated with prognosis only for patients who had immune cell infiltration in either PT or in LNM, but not for both, are interesting. Subsequently, patients who had $CD169^+$ TAMs or TLLS infiltration in both PT and LNM had similar survival compared to patients that lacked the same in both PT and LNM. This result either implies that the harmful prognostic effect of immune cell infiltration seen in PTs cancels out the beneficial prognostic effect seen in LNMs, or that infiltration of $CD169^+$ TAMs into PTs is concurrent with the depletion of $CD169^+$ macrophages from the LNMs in breast cancer patients with a higher tumor grade, therefore giving rise to worse prognosis within our cohort that only includes patients with advanced breast cancer.

5. Conclusions

In conclusion, $CD169^+$ macrophages present in breast cancer PTs and LNMs correlate to the presence of TLS, T_{reg} and B_{reg} signatures for a subset of patients. This was associated to worse survival when present in PTs, while conferring a better prognosis when present in LNMs (Figure 5). We propose that attraction and polarization of $CD169^+$ TAMs occur in tumors where formation of TLSs occurs, and that these inflamed environments cause enrichment of immunosuppressive regulatory lymphocytes, T_{regs} and B_{regs}, thus fueling even more immunosuppressive environments and breast tumor progression.

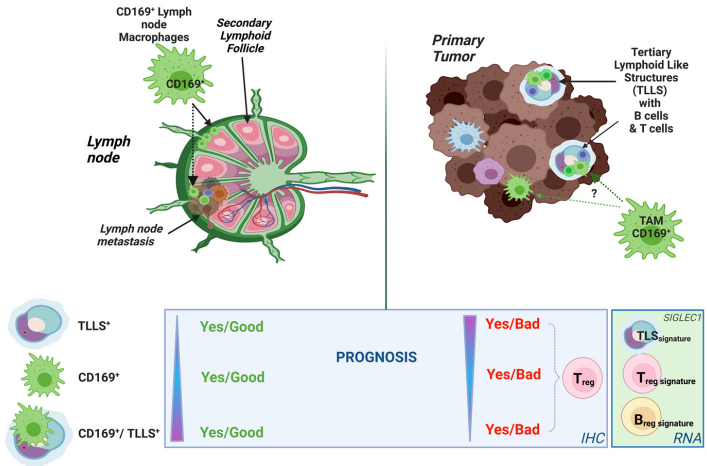

Figure 5. Schematic summary of results. Made in biorender.com. https://biorender.com (accessed on 9 February 2023).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers15041262/s1, Figure S1: Kaplan–Meier survival showing the differences in 25-year breast cancer specific survival (BCSS) and recurrence free interval (RFI) for specific immune cell populations infiltrating lymph node metastases (LNMs); Figure S2: Kaplan–Meier analyses performed on matched donors; Figure S3: Forest plots with Cox regression analyses (BCSS); Figure S4: Forest plots with Cox regression analyses (RFI); Figure S5: Complete heat maps containing sample hierarchical clusters with Pearson correlations between *SIGLEC1* (CD169) and gene signatures for tertiary lymphoid structures (TLSs), B_{regs} or T_{regs}; Table S1: Odds ratio table comparing $CD169^+$ /$TLLS^+$ co-infiltration and $TLLS^+$ infiltration with tumor and metastasis clinicopathological features and other immune cell infiltration.

Author Contributions: The work reported in the paper was performed by the authors. O.B., E.K. and K.L. performed the annotation of CD169, CD20 and CD3 IHC in the TEX cohort. O.B. was responsible for managing the data analyses in SPSS and TIGR MeV, for interpretation of results and drafting of the manuscript. S.K., I.H. and T.H. collected clinicopathological traits and outcome data for all patients and constructed TMAs for the TEX cohort. S.V. supported the RNA sequencing analysis and batch correction in the SCAN-B cohort. J.S. and C.H. were responsible for the annotation and analysis of FoxP3 in the TEX cohort. T.H. was responsible for the design of the TEX cohort and follow-up. K.L. was responsible for designing this study, interpretation of the results (together with OB) and drafting of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki, and ethical approvals for the clinical trials were obtained from the regional ethics committees in Sweden: Stockholm, Dnr KI 02-206 and KI 02-205 (date of approval 14 November 2002), and Lund Dnr 2009/658 (date of approval 17 December 2009) [28,29].

Informed Consent Statement: A written informed consent was received from the included patients in the clinical trials presented in this study.

Data Availability Statement: Gene expression data are available at Mendeley Data as a publicly accessible dataset (https://data.mendeley.com/datasets/yzxtxn4nmd, accessed on 17 May 2022) (https://doi.org/10.17632/yzxtxn4nmd.1) [49].

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Conflicts of Interest: K.L. is a board member of Cantargia A.B., a company developing IL1RAP inhibitors. Cantargia A.B. had no role in the funding of the study or in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results. The authors otherwise declare no competing interest. The funders had no role in the design of the study; in the collection, analyses or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Table S1: Odds ratio table comparing CD169$^+$/TLLS$^+$ co-infiltration and TLLS$^+$ infiltration with tumor and metastasis clinicopathological features and other immune cell infiltration.

Clinicopathological features		CD169$^+$/TLLS$^+$ PT				CD169$^+$/TLLS$^+$ LNM				TLLS$^+$ PT				TLLS$^+$ LNM			
		OR	95% CI	P-value[a]	N	OR	95% CI	P-value[a]	N	OR	95% CI	P-value[a]	N	OR	95% CI	P-value[a]	N
Age	*>50 y*	1			180	1			74	1			85	1			48
	<50 y	0.65	0.192 – 2.214	0.54	11	0.64	0.295 – 1.383	0.25[b]	41	1.97	0.81 – 4.78	0.14[b]	106	0.84	0.39 – 1.79	0.70[b]	67
Overall Survival	*>5 y*	1			180	1			74	1			67	1			42
	<5 y	**0.29**	0.080 – 1.014	**0.053**	11	**3.51**	1.429 – 8.603	**0.005**[b]	41	**0.41**	0.18 - 0.94	**0.045**[b]	124	**2.63**	1.20 – 5.76	**0.018**[b]	73
Relapse free interval	*>5 y*	1			170	1			71	1			108	1			77
	<5 y	0.31	0.065 – 1.478	0.20	11	1.75	0.773 – 3.971	0.18[b]	41	**0.31**	0.11 - 0.85	**0.018**[b]	73	1.64	0.70 – 3.81	0.30[b]	35
Tumor Size	*T1*	1			179	1			74	1			80	1			34
	>T1	1.29	0.365 – 4.569	0.76	11	0.48	0.211 – 1.101	0.081[b]	40	1.19	0.51 – 2.78	0.83[b]	110	0.62	0.27 – 1.43	0.30[b]	80
Ki67 PT	*Low*	1			167	1			66	1			115	1			75
	High	**5.43**	1.386 – 21.276	**0.018**	11	1.09	0.448-2.635	0.85[b]	38	2.04	0.881-4.725	0.092[b]	63	0.94	0.395-2.258	0.90[b]	29
Ki67 LNM	*Low*	1			87	1			64	1			62	1			69
	High	3.33	0.526 – 21.114	0.33	5	0.57	0.210-1.530	0.26[b]	29	2.90	0.718-11.712	0.122[b]	30	1.30	0.530-3.169	0.57[b]	30
ER PT	*Neg*	1			172	1			68	1			36	1			21
	Pos	**0.26**	0.076-0.920	**0.042**	11	1.51	0.532-4.285	0.44[b]	38	0.49	0.19 - 1.24	0.18[b]	147	0.92	0.35 – 2.46	0.87	85
ER LNM	*Neg*	1			89	1			67	1			24	1			28
	Pos	**0.10**	0.010-1.043	**0.051**	4	0.50	0.119-1.241	0.13[b]	31	**0.17**	0.04 - 0.79	**0.025**[b]	69	0.48	0.19 – 1.22	0.12[b]	70
PR PT	*Neg*	1			171	1			65	1			79	1			42
	Pos	0.92	0.269-3.116	1	11	0.92	0.407-2.067	0.83[b]	38	0.51	0.22 - 1.18	0.14[b]	103	0.66	0.30 – 1.47	0.32[b]	61
PR LNM	*Neg*	1			89	1			66	1			57	1			60
	Pos	0.37	0.040-3.429	0.65	5	0.56	0.222-1.388	0.21[b]	31	0.63	0.15 - 2.61	0.74[b]	37	**0.43**	0.18 – 0.99	**0.044**[b]	37
HER2 PT	*Neg*	1			171	1			171	1			166	1			95
	Pos	2.49	0.490 – 12.68	0.25	11	2.49	0.490 – 12.678	0.25	11	2.18	0.65 – 7.37	0.25	16	0.92	0.24 – 3.47	1.0	10
HER2 LNM	*Neg*	1			79	1			79	1			69	1			77
	Pos	2.79	0.234 – 33.264	0.41	3	2.79	0.234 – 33.264	0.41	3	2.96	0.48 - 18.12	0.24	12	0.71	0.21 – 2.41	0.76	12
Cell infiltration association																	
FoxP3 PT	*Neg*	1			164	1			60	1			68	1			40
	Pos	**0.59**	0.515- 0.666	**0.007**	11	1.45	0.619-3.384	0.39[b]	36	**8.54**	1.94 - 37.63	**0.001**[b]	107	1.91	0.83 – 4.41	0.13[b]	56
FoxP3 LNM	*Neg*	1			70	1			61	1			24	1			26
	Pos	0.66	0.555- 0.778	0.55	3	1.47	0.466-4.641	0.59	19	2.61	0.29 - 23.71	0.66	49	1.83	0.71 – 4.72	0.21[b]	54
CD169 PT	*Neg*	1			180	1			65	1			138	1			79
	Pos	---	---	---	11	2.43	0.514 – 11.505	0.25[b]	38	**3.77**	1.61 - 8.82	**0.004**[b]	53	1.02	0.40 – 2.62	0.97[b]	24
CD169 LNM	*Neg*	1			96	1			74	1			50	1			57
	Pos	2.5	0.462 – 13.521	0.44	7	---	---	---	41	2.04	0.58 – 7.27	0.36[b]	53	**4.76**	2.12 – 10.71	**0.0001**[b]	58

Abbreviations: PT = Primary Tumor. LNM = Lymph node metastases. OR = Odds ratio. 95% CI = 95% confidence interval. N = Number of patients. ER = Estrogen Receptor. PR = progesterone Receptor. HER2 = Human epidermal growth factor receptor 2.

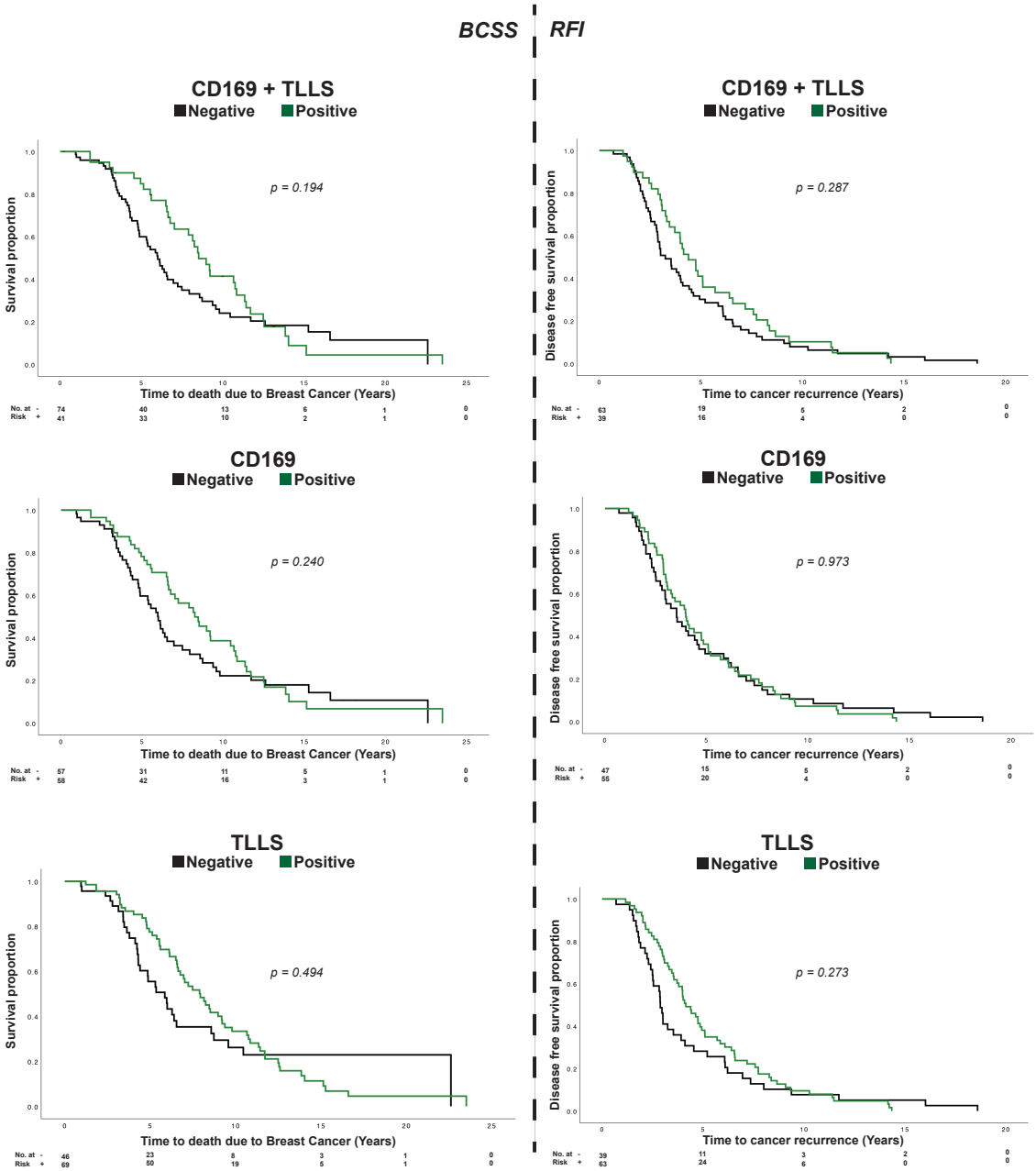
a= Fisher exact test unless otherwise stated. two tailed

b= Pearson's chi square test. two-tailed

Supplementary Figures

Figure S1

Lymph Node Metastases
Long term prognosis



Supplementary Figure legends

Figure S1:

Kaplan Meier survival showing the differences in 25 year breast cancer specific survival (BCSS) and recurrence free interval (RFI) for specific immune cell populations infiltrating lymph node metastases (LNM). *P* values by the log rank test are highlighted in bold when significant. On the left, the impact of immune cell infiltration for $CD169^+$ TAMs, TLLS and $CD169^+$ TAMS/TLLS was investigated as prognostic markers for BCSS. On the right, the impact of $CD169^+$ TAMs, TLLS and $CD169^+$ TAMS/TLLS was investigated as a prognostic marker for RFI. Green lines indicate LNMs with $CD169^+$ TAMs, TLLS or $CD169^+$ TAMS/TLLS infiltration and black lines indicate the absence of $CD169^+$ TAMs, TLLS or $CD169^+$ TAMS/TLLS.

Figure S2

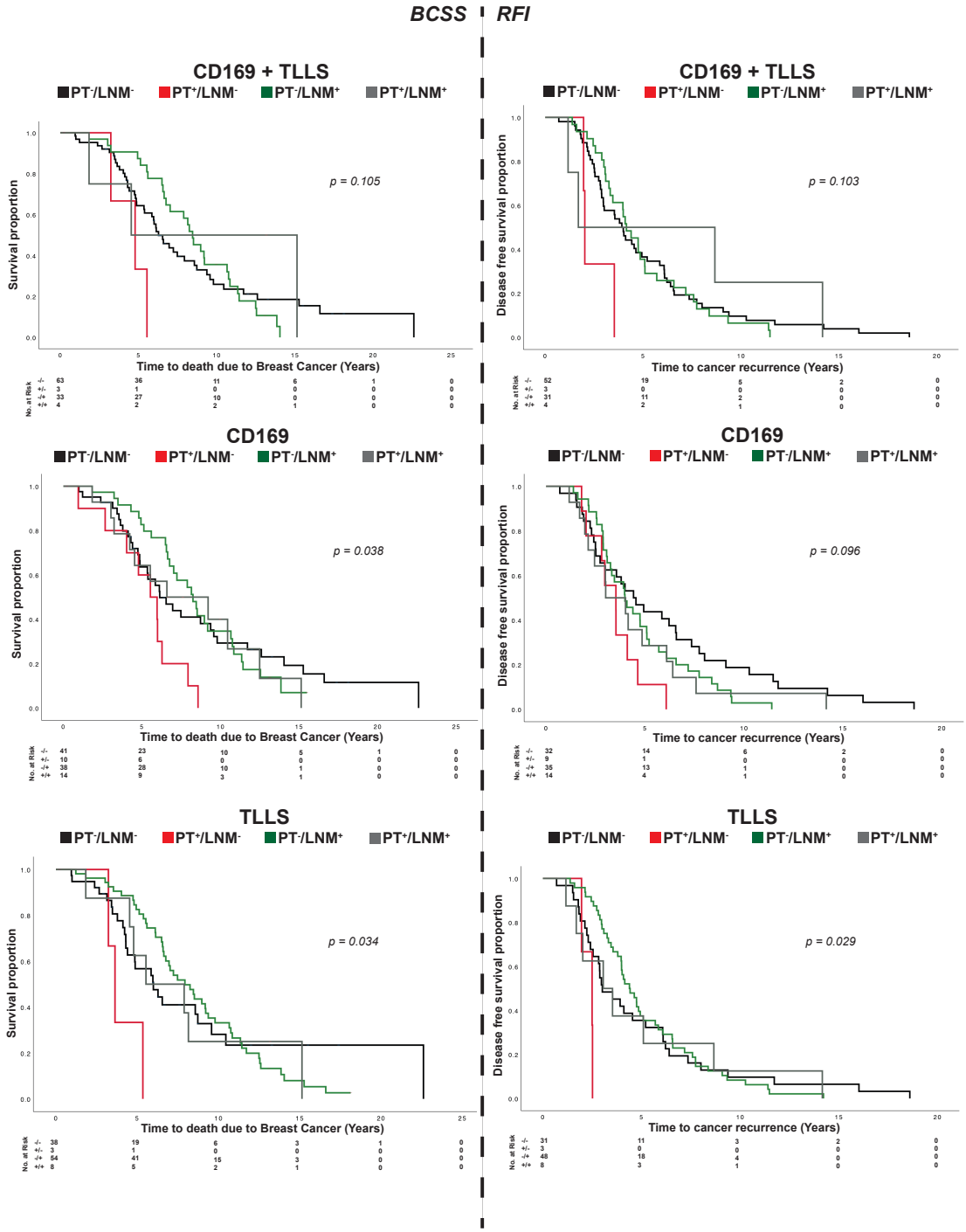

BCSS
RFI
CD169 + TLLS
PT-/LNM-
PT+/LNM-
PT-/LNM+
PT+/LNM+
Survival proportion
Disease free survival proportion
p = 0.105
p = 0.103
Time to death due to Breast Cancer (Years)
Time to cancer recurrence (Years)
No. at Risk
CD169
p = 0.038
p = 0.096
TLLS
p = 0.034
p = 0.029

Figure S2:

Kaplan Meier analyses performed on matched donors. Four types of associations were compared: patients lacking immune cells infiltration in both PT and LNM, patients having immune cells infiltrating in both PT and LNM and patients having cell infiltration in either PT or LNM. In the left panel, BCSS was investigated for all three types of cell infiltration. In the right panel, the same analysis was performed for RFI. *P* value by the log rank test.

Figure S3

Multiple Cox regression analysis

Forest Plots - Breast cancer specific survival

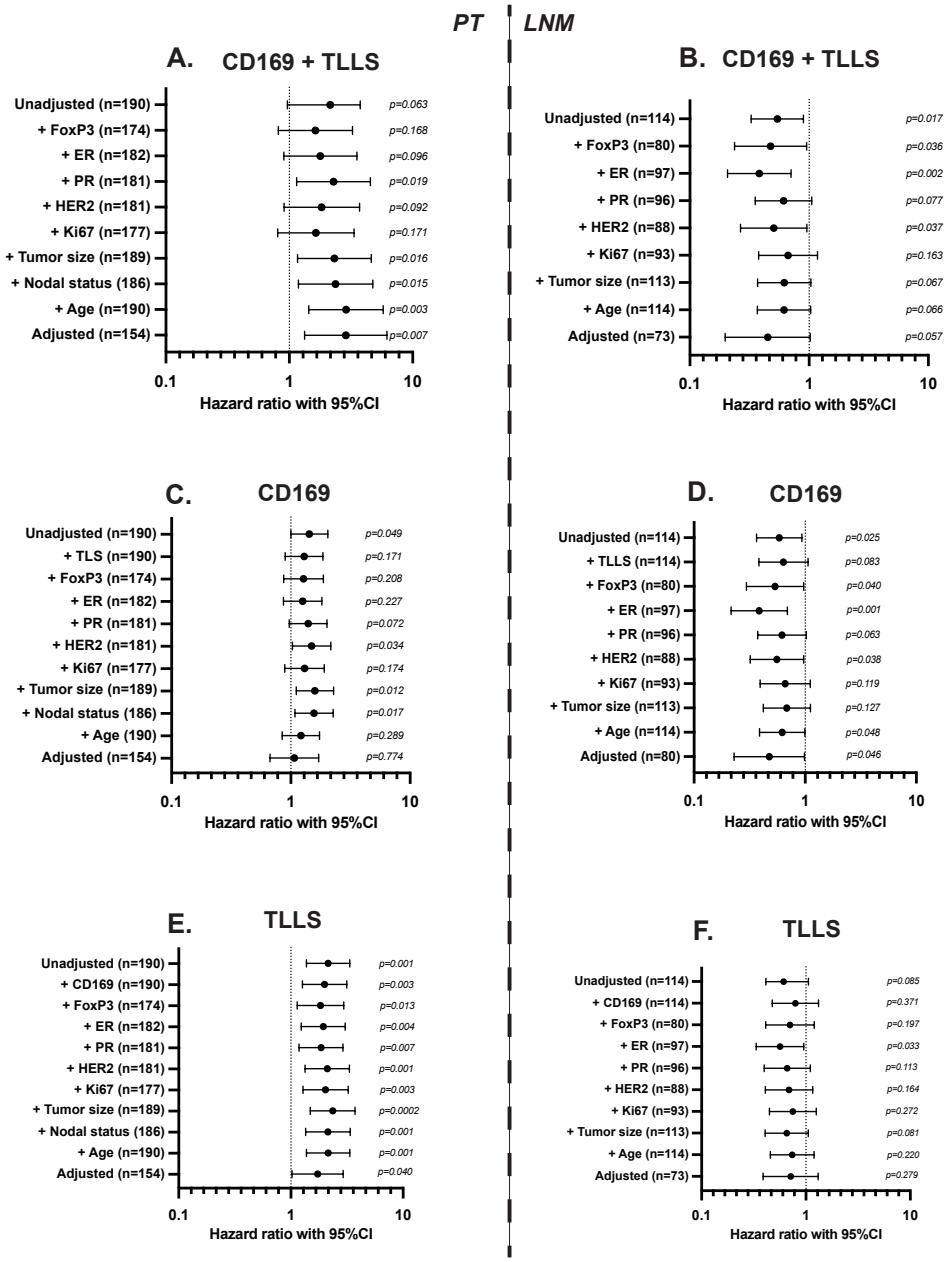


Figure S3:

Forest plots with Cox regression analyses (BCSS). Forest plots showing Cox regression analysis on 25 year BCSS (PT) and 10 year BCSS (LNM) in breast cancer patients with $CD169^+$ TAMs, TLLS or $CD169^+$ TAMS/TLLS presence, adjusted individually and all together for confounders such as receptor expression status (ER, PR, HER2), T_{regs} presence, Ki67 levels, tumor size and age at diagnosis. Hazard ratios are indicated with dots together with horizontal lines representing the 95% confidence interval.

Figure S4

Multiple Cox regression analysis
Forest Plots - Recurrence free interval

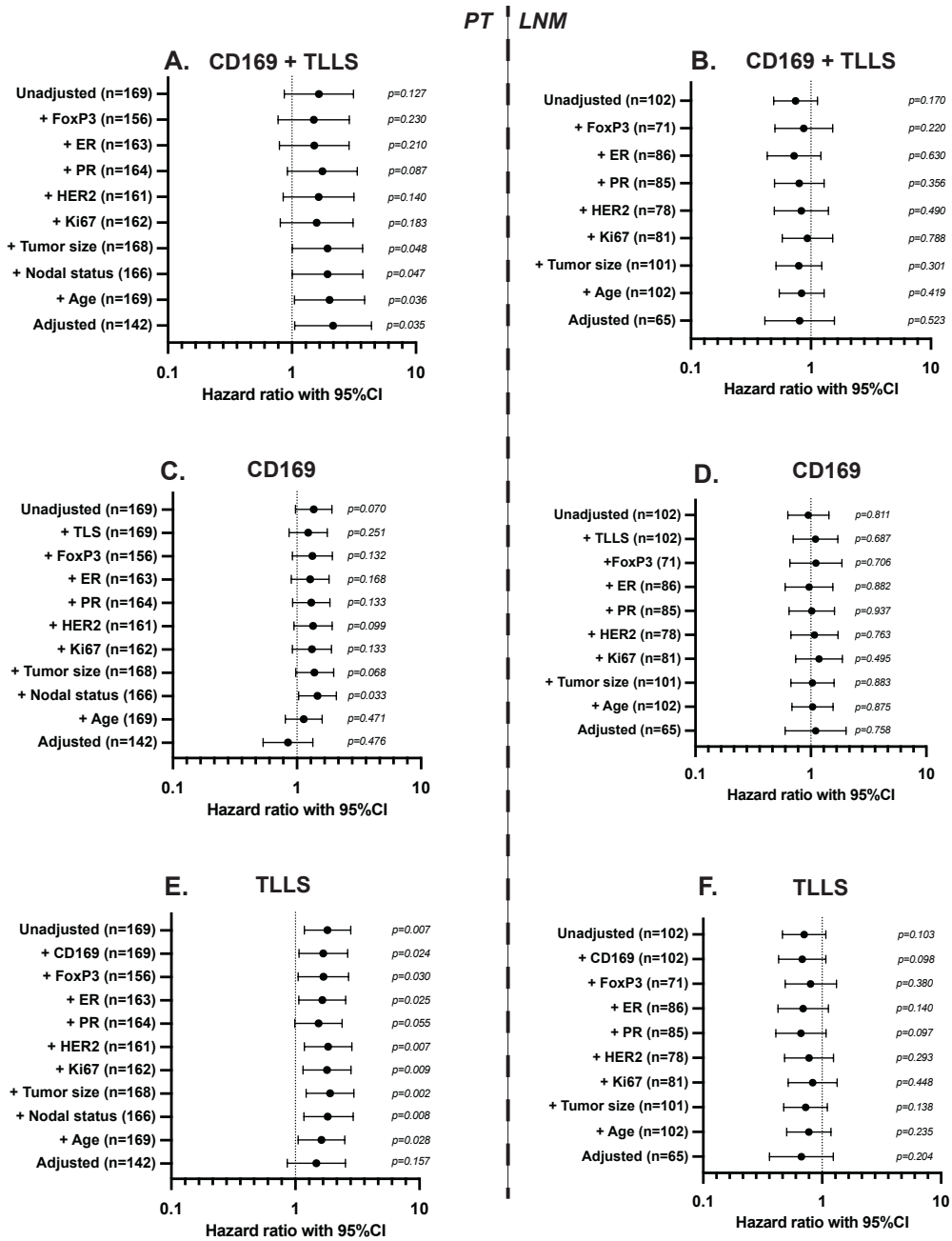


Figure S4:

Forest plots with Cox regression analyses (RFI). Forest plots showing Cox regression analysis on 25 year RFI (PT) and 10 year RFI (LNM) in breast cancer patients with $CD169^+$ TAMs, TLLS or $CD169^+$ TAMS/TLLS presence, adjusted individually and all together for confounders such as receptor expression status (ER, PR, HER2), T_{regs} presence, Ki67 levels, tumor size and age at diagnosis. Hazard ratios are indicated with dots together with horizontal lines representing the 95% confidence interval.

Figure S5

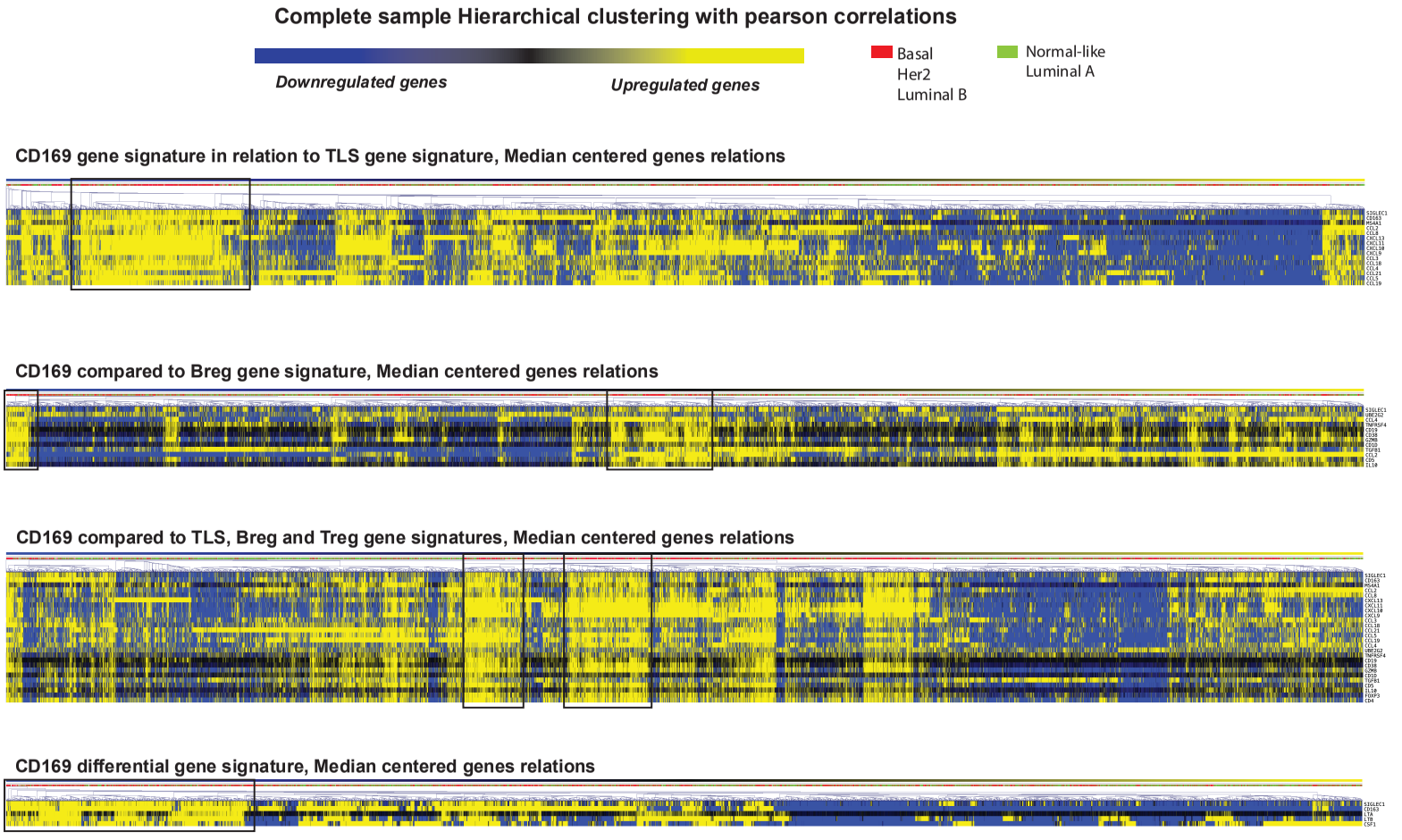

Complete sample Hierarchical clustering with pearson correlations
Downregulated genes
Upregulated genes
Basal
Her2
Luminal B
Normal-like
Luminal A
CD169 gene signature in relation to TLS gene signature, Median centered genes relations
CD169 compared to Breg gene signature, Median centered genes relations
CD169 compared to TLS, Breg and Treg gene signatures, Median centered genes relations
CD169 differential gene signature, Median centered genes relations

Figure S5:

Complete heat maps, containing sample hierarchical clusters with Pearson correlations between *SIGLEC1* (CD169) and gene signatures for tertiary lymphoid structures (TLS), B_{regs} or T_{regs}. Patients are characterized based on their molecular subtype of breast cancer, aggressive subtypes in red (Basal-like, $HER2^+$ and Luminal B) and luminal A or normal-like subtypes in green. Upregulated genes are shown in yellow while downregulated genes are shown in blue. The threshold for upregulation/downregulation was set at 1/-1 based on median centered gene relations. The highlighted areas represent clusters with positive cell infiltration correlations shown in Figure 4.

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Breast cancer associated CD169$^+$ macrophages possess broad immunosuppressive functions but enhance antibody secretion by activated B cells

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CD169$^+$ resident macrophages in lymph nodes of breast cancer patients are for unknown reasons associated with a beneficial prognosis. This contrasts CD169$^+$ macrophages present in primary breast tumors (CD169$^+$ TAMs), that correlate with a worse prognosis. We recently showed that these CD169$^+$ TAMs were associated with tertiary lymphoid structures (TLSs) and T_{regs} in breast cancer. Here, we show that CD169$^+$ TAMs can be monocyte-derived and express a unique mediator profile characterized by type I IFNs, CXCL10, PGE_2 and inhibitory co-receptor expression pattern. The CD169$^+$ monocyte-derived macrophages (CD169$^+$ Mo-M) possessed an immunosuppressive function *in vitro* inhibiting NK, T and B cell proliferation, but enhanced antibody and IL6 secretion in activated B cells. Our findings indicate that CD169$^+$ Mo-M in the primary breast tumor microenvironment are linked to both immunosuppression and TLS functions, with implications for future targeted Mo-M therapy.

KEYWORDS

breast cancer, macrophage, CD169, tolerance, type I IFN, B cell, TLS

1 Introduction

Macrophages are a heterogeneous population of innate immune cells. They can be divided into resident macrophages, originating from the yolk sac, liver, or bone-marrow during the fetal stage (1, 2), or recruited macrophages that are monocyte-derived (2, 3). The characteristic chronic inflammatory microenvironment in a tumor result in the majority of

tumor-associated macrophages (TAMs) being monocyte-derived recruited macrophages (Mo-M) (2–4). TAMs are generally associated with worse prognosis for cancer patients (3). However, tumor infiltration of resident macrophages as an alternative source of TAMs has also been shown (2–4). The tumor microenvironment may affect the polarization of TAMs differently, leading to a plethora of subclasses of TAMs with a range from pro- to anti-inflammatory functions. To successfully target TAMs in cancer patients, it is becoming urgently important to understand the biology of various TAM subpopulations with regard to origin, phenotype, function, and the microenvironmental signals (localization, cellular microenvironment, or tumor type) that affect these traits.

Macrophages are generally associated with a worse prognosis in cancer patients. There is however one clear exception, the lymph node CD169$^+$ resident macrophages. Presence of resident CD169$^+$ macrophages in lymph nodes has been correlated to an improved prognosis in patients with a variety of cancer types (5–9). The exact role of CD169$^+$ macrophages in cancer patients remains unknown. Lymph node resident CD169$^+$ macrophages can be divided into two distinct populations, the subcapsular sinus-macrophages (CD169$^+$CD163$^-$) and the medullary macrophages (CD169$^+$CD163$^+$), with somewhat varying functions (10, 11). While CD169$^+$ subcapsular sinus macrophages are derived from fetal yolk sac or recruited from monocytes during adult life, less is known about the origin of CD169$^+$ medullary macrophages (12, 13). The CD169$^+$ medullary resident lymph node macrophages are efficient at sensing lipids, pathogen clearance, phagocytosis, and at inducing tissue destruction (13, 14). The role of CD169$^+$ subcapsular sinus macrophages is to act as gatekeepers for soluble, lymph-borne, particulate antigens (virus, bacteria or tumor antigens), to deliver antigens to activate B cells present in the lymphoid follicles, and they are also assigned as crucial antigen-presenting cells (APCs) for high-affinity B cell responses (15, 16). In mice, lymph node CD169$^+$ macrophages have been associated with both activating (B, T and NK cell activation), and regulating (T_{regs}) immune responses (17–21). In viral infections CD169$^+$ subcapsular sinus macrophages induce type I IFNs that promote PDL1 expression, resulting in a local T cell exhaustion (20).

We recently showed that CD169$^+$ macrophages are found in primary breast tumors (CD169$^+$ TAMs), co-localize with the expression of PDL1 (9) and are spatially associated with tertiary lymphoid like structures (TLLS) and T_{regs} (22). While the CD169$^+$ TAM/TLLS infiltration in primary tumors associated to a worse prognosis for breast cancer patients, their presence in metastatic lymph nodes were contrastingly associated to a beneficial prognosis (9, 22). These intriguing findings led us to here investigate the role for CD169$^+$ TAMs in the primary breast tumor environment and their functional relation to other infiltrating immune cells. We show that human CD169$^+$ TAMs in breast cancer can be monocyte-derived macrophages with broad immunosuppressive functions. In conjunction with activated B cells however, they promote B cell antibody and IL6 secretion. Our findings illuminate the role for CD169$^+$ TAMs in primary breast cancers and may explain the spatial association between CD169$^+$ TAMs and TLSs found in primary tumors and lymph node metastases.

2 Material and methods

2.1 Breast cancer patients and tumor tissue microarray

Two breast cancer patient cohorts were used for this study, hereafter referred to as the large and small cohort. The large breast cancer cohort presented in this study consisted of 304 patients diagnosed with locally advanced, inoperable, or metastatic breast cancer in Sweden between 2002 and 2007 included in the randomized phase III trial (TEX) (23). A detailed description regarding the trial and the patient cohort has been described previously (22, 24–26). Ethical approval was obtained from corresponding Regional Ethics committees in Sweden of each of the clinics involved in the trial (23–26). Primary tumor material from 231 patients, ages ranging from 27 to 71 years of age, was included in the final analysis due to missing clinicopathological information or low quality of TMA cores for the remaining cases.

The small breast cancer cohort presented in this study consisted of 23 patients diagnosed with invasive primary breast cancer with lymph node and/or distal metastasis, at the South-Swedish Health Care Region between 1976-2005. The clinical material was collected retrospectively from paraffin embedded tissue. Ethical approval was obtained from Regional Ethic committee Lund, Sweden (Dnr 2010/477), according to the Declaration of Helsinki. ER-positivity was defined as >10%, in line with current diagnostic routines in Sweden. Cores from primary tumor, lymph node metastasis and/or distal metastasis were collected and mounted in a tissue microarray (TMA).

2.2 Immunohistochemistry

The cores were 1 mm Ø (small cohort) or 0.6 mm Ø (large cohort), and blocks were sectioned at a thickness of 4 µm prior to mounting. TMA sections were automatically pre-treated using the PT Link system and then stained in an Autostainer Plus (DAKO) at pH9 with an overnight staining protocol. Immunohistochemical (IHC) staining was performed on sections using antibodies specific for B-cells (CD20; dilution 1:100; Abcam; clone L-26), T-cells (CD3; dilution 1:100; Abcam; clone 11084), CD169$^+$ macrophages (CD169$^+$; dilution 1:100; Invitrogen; clone SP216), NK-cells (CD56; dilution 1:100; Novus Biologicals (Centennial, CO, USA); clone NBP2-34280) and a TripleStain IHC kit was used (Abcam, Cambridge, UK). For double CD169/PDL1 staining of xenografts the antibodies anti-CD169 (dilution 1:500, Spring M5160) and anti-PDL1 (dilution 1:500, Cell Signaling 29122) and as secondary antibody staining protocol, a Double Stain Polymer Kit from Nordic Biosite (anti-mouse HRP (brown) and anti-rabbit AP (pink)) was used according to the manufacturer´s guidelines. The glass slides were fixed and mounted using xylene and Cyto Seal (DAKO). All material was scanned using Aperio slide scanner (Leica Biosystems). The material could then be viewed in Aperio ImageScope (v.12.4.3.5008). Separate staining and annotation for CD3 (T cells) had been performed previously (26). For immunofluorescence (IF), anti-mouseCD169 (Alexa488-conjugated;

clone 3D6.112; Biolegend) and -F4/80 (Alexa647-conjugated; clone BM8; Biolegend) was used on frozen sections from mouse.

2.3 Animal procedures and the NSG co-xenograft model

The paraffin embedded NSG co-xenograft material presented in this study, originated from our previously performed NSG co-xenografts (27). Briefly, female 8-week-old NSG mice (NOD.Cg-Prkdc(scid)Il2rg(tm1Wji)/SzJ strain, The Jackson Laboratory, USA) were housed in a controlled environment. Mice were anesthetized by isoflurane and injected with human breast cancer cells (SUM159) or (MDA-MB-231) at $1x10^6$ cells/mouse on the right flank, alone or in combination with primary human monocytes ($1x10^6$ cells/mouse) as previously described (27). Tumors were excised on day 21 after injection and subsequently fixed in 4% paraformaldehyde and embedded in paraffin. Five (N=5) mice were used in each group. All procedures were approved by the regional ethics committee for animal research at Lund University, Sweden (M11-15). Frozen sections of Balb/c spleen and 4T1-tumors were used for the IF, approved by the regional ethics committee for animal research at Lund University, Sweden (approval M149-14). For the 4T1-model, in brief $1x10^5$ 4T1 cells were injected in the mammary fat pad of a Balb/c mouse and dissected on day 21. The animal work was performed in accordance with the ARRIVE reporting guidelines.

2.4 Isolation of primary human immune cells

Ethical permit for the use of human leukocytes was obtained from the regional ethical committee at Lund University (Dnr 2021/04792). Concentrated leukocytes were obtained from healthy donors. Ficoll-Paque Plus (GE Healthcare Bio-sciences) gradient was used to isolate peripheral blood mononuclear cells (PBMC). Monocytes, T cells, B cells and NK cells were isolated from PBMCs by magnetic cell sorting (MACS) using: Classical Monocyte Isolation kit, human; anti-CD3-FITC anti-FITC isolation for T cells, Naïve $CD4^+$ T cell isolation kit, human; B cell isolation kit II, human; and NK cell isolation kit, human (Miltenyi Biotec), according to manufacturer's protocol. T_{regs} were isolated using Dynabeads™ Regulatory $CD4^+CD25^+$ T cell kit (Invitrogen Thermo Fisher Scientific).

2.5 Cell cultures and Compounds

Monocytes were differentiated into M1-like, M2-like or M2/type I IFN induced $CD169^+$ macrophages, in OptiMEM supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) using recombinant human (rh) GM-CSF (10 ng/ml) for M1-like macrophages and rhM-CSF (10ng/ml) for M2-like and CD169 expressing macrophages for 5 days, followed by polarization for 2-3 days using: LPS (100ng/ml) and rhIFNγ (20 ng/ml) for M1-like; rhIL-4 (20 ng/ml) for M2-like; and rhIL-4 (20 ng/ml) and IFNα (670 units/ml) for CD169 expressing macrophages. Macrophages were grown in low adherent plates and harvested using non-enzymatic cell dissociation buffer (Sartorius). All cytokines were from R&D Systems, except for IFNα from PBL assay Science, USA. For co-culture experiments, primary macrophages were harvested on day 7 of culture, reseeded in 96 well plates, and incubated with freshly isolated lymphocytes. All co-cultures were performed in OptiMEM media. For T cell suppression assay (TSA); naïve $CD4^+$ T cells were activated using CD3/CD28 Dynabeads™ (Gibco), and then plated with macrophages at stimulator-responder ratio ranging from 1:2 to 1:8. For mixed lymphocyte reaction (MLR), macrophages and T cells were plated at a stimulator-responder ration ranging from 1:1 to 1:100, without addition of Dynabeads™. For B cell and B/T cell co-cultures, macrophages and lymphocytes were plated at a stimulator-responder ratio 1:5, without addition of Dynabeads™. Cells were incubated at 37°C for 5 days. For B_{reg} cell differentiation culture, macrophages were cultured with B cells for 2 days, while for B cell activation (plasma cell differentiation cultures), B cells were pretreated with anti-IgM for 4 hours, whereafter macrophages were co-cultured with B cells for 6 days as previously described (28), and as positive control for plasma cell differentiation CpG (2.5 µg/ml) (Invitrogen), IL-21 (50 ng/ml) and CD40L (1 µg/ml) (R&D Systems) was added. Inhibitors for HLA-G (10 µg/ml) (HLA-G monoclonal antibody, Thermo Fisher) and PDL1 (10 µg/ml) (Atezolizumab, Chemtronica AB) were added on first day of incubation and on day 3. ^3H incorporation was measured using 1 µl Ci [methyl3H] Thymidine (PerkinElmer) for 18h, a MicroBeta Filtermat-96 Cell Harvester (PerkinElmer) and a Wallace 1450 MicroBeta TriLux Liquid Scintillation and Luminescence counter (PerkinElmer).

2.6 Functional cell assays

For cytotoxicity assay, lactate dehydrogenase (LDH) activity was measured using a Cytotoxicity detection kit (Roche Diagnostics) according to manufacturer's protocol. For pinocytosis assay cells were incubated with 0.25 mg/ml FITC-Dextran (Sigma-Aldrich) at 37°C for 20 minutes and subsequently analysed using flow cytometry. TLR3 agonist Polyinosinic-polycytidylic acid sodium salt (Poly(I:C)) (20 µg/ml) (Sigma-Aldrich) was added to Mo-M cultures on day 5. For migration assay of T cells, T_{regs} and B cells, a SPLInsert™ Hanging 3µm pore size (SPL Life Sciences) migration chamber was used. $2x10^5$ isolated T cells, T_{regs} or B cells were allowed to migrate towards conditioned media from M2 or M2/IFN treated $CD169^+$ Mo-M for 18h h, or serum as positive control, with subsequent 4% PFA fixation of transmigrated cells and subsequent Cytospin with H/E staining was performed prior to counting. For cell lines, MDA-MB-231 (ATCC) and SUM159 (a kind gift from Professor S. Ethier (27) and bought from BioIVT, NY, US) TNBC breast cancer cells were used.

2.7 Flow cytometry, chemokine and cytokine assays

For flow cytometry, FcR Blocking Reagent (Miltenyi Biotec) and antibodies found in Supplementary Table 2 were used. All antibodies used were purchased from BD Biosciences and samples were run on a FACS Verse flow cytometer (BD Biosciences) with data analysis performed using FlowJo (Tree Star). Supernatants from macrophage cultures were collected on day 7-8, and cytokines were measured using a V-PLEX Human Cytokine 36-Plex (Meso Scale Diagnostics), or IL15 and IgG ELISA (R&D Systems), or for measuring levels of TNFα Human Inflammatory Cytokine bead array (BD Biosciences), all according to manufacturer's protocols. Gating strategies are shown in Supplementary Data File 1.

2.8 RNA extraction, cDNA synthesis and reverse transcription qPCR (RT-qPCR)

Total RNA was extracted and purified using total RNA purification kit (Norgen Biotek Corp) and RevertAid RT Reverse Transcription Kit (Thermo Scientific) was used to generate cDNA according to manufacturer's protocols. qRT–PCR was performed in triplicates using Maxima SYBR Green/Rox (Thermo Scientific) and the Mx3005 P QPCR system (Agilent Technologies), and the relative mRNA expression was normalized to *GAPDH*, *SDHA* and *YWHAZ* housekeeping genes and calculated using the comparative Ct method. List of primer sequences can be found in Supplementary Table 3.

2.9 Nanostring GeoMX

The proteome analyses were performed on CD169$^+$ cells adjacent to lymph node metastases from the small TMA cohort using the Nanostring kits; Solid tumor TME kit, Immune cell profiling/IO drug target/Immune activation status/Immune cell typing - cores, together with a labelled CD169 antibody using the Alexa Fluor^{TM} 647 Antibody labeling kit (Invitrogen Thermo Fischer Scientific), all according to manufacturer's instructions.

2.10 Statistical analysis

Student's t-test, paired ratio t-test or Analysis of variance (ANOVA) according to figure legends were performed using Graph Pad Prism software. Pearson Chi Square and Linear by Linear association were performed using IBM SPSS Statistics version 26 (SPSS Inc). Correlation between *SIGLEC1* expression, the gene corresponding to CD169 and overall survival, and the correlation between *SIGLEC1* and *CXCL10, IL10, IFNA4* and *IFNB1* in the human breast cancer 1097 TGCA database was performed *via* R2: microarray analysis and visualization platform http://2r.amc.nl. Single cell analyses were performed using the public data set of Human breast tumor single cell RNA Seq data from miPanda (https://mipanda.med.umich.edu/gene/Coexpression) (29).

3 Results

3.1 Spatial association between CD169$^+$ TAMs and lymphocytes in breast cancer

We have recently shown that presence of CD169$^+$ TAMs in primary human breast tumors showed evidence for being associated with a worse prognosis (9, 22). These previous results are here supported by data using the TCGA database in R2: Genomics Analysis and Visualization platform (www.hgserver1.amc.nl), where high mRNA expression levels of *SIGLEC1* in primary human breast cancers correlated significantly with worse overall survival (Figure 1A) (P=0.020). The prognostic impact of *SIGLEC1* using TCGA differed slightly when categorizing into ER$^+$ (P=0.035) and ER$^-$ (P=0.098) tumors.

In our recent study we also showed that CD169$^+$ TAMs associated with B cells alone, TLLS, T_{regs} and a B_{reg} signature (22). To understand why CD169$^+$ TAMs in primary human breast tumors were associated to these cell types and also to a worse prognosis (22), we set out to expand our analysis on the spatial associations between CD169$^+$ TAMs and a broader panel of lymphocytes. Representative images for immunohistochemical (IHC) stainings are shown in Figures 1B–E. We performed stainings with antibodies specific for: CD169, CD3 (T cells), CD20 (B cells) and CD56 (NK cells) to investigate CD169$^+$ TAMs in relation to T cells and B cells (tertiary lymphoid like structures (TLLS; CD20$^+$ B cell clusters with CD3$^+$ T cells)); or CD169$^+$ TAMs in relation to NK cells and B cells. Supporting our recently published data using a large breast cancer cohort (22), CD169$^+$ TAMs associated significantly with TLLS also in a small breast cancer tissue array (TMA) test cohort consisting of 23 patients, (Supplementary Table 4; Pearson Chi-Square, Linear by Linear association *p=0.048*). CD169$^+$ TAMs did however not show any spatial associations with NK cells in the small breast cancer cohort (Pearson Chi-Square, Linear by Linear association p=0.449; Supplementary Table 4), indicating that NK cells and CD169$^+$ macrophages do not usually interact in primary tumors. We therefore did not proceed with further NK cell analysis in the large cohort. However, a significant spatial association between CD169$^+$ TAMs in the primary tumors (CD169 PT) and only T cells (CD3) was found using the large breast cancer cohort (Supplementary Table 5; Pearson Chi-Square, Linear by Linear association *p=0.018*).

Together with our previous findings we can summarize that tumor infiltrating CD169$^+$ TAMs in primary breast tumors are associated with TLLS, T_{regs} and B cells (22) and also with T cells alone as shown here in this study, but not with NK cells.

3.2 CD169$^+$ TAMs in breast cancer originate from monocytes

We next performed *in vivo* analyses of the cellular origin of CD169$^+$ TAMs. To this end, we performed immunohistochemistry on material from our previously published xenograft co-

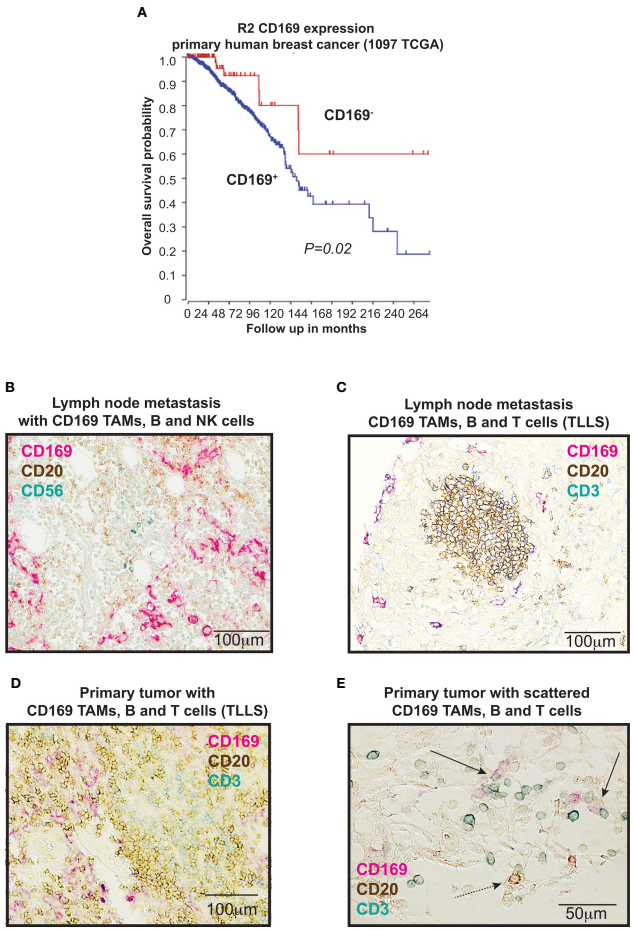

FIGURE 1
CD169$^+$ macrophages and lymphocytes in breast cancers. **(A)** Kaplan-Meier curves illustrating differences in overall survival according to CD169 expression in primary tumors of breast cancer patients using the TCGA data base in R2 (r2.amc.nl). *P* value by log-rank test. **(B-E)** Immunohistochemical (IHC) triple staining of breast tumor tissue array cohort (TMA), using specific antibodies for CD169, CD3 (T cells), CD20 (B cells), and CD56 (NK cells). **(B)** Staining of lymph node metastasis for CD20 (brown), CD169 (red) and CD56 (blue). **(C)** Staining of lymph node metastasis for CD20 (brown), CD169 (red) and CD3 (blue). **(D)** Primary tumor tertiary lymphoid like structure (TLLS) with presence of CD169$^+$ tumor associated macrophages (TAMs) stained for CD20 (brown), CD169 (red) and CD3 (blue). **(E)** Magnification of primary tumor with TLLS with presence of CD169$^+$ TAMs, T cells and B cells, stained for CD20 (brown), CD169 (red) and CD3 (blue). Solid black arrows point to CD169$^+$ TAMs (red) in contact with CD3$^+$ T cells (blue), while dashed black arrow points to CD169$^+$ TAM in contact with CD20$^+$ B cell.

transplantations using primary human monocytes and the human triple negative breast cancer (TNBC) cell lines, SUM159 and MDA-MB-231 (27), in NSG mice (Figures 2A, B and Supplementary Figure 1A). NSG mice lack functional lymphocytes, have defective macrophages and dendritic cells as a consequence of common gamma chain (γ_c) deletion, but produce monocytes and neutrophils (30). TNBCs are generally associated with TAM infiltration and PDL1 expression (9, 31, 32). SUM159 and MDA-MB-231 tumor cells in xenografts express PDL1 (Figure 2A left and Supplementary Figure 1A). When SUM159 tumor cells were co-transplanted with primary human monocytes for 21 days, these monocyte-derived TAMs upregulated CD169 and potentially co-expressed PDL1 (Figures 2A, B right), indicating that human CD169$^+$ TAMs can be monocyte-derived. The expression of CD169 was however not seen in the other TNBC xenograft using MDA-MB-231 cells (Supplementary Figure 1A), indicating that

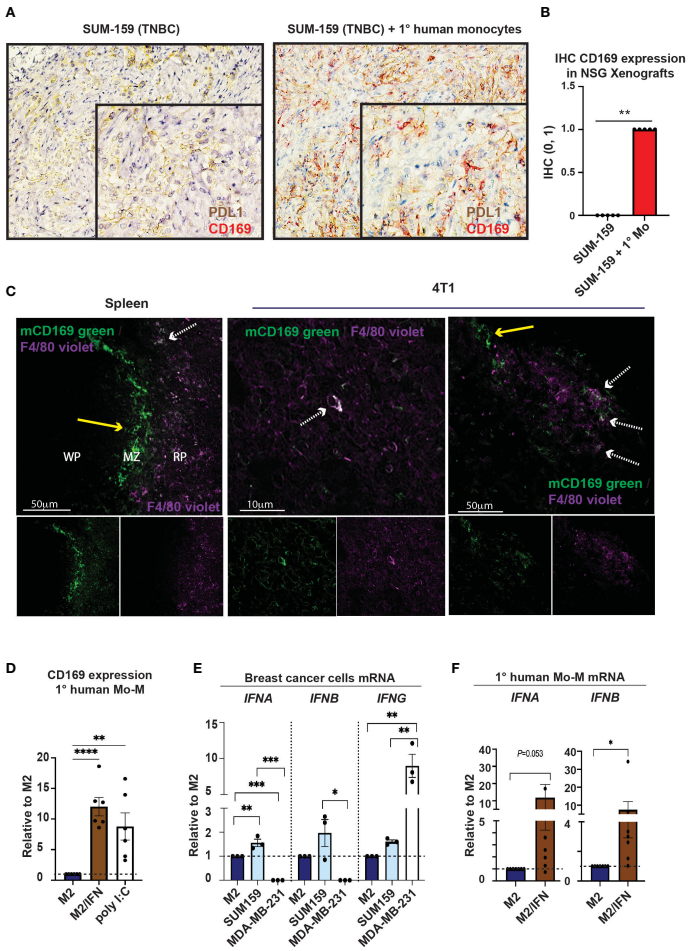

FIGURE 2
Monocytes can give rise to CD169$^+$ TAMs. **(A)** Tumor xenografts in NSG mice were performed previously (27). Primary human monocytes were co-transplanted with SUM159 breast cancer cell lines in NSG mice for 21 days. Controls were transplanted with SUM159 cells alone. CD169$^+$ cells (red) were only seen in the SUM159 + monocyte xenografts (right) while PDL1 (brown) was seen in both SUM159 (left) and SUM159 + monocyte (right) xenografts, with CD169/PDL1 co-expression observed in co-transplanted xenografts (right). **(B)** Immunohistochemistry statistics of **(A)** using Mann-Whitney t-test, N=5 in each group, ** $p < 0.01$. **(C)** Immunofluorescence (IF) staining of a Balb/c mouse spleen (left) and 4T1 tumor model (middle, right). mCD169 shown in green and F4/80 in purple. Arrows point to macrophages only positive for mCD169 (dashed white), or double positive for mCD169 and F4/80 (yellow; approximately 10-20% of the CD169$^+$ TAMs), indicating CD169$^+$ infiltrating macrophages of both monocyte-derived and possibly resident origin. **(D)** Surface expression of CD169 on primary human monocyte-derived macrophages with addition of type I IFN or the TLR3 ligand Poly(I:C) on day 5 of culture, compared to M2 cultured macrophages as a negative control, N = 6. **(E)** Relative mRNA levels of *IFNB* and *IFNB* in breast cancer cells (SUM159 and MDA-MB-231) and compared to M2 primary human monocyte-derived macrophages (Mo-M) as measured by RT-qPCR, N = 3. **(F)** Relative mRNA levels of *IFNB* and *IFNB* in primary human Mo-M as measured by RT-qPCR, N = 7. For D-E panels: One-way ANOVA multiple comparison Dunnett's test. For panel F: Ratio paired t-test. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

different TNBC tumor cells and microenvironments may have different effects on CD169 upregulation.

To investigate the potential cellular origin of murine CD169$^+$ TAMs in breast tumors, we used the immunocompetent and syngeneic murine breast cancer model 4T1 (Figure 2C middle and right). In mice, monocyte-derived macrophages (Mo-M) express F4/80 at fluctuating levels during maturation but are often F4/80$^{-/low}$ (33). CD169$^+$ resident lymph node subcapsular

sinus and spleen marginal zone macrophages respectively, express high levels of mCD169 but lack or express low levels of the murine macrophage marker F4/80, while CD169$^+$ resident lymph node medullary/spleen red pulp macrophages express F4/80 (33, 34). We found that approximately 10-20% of CD169$^+$ TAMs present were also positive for F4/80 (F4/80$^+$; white dashed arrow), but they were mostly negative for F4/80 (F4/80$^-$; yellow arrow), indicating infiltrating macrophages of Mo-M origin or possibly resident origin (Figure 2C middle and right). Balb/c mouse spleen was used as a staining control (Figure 2C left), showing CD169$^+$ white pulp (WP) and marginal zone (MZ) macrophages being F4/80$^{-/low}$ (34) (yellow arrow; Figure 2C left), and red pulp (RP) macrophages being F4/80$^+$ (white dashed arrows; Figure 2C left).

In summary, a recruited monocyte-derived origin of human CD169$^+$ TAMs in breast tumors is likely, but resident-recruited macrophages should not be disregarded (33). Furthermore, the breast cancer type may affect CD169 upregulation on Mo-M differently depending on the microenvironment and mediators being produced.

3.3 Type I IFN is associated with CD169$^+$ Mo-M

To understand what causes the unique CD169$^+$ phenotype on distinct TAM populations in human breast tumors (9, 22), we next evaluated different inflammatory or tumor-derived mediators on primary human Mo-M, in an *in vitro* M2 tumor microenvironment-setting. We selected relevant mediators that would be able to induce expression of CD169. Type I IFNs have previously been shown to induce CD169 on macrophages (35). CD169$^+$ subcapsular sinus macrophages are further themselves high producers of type I IFNs in viral immune responses (20, 36) and found responsible for the PDL1 expression on nearby cells (20), a feature that would fit with the CD169$^+$/PDL1$^+$ TAM phenotype observed in human breast tumors. As expected, the Mo-M upregulated CD169 specifically in the M2 tumor microenvironment/type I IFN setting (M2/type I IFN) (Figure 2D; CD169$^+$ Mo-M). The type I IFN inducer TLR3 ligand Polyinosinic:polytidylic acid (Poly(I:C)) also induced CD169 expression on Mo-M (37, 38) (Figure 2D). Using qPCR we could show that endogenous *IFNA* and *IFNB* was expressed at very low levels in the breast cancer cell line SUM159, but not in MDA-MB-231 cells (Figure 2E), whereas *IFNG* was more expressed in MDA-MB-231 cells (Figure 2E). We could further show that CD169$^+$ Mo-M are actually capable of expressing type I IFNs (*IFNA* and *IFNB*) themselves *in vitro* whereas M2 macrophages did not (Figure 2F), however at lower levels than monocytes and M1 macrophages (Supplementary Figure 1B). A possible relationship between type I IFNs and CD169-expression on Mo-M was supported by mRNA data from primary human breast tumors, where mRNA expression for the gene *SIGLEC1* encoding CD169 significantly correlated with *IFNA4* (P=6.25e-04) and *IFNB1* (P=5.53e-41) (Supplementary Figure 1C). Although M1 macrophages expressed type I IFNs, they did not upregulate CD169 (Figure 2D, Supplementary Figure 1B and Supplementary Data File 1), indicating the type I IFN primarily led to CD169 upregulation in an M2/IFN environment like tumors.

Together this indicates that CD169 can be induced on recruited monocyte-derived macrophages (CD169$^+$ Mo-M) in a breast tumor microenvironment, and that this is associated with type I IFN production.

3.4 CD169$^+$ Mo-M have a nique phenotype

We next set out to perform a broad phenotypic analysis of CD169$^+$ macrophages in primary human breast tumors compared to those in lymph node metastases. Proteome analysis of CD169$^+$ macrophages in lymph nodes with breast tumor metastases using Nanostring GeoMX (Figure 3A) were compared to gene expression of the CD169$^+$ (*SIGLEC1*) clusters in a public data set of Human breasts tumor single cell RNA Seq data from the Michigan Portal for the Analysis of NGS Data (MiPanda) (29) (Figure 3B). The primary breast cancer CD169$^+$ TAMs were too few to analyse using the chosen Nanostring GeoMX proteome analysis method. We could however show, that CD169$^+$ macrophages in association with lymph node metastasis expressed CD163. They also expressed higher levels of the proteins STING, CD80, VISTA, IDO1 and Ox40L, in relation to CD45$^+$ cells in general, and the inhibitory co-receptors PD-L1, B7H3, LAG3 and Tim-3. Of note, in the proteome analysis we compared relative protein expression of CD169$^+$ macrophages, with CD45$^+$ expressing cells located in follicles in general (Figure 3A). The majority of CD45$^+$ cells in follicle areas are B cells. This can explain the seemingly low expression levels of *HLADR* and *CD40* on primary breast cancer CD169$^+$ TAMs in the proteome analysis (Figure 3A) since B cells express high levels of HLADR and CD40 in general. HLADR and CD40 is therefore probably expressed at similar levels on CD169$^+$ macrophages and B cells, being antigen presenting cells (APCs) (Figure 3A). In the Human primary breast tumor single cell RNA Seq data CD169$^+$ (*SIGLEC1*) cluster (Figure 3B), the corresponding genes were also expressed, shown using the public data set of Human breast tumor single cell RNA Seq data from miPanda (https://mipanda.med.umich.edu/gene/Coexpression (29)), as was the gene for *MARCO*.

The *in vitro* generated CD169$^+$ Mo-M showed a similar cell surface phenotype with prominent PDL1 expression (Figure 3C and Supplementary Figure 1D), slight CD163 expression (Figure 3C), slightly higher levels of STING and VISTA (Figure 3D), and significantly higher levels of Ox40L (Figure 3D), CD80 and HLA-DR (Figure 3E), in relation to M2-like macrophages. Indeed, the CD169$^+$ Mo-M also showed a mixed macrophage cell surface phenotype representing both M1- and M2-like macrophages (CD14^{hi}HLADR^{hi}CD80^{hi}CD1a$^-$CD206$^-$PDL1$^+$CD163$^{-/+}$) as seen in Figures 3C, E and Supplementary Figures 1E, F and Supplementary Data File 1.

Together this suggests that CD169$^+$ macrophages generated from monocytes in a type I IFN tumor microenvironment *in vitro* (CD169$^+$ Mo-M), possess a unique phenotype, much resembling CD169$^+$ TAMs in breast tumors and CD169$^+$ lymph node macrophages.

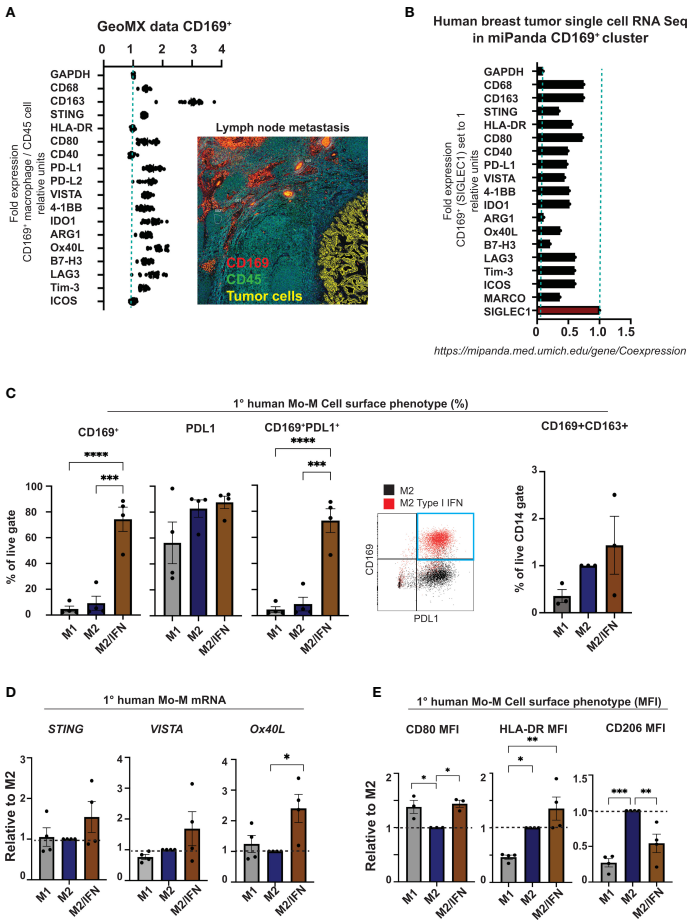

FIGURE 3
CD169$^+$ macrophage phenotype analysis. **(A)** Nanostring GeoMX proteome analysis of CD169$^+$ macrophages in lymph node with breast cancer metastases. Regions of CD169$^+$ macrophages (red) adjacent to metastasis (yellow) in lymph node were chosen, and expression levels of proteins presented in panel **(A)** were compared in relation to their expression in nearby areas of CD45$^+$ cells (green) in follicle structures, representing mostly B cells. **(B)** Single-cell RNA Seq data showing the corresponding genes from panel **(A)** in the CD169$^+$ macrophage cluster (CD169$^+$ TAMs) from the Human breast cancer data set in miPanda (https://mipanda.med.umich.edu/gene/Coexpression) (29). **(C)** Comparison of CD169 surface expression (left), PDL1, co-expression of CD169 and PDL1 (centre) or CD169 and CD163 (right) on primary human monocyte-derived macrophages. Representative dot-plot showing expression of CD169 and PDL1 on M2 treated control primary human macrophages (black) compared to IFN treated (red) (right), N = 4 and N = 3. **(D)** Relative mRNA expression of *STING, VISTA* and *OX40L* on primary human monocyte-derived macrophages (N=4). **(E)** Ratio of MFI of cell surface markers CD80, HLA-DR and CD206 on human primary macrophages with M2 as control, N = 4. For flow cytometry gating strategies see Supplementary Data File 1. For figures C-E: One-way ANOVA multiple comparison Dunnett's test. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.5 CD169$^+$ Mo-M have a distinctive mediator profile

The cytokine and chemokine profile of CD169$^+$ MoM was next analysed using the V-PLEX system (Figures 4A, B and Supplementary Table 1). The *in vitro* type I IFN/M2 tumor microenvironment generated CD169$^+$ Mo-M with a distinctive chemokine profile in comparison to paired donor M2 macrophages. Of the 36 cytokines and chemokines analysed, CXCL10 showed a pronounced, significant upregulation and IL15 was slightly upregulated, while CCL2, CCL17 and IL6 were secreted at a notably higher level by CD169$^+$ Mo-M although not significant (Figures 4A, B and Supplementary Table 1). In contrast CCL3, CCL4 and CCL22 were all secreted at lower levels (Figure 4A and Supplementary Table 1). Using independent methods,

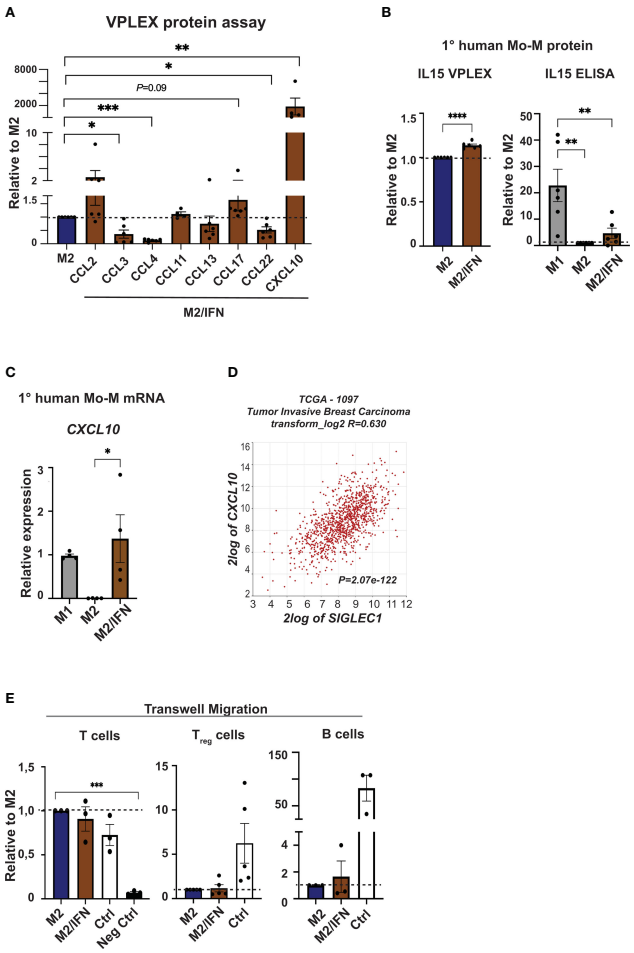

FIGURE 4
Chemokine profile of CD169$^+$ Mo-M. **(A)** Chemokine analyses were performed using V-PLEX, on supernatants from M2 and M2/IFN (CD169$^+$ Mo-M). CCL2, CXCL10, CCL11 and CCL17 were induced in the CD169$^+$ Mo-M, while CCL3, CCL4, CCL13 and CCL22 were downregulated. **(B)** Cytokine secretion measured with VPLEX of IL15, N = 6. Cytokine secretion of IL15 as measured by ELISA, ratio of concentration with M1 as control, N = 6. **(C)** Relative mRNA expression of and *CXCL10* as measured by RT-qPCR, N = 4. **(D)** *SIGLEC1* association to *CXCL10* mRNA expression in primary tumors of breast cancer patients using the TCGA data base in R2 (r2.amc.nl). **(E)** Transwell migration assay of isolated T cells, T_{regs} and B cells towards serum free conditioned media from M2 or M2/IFN (CD169$^+$ Mo-M), with serum as positive control. For panels A-B and E: Ratio paired t-test. For panel D: One-way ANOVA multiple comparison Dunnett's test. Error bars indicate SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

we observed that CXCL10 expression was further significantly upregulated in the CD169$^+$ Mo-M at mRNA levels (Figure 4C) and confirmed results for IL15 and IL6 as measured by ELISA or CBA (Figure 4B and Supplementary Figure 2A). Interestingly, while M1 macrophages secreted high levels of TNFα, neither M2 nor CD169$^+$ Mo-M did (Supplementary Table 1 and Supplementary Figure 2B).

CXCL10 is a chemokine that attracts T cells to tumor sites and is induced by IFNγ and Type I IFNs (39–41). We could show that *CXCL10* is strongly associated with *SIGLEC1* expression in breast cancer specimens in primary tumors using the TCGA database in R2 (r2.amc.nl) (R=0.630, *P*=2.07e-122; Figure 4D). Using the Michigan Portal for the Analysis of NGS Data (MiPanda), we saw that *CXCL10* was again highly associated with *SIGLEC1* in primary breast cancer (Pearson correlation *P=2.53e-12*) while it was not associated in normal breast tissue (Pearson correlation *P=0.66*) (29). Nevertheless, the CD169$^+$ Mo-M did neither attract T cells or T_{regs} more than M2-like macrophages *in vitro*, nor B cells according to transwell migration assays performed using supernatant from

cultured M2-like macrophages, CD169$^+$ Mo-M or a serum control (Figure 4E).

In summary, our data indicate that CD169$^+$ Mo-M produce high levels of the chemokine CXCL10, but do not attract T cells, T_{regs} nor B cells more than M2-like macrophages, ruling out that the spatial association found between CD169$^+$ TAMs, TLLS and T_{regs} is caused by chemotactic processes alone.

3.6 CD169$^+$ Mo-M have immunosuppressive functions

We next asked which functional phenotype the CD169$^+$ Mo-M generated *in vitro* in an M2/type I IFN tumor microenvironment setting would have. We analysed pinocytic and immune-activation or suppression capacity in relation to T cells, B cells and NK cells. Firstly, the CD169$^+$ Mo-M had a significantly reduced pinocytic capacity compared to M2-like macrophages, but still slightly better than M1-like macrophages (Figure 5A). Co-culture of macrophages with MDA-MB-231 breast cancer cells, did not show cytotoxic activity for CD169$^+$ Mo-M as compared to M1-like macrophages (Figure 5B), indicating an M2-like function. Using co-cultures of macrophages and autologous NK cells together with MDA-MB-231 breast cancer cells revealed that presence of CD169$^+$ Mo-M significantly reduced NK cell tumoricidal capacity, in contrast to presence of M1-macrophages (Figure 5C). The CD169$^+$ Mo-M further acted immunosuppressive in relation to T cells (Figures 5D, E), a typical M2-like function. Importantly, the CD169$^+$ Mo-M even acted immunosuppressive towards non-activated B cells and B/T cell co-cultures, a trait that neither M1- or M2-like macrophages had (Figure 5F). This inhibitory effect was not caused by graft versus host cytotoxicity as CD169$^+$ Mo-M did not kill allogeneic CD4$^+$ T cells (Figure 5G).

In summary, given that the CD169$^+$ TAMs in breast cancer associate with lymphocytes and TLSs in primary tumors, our functional data propose that CD169$^+$ TAMs do not promote NK, T or B cell proliferation or activation, but rather inhibit them.

3.7 CD169$^+$ Mo-M express immunosuppressive mediators

To investigate possible immunosuppressive mediators expressed by the CD169$^+$ Mo-M, other than PDL1 and Ox40L (Figure 3), we next performed ELISA and qPCR analyses of various NK, T and B cell inhibitory effector molecules (Figure 6). The NK and T cell inhibitory mediators PGE2 (*PTGES2)* (42, 43) and HLA-G (*HLA-G)* (44) were both specifically upregulated at mRNA level in the CD169$^+$ Mo-M, compared to both M1- and M2-like macrophages (Figure 6A). Arginase (*ARG1*) and Indoleamine 2,3-dioxygenase (*IDO1*) were not significantly upregulated at mRNA level in the CD169$^+$ Mo-M (Figure 6A), also in line with the single cell data in Figure 3. The increased level of IDO1 in M1 macrophages can be explained by the fact that IFNγ upregulates IDO1 on M1-like macrophages (45). Inhibition of HLA-G or PDL1 did however not alleviate the suppressive effect that CD169$^+$ Mo-M had on T cells (Figure 6B), nor affect NK cell cytotoxicity in co-cultures with macrophages and breast cancer cells (Supplementary Figure 2D). Instead, the immunosuppressive cytokine *IL10* mRNA was significantly upregulated by CD169$^+$ Mo-M as compared to both M1 and M2-macrophages (Figure 6C). This finding was supported by a strong correlation between *IL10* and *SIGLEC1* expression in primary tumors from breast cancer patients using the TCGA data base in R2 (r2.amc.nl) (R=0.63; P=1.54e-122) (Figure 6D), but not by the IL10 V-PLEX protein analysis data due to low detection levels (N=3; Supplementary Table 1), a finding that also could be explained by natural polymorphism in the human *IL10* gene promoter (46). The ROS inhibitor Catalase alleviated the suppressive effect caused by the CD169$^+$ Mo-M on T cell proliferation, as compared to M2-like macrophages (Figure 6E).

Together this indicates that CD169$^+$ Mo-M generated in a tumor microenvironment *in vitro*, act immunosuppressive in relation to NK and T cells *via* typical M2-like mediators (PGE2, ROS and IL10).

3.8 CD169$^+$ Mo-M promote IgG and IL6 secretion by activated B cells

M2-like mediators (PGE2, ROS and IL10) may affect the accumulation or differentiation of T_{regs}. However, *in vitro* CD169$^+$ Mo-M did not promote T_{reg} differentiation (Supplementary Figure 2D) nor IL10-producing B_{reg} cells (Supplementary Figure 2E). We finally co-cultured the CD169$^+$ Mo-M with previously activated primary human peripheral blood B cells for six days, to investigate their effect on B cell activation and differentiation. To our surprise, we now found that the CD169$^+$ Mo-M promoted antibody (IgG) and IL6 secretion by activated B cells (Figures 6F, G), a feature that has previously been associated to spontaneous, local germinal centre formation (47).

This indicates that CD169$^+$ Mo-M are immunosuppressive, acting *via* typical M2-like mediators (PGE2, ROS and IL10), but at the same time aid in antibody and IL6 secretion from activated B cells. This would explain the functional reason for being localized near TLSs in breast tumors. It could also indicate that CD169$^+$ Mo-M and CD169$^+$ TAMs studied here, are indeed functionally similar to CD169$^+$ subcapsular sinus macrophages in lymph nodes.

4 Discussion

The importance of lymph node resident CD169$^+$ macrophages as beneficial immune cells in cancer patients has come to light lately (48). Although their role during viral infections is becoming clearer, there is still a large gap of knowledge regarding their mechanisms of action in cancer patients. Indeed, CD169$^+$ lymph node macrophages have been shown to have both immunogenic and tolerogenic functions (15–21, 35, 36, 49), thus more data is needed to understand their involvement in cancer. When CD169$^+$ macrophages are present in primary breast tumors (CD169$^+$ TAMs), they are closely linked to a worse prognosis (9, 22). It is still unknown whether resident CD169$^+$ lymph node macrophages in cancer patients are associated with a beneficial prognosis because

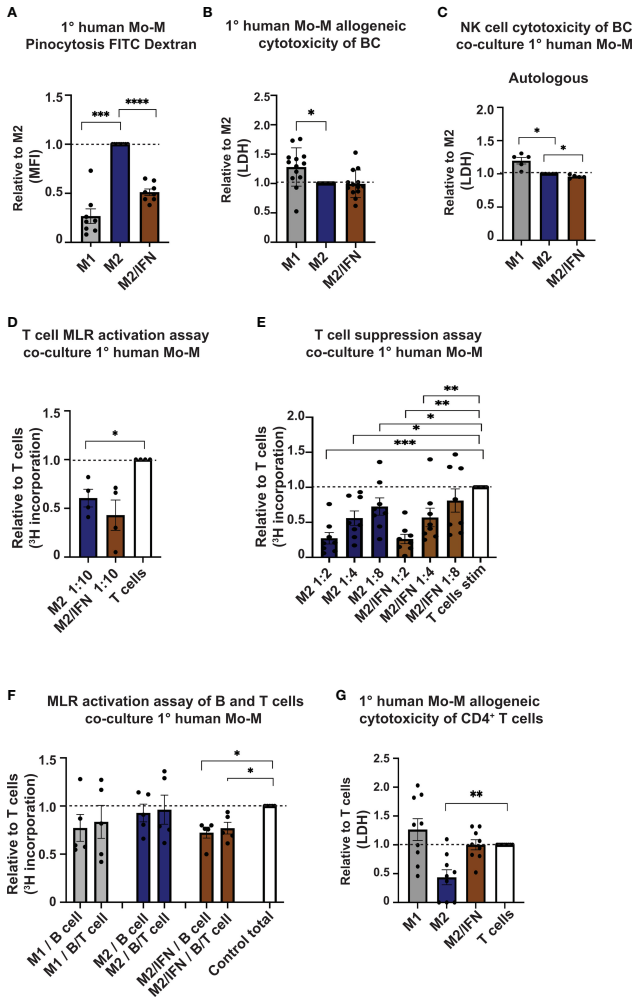

FIGURE 5
Immune suppressive functions of CD169$^+$ Mo-M. **(A)** Pinocytosis capacity as measured by FITC-Dextran uptake with M2 macrophages as control, N = 8. **(B, C)** Allogeneic Cytotoxicity assay as measured by LDH activity released from cytosol of damaged cells. **(B)** Primary human monocyte-derived macrophages cytotoxicity of MDA-MB-231 breast cancer cell line, N = 16. **(C)** Effect of primary human monocyte-derived macrophages on cytotoxicity by primary human autologous NK cells on MDA-MB-231 breast cancer cell line, N=5. **(D)** Allogeneic MLR of primary human monocyte-derived macrophages and primary human CD4$^+$ T cells as measured by [^{3}H] incorporation at ratio 1:10. Ratio with base T cell [^{3}H] incorporation, represented by dashed line, N = 3. **(E)** Allogeneic T cell suppression assay of primary human monocyte-derived macrophages and primary human CD4$^+$ T cells activated with CD3/CD28 beads. Dashed line represents base activated T cell [^{3}H] incorporation, N = 8. **(F)** Allogeneic MLR of primary human monocyte-derived macrophages and primary human B cells or B cells/CD4$^+$ T cells as measured by [^{3}H] incorporation at ratio 1:5, N=5. Ratio with base T cell or B cell [^{3}H] incorporation, represented by dashed line. **(G)** Cytotoxicity assay as measured by LDH activity released from cytosol of damaged cells of allogeneic primary human monocyte-derived macrophages cytotoxicity on CD4$^+$ T cells. N=9. For panels A-F: Ratio paired t-test. Panel G: One-way ANOVA multiple comparison Dunnett's test due to values 0. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

of their function, or simply because of their mere presence in lymph nodes at an early tumor stage, thus being a prognostic biomarker. In this study, we aimed to investigate the functional biology of CD169$^+$ TAMs in a breast tumor environment, in relation to lymph node resident CD169$^+$ macrophages, to understand why CD169$^+$ TAMs are associated with a worse outcome.

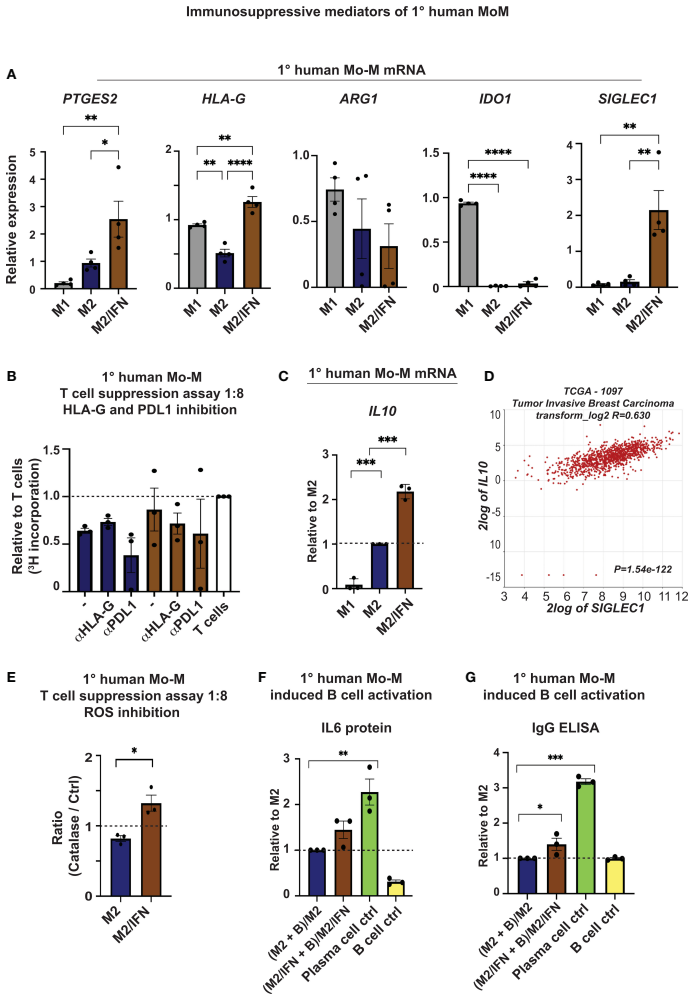

FIGURE 6
Immunosuppressive mediators of CD169$^+$ Mo-M. **(A)** Relative mRNA expression of PGE2 (*PTGES2*), HLA-G (*HLA-G*), Arginase (*ARG1*), Indoleamine 2,3-Dioxygenase 1 (*IDO1*) and CD169 (*SIGLEC1*) as control. N = 4. One-way ANOVA. **(B)** Allogeneic T cell suppression assay of primary human monocyte-derived macrophages and primary human CD4$^+$ T cells as measured by [^{3}H] incorporation at ratio 1:8 with M2 macrophages (blue) and M2/IFN macrophages (brown), with inhibitors for HLA-G (10 µg/ml) and PDL1 (Atezolizumab, 10 µg/ml). Ratio with base activated T cell [^{3}H] incorporation, represented by dashed line, N = 3, Ratio paired t-test. **(C)** Relative mRNA expression of *IL10* as measured by RT-qPCR, N = 3. **(D)** *SIGLEC1* association to *IL10* mRNA expression in primary tumors of breast cancer patients using the TCGA data base in R2 (r2.amc.nl). **(E)** Allogeneic T cell suppression assay of primary human monocyte-derived macrophages and primary human CD4$^+$ T cells activated with CD3/CD28 beads as measured by [^{3}H] incorporation at stimulator-responder cell ratio 1:8 with and without the ROS inhibitor Catalase. Dashed line represents base activated T cell [^{3}H] incorporation without the ROS inhibitor Catalase for each group, N=3. Paired t-test. **(F, G)** B cell activation cultures of primary human monocyte-derived macrophages and anti-IgM stimulated primary human B cells as measured by **(F)** IL6 and **(G)** IgG secretion using ELISA, N=3. One-way ANOVA multiple comparison Dunnett's test. For all figures: Error bars indicate SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

TAMs in primary tumors are generally associated with recruited Mo-M, of various alternative activation types (2, 3). The majority of TAMs in breast cancer express the typical M2 marker CD163 (50). Only a small minority of TAMs in primary human breast cancers express CD169 (9, 51). Why TAMs would adapt the CD169$^+$ phenotype is still unclear. What is clear as shown here however, is that two different TNBC xenografts, presumably having rather similar tumor microenvironments, can give rise to CD169

expression in one situation and not in the other. We suggest that CD169 expression on TAMs can be induced in a certain tumor microenvironment. Type I IFN signalling pathways and signalling leading to type I IFN production may be one cause. TLR3 signalling has previously been shown to induce antitumoral function of macrophages and upregulate secretion of inflammatory cytokines and chemokines such as CXCL10 (38) and type I IFN (37). Tumor specific ligands for TLR3 in the form of damage associated molecular patterns (DAMPs) released from tumor and necrotic cells have also previously been identified (52–54). In this present study we did observe an upregulation of CD169 on M2/Type I IFN treated primary human macrophages, as well as on TLR3 agonist Poly(I:C) treated primary human macrophages. M1 macrophages did however not upregulate CD169 despite showing expression of type I IFNs, indicating that CD169 is upregulated primarily in a tumor M2/IFN microenvironment. Which other specific mediators in the tumor microenvironment that induce CD169 expression on TAMs needs further investigation. We also propose that human $CD169^+$ TAMs in breast cancer can be monocyte-derived, just like other TAMs, but a resident origin should not be excluded. This is in line with previous data on hepatocellular carcinoma showing that tumor infiltrating $CD169^+$ macrophages originate from monocytes (55), and a study where $CD169^+CD14^+$ TAMs were characterized using CyTOF (56). Another study using the syngeneic 4T1 tumor model, argued that resident $CD169^+$ macrophages infiltrate murine mammary tumors (49). We here show using the same model that a proportion of them may indeed rather be monocyte-derived as judged by lack of F4/80, thus indicating a mixed origin. Nevertheless, the expression of CD169 seems to be rare, as compared to other TAM subpopulation markers, and associated with certain breast tumor environments.

The phenotype of $CD169^+$ TAMs and $CD169^+$ Mo-M indicates similarities to lymph node resident $CD169^+$ macrophages. $CD169^+$ TAMs may be $CD163^+$ just like the proteome and single cell data presented here suggest. However, our experience from working with breast cancers is that infiltrating $CD163^+$ TAMs are quite frequent, but $CD169^+$ TAMs are not, indicating that only a minority of the $CD163^+$ TAMs (M2-like TAMs) are $CD169^+$ (9, 51). A recent study presented similar data as ours regarding $CD169^+CD163^+$ TAMs in breast cancer, associating them with a worse prognosis but indicating a connection to TNFα production, which we did not find (57). Instead, we found expression of IL15 and CXCL10. IL15 is important for T and NK cell activity (58) a mechanisms that our $CD169^+$ Mo-M did not have *in vitro*, and for antitumor immunity (59) which does not support our prognostic data regarding $CD169^+$ TAMs in primary breast cancers (9, 22). As shown here, the $CD169^+$ TAMs did not associate with NK cells in breast tumors, thus ruling out an important functional relation between them in breast cancer. Of note, IL15 may also have immunosuppressive effects in a GM-CSF environment (60). CXCL10, which was secreted at high levels in our *in vitro* cultured $CD169^+$ Mo-M, has previously been correlated with infiltration of both $CD8^+$ and $FOXP3^+$ TILs, as well as $PDL1^+$ immune cells in breast cancer (61), but also with cell proliferation, migration and epithelial-mesenchymal transition of breast cancer cell lines. Most studies on CXCL10 in breast cancer have focused on CXCL10 expression in the breast cancer cells, rather than the effect of macrophage derived CXCL10. One exception is the tumor driven macrophage expression of CXCL10 in osteolytic bone metastasis that was associated with increased metastases (62). In a study using single cell RNA seq analysis of a breast cancer, *CXCL10* was also found to be expressed in a TAM subpopulation, but these TAMs did not express *SIGLEC1* (51), indicating the possibility of even further macrophage subtypes. Hence, CXCL10 could theoretically explain the correlation between $CD169^+$ cells and a worse prognosis found in our patient cohort. We also found slightly elevated levels of CCL17, a chemokine that recently was shown to be expressed in tumor associated tissue resident macrophages of NSCLC and linked to chemoattraction, differentiation and proliferation of T_{regs} (63). A broader analysis of CXCL10 and CCL17 in $CD169^+$ TAMs originating from various breast cancer subtypes and stages will be needed to assess their relationship in more detail.

When we analysed the spatial localization of $CD169^+$ TAMs in primary tumors in relation to other immune cells, we found that $CD169^+$ macrophages were spatially associated with T cells, B cells, tertiary lymphoid like structures (TLLS), immunosuppressive T_{regs}, and even a B_{reg} signature (22) in the primary tumor. TLS formation has previously been postulated to be important for anti-tumor immune reactions (64, 65), however we recently published that in patients with advanced breast cancer the opposite is seen (22). We speculated on whether this could be caused by presence of T_{regs} in the TLSs (22), a finding that previously has been described to associate with worse outcome for cancer patients, including breast cancer patients (22, 64, 66, 67). Our *in vitro* data indicate that in contrast to having an anti-tumoral function, the *in vitro* cultured $CD169^+$ Mo-M are immunosuppressive in relation to NK cells, T cells and non-activated B cells, with the three most likely inhibitory mediators being PGE_2, ROS or IL10, probably also involving the inhibitory co-receptors B7H3, LAG3 and Tim-3, here shown to be expressed by the $CD169^+$ macrophages. The immunosuppressive mechanism of $CD169^+$ TAMs is supported by a study using the CD169-DTA 4T1 tumor model, showing that $CD169^+$ macrophages induce tumor progression (49). A similar finding was shown in lung cancer models (68), but the opposite was found in glioblastoma (69) indicating a possible functional variation for $CD169^+$ TAMs between different tumor types.

Our findings also indicate that $CD169^+$ Mo-M promote antibody and IL6 secretion from *in vitro* activated B cells, which hints in the direction that $CD169^+$ macrophages actually may promote spontaneous germinal centre B cell formation locally (47). It is therefore likely that the association between $CD169^+$ TAMs and TLLS, T cells and T_{regs} in breast tumors has a functional interrelation, where $CD169^+$ TAMs could promote TLLS formations. The breast cancer patient cohort used in our study was from advanced breast cancer patients (23–26), where TLS and T_{regs} associated with a worse prognosis (22, 26), as did $CD169^+$ TAMs (22). It is therefore interesting to note that the $CD169^+$ Mo-M have functional similarities to lymph node resident $CD169^+$ subcapsular sinus macrophages, with both B cell stimulatory and immunosuppressive potential (15–21, 35, 36, 49). This would indicate that the spatial colocalization with TLSs in primary breast tumors is not a coincidence and that $CD169^+$ TAMs have unique functions in breast tumors. Alternatively, it is the B cells present in TLSs that could aid $CD169^+$ TAMs differentiation as has been shown in lymph nodes (70).

In conclusion, we propose that CD169$^+$ TAMs present in primary human breast cancer, are monocyte-derived macrophages generated in certain breast cancer microenvironments involving type I IFN signalling pathways. They possess immunosuppressive functions but simultaneously promote antibody and IL6 secretion by activated B cells. In advanced breast cancer patients, the CD169$^+$ TAMs associate with TLSs containing T_{regs}, with possible detrimental effects for these patients (22). The phenotypic and functional similarities between CD169$^+$ Mo-M and lymph node resident CD169$^+$ macrophages in cancer patients are intriguing and reflect a possible similar mode of action despite having opposite prognostic impact. The finding regarding opposite prognostic impact warrants further studies to understand whether lymph node resident CD169$^+$ macrophages actually possess anti-tumorigenic features, or whether they rather disappear from late-stage lymph nodes containing metastasis. Their beneficial prognostic impact would then be related to CD169$^+$ lymph node macrophages being present more often in lymph nodes of cancer patients with early-stage breast cancer, therefore linking them to a beneficial prognosis. More knowledge is therefore needed before we know whether CD169$^+$ macrophages should be viewed as a therapeutic target.

Data availability statement

Gene expression data were derived from the TCGA database in R2: Genomics Analysis and Visualization platform (www.hgserver1.amc.nl) and the public data set of Human breast tumor single cell RNA Seq data from the Michigan Portal for the Analysis of NGS Data (MiPanda) (https://mipanda.med.umich.edu/gene/Coexpression) (29).

Ethics statement

The studies involving human participants were reviewed and approved by the regional Ethics Committees in Sweden; for the small breast cancer cohort from Lund Dnr 2010/477, and for the large clinical trial cohort Stockholm, Dnr KI 02-206 and KI 02-205 (22–26). A written informed consent was received from the included patients in the clinical trials presented in this study. The study was conducted in accordance with the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Djurförsöksetiska nämnden i Malmö/Lund Lund University, Sweden.

Author contributions

The work reported in the paper has been performed by the authors. FG performed the majority of experiments with the help from RG, CR, MW and CM, while OB, AL and EK performed a substantial amount of the experiments. CA, and HV were responsible for the annotation of CD169, CD20, CD3 and CD56 IHC in the small TMA cohort. CH was responsible for collecting clinicopathological traits and outcome data for patients for the small TMA cohort. IH was responsible for collecting clinicopathological traits and outcome data for all patients for the large TMA cohort. MJ was responsible for professional guidance on performing and interpretations of IHC and annotations of TMAs. DB was responsible for performing the xenografts. OB and KL was responsible for managing the data analyses in SPSS. FG, OB, EK, CH, IH and KL were responsible for interpretation of final results. FG and KL was responsible for designing this study and drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

KL is a board member of Cantargia AB, a company developing IL1RAP inhibitors. This does not alter the Author's adherence to all guidelines for publication.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1180209/full#supplementary-material

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Supplementary tables:

Supplementary Table 1. V Plex assay with cytokines secreted by M2 like macrophages and M2/type I IFN ($CD169^+$ Mo-M) macrophages measured.

Cytokine/ Chemokine	M2 (pg/ml)	M2/type I IFN (pg/ml)	P value*
CCL2	18058,81	26258,18	0,088
CCL3	4408,45	544,15	0,30
CCL4	14568,58	2313,65	0,074
CCL11	31,36	52,70	0,091
CCL13	8110,83	2588,21	0,13
CCL17	1266,14	1800,93	0,059
CCL20	16,71	16,55	0,95
CCL22	74285,06	26530,56	0,25
CCL26	28,38	41,09	0,52
CXCL10	48,96§	34823,64	**0,005**
IFNγ	0,78#	3,83#	0,36
IL1α	164,41	178,74	0,50
IL1β	193,23	256,41	0,25
IL2	-	-	-
IL5	-	-	-
IL6	1007,46	1332,13	0,079
IL7	4,83	5,34	0,80
IL8	5475,78	5507,61	0,55
IL10	429,43#	423,01#	0,36
IL12p70	0,37#	0,01#	0,36
IL-12/IL-23p40	82,81	78,31	0,64
IL13	4,56	6,23	0,25
IL15	9,19	10,43	**0,0002**
IL16	87,54	84,54	0,40
IL17A	10,50	8,81	0,48
IL21	15,54	13,20	0,43
IL22	-	-	-
IL23	-	-	-
IL27	81,80	87,73	0,15
IL31	-	-	-
TNFα	123,37	152,31	0,49
TNFβ	0,83	1,42#	0,73
VEGF	0,30#	-	-

- : Not detected
* P-value calculated by paired t-tests N=6
# Undetectable in 5 out of 6 samples
§ Value below lowest standard but measurable

Supplementary Table 2. Flow cytometry antibodies

Antibody	Fluorochrome	Clone
CD169	PE	7-239
CD169	Alexa fluor 647	7-239
CD14	PECy7	M5E2
CD14	FITC	M5E2
HLA-DR	FITC	L243
IL15Ra	PE	JM7A4
IL15	APC	34559
CD1a	PE	HI149
CD206	APC	19.2
PDL1	APC	MIH1
CD163	PE	GHI/61
CD80	PE	L307.4

Supplementary Table 3. Primer sequences for RT-qPCR

Gene	Sequence
SIGLEC1	F: 5'-GGCTGTTACGATGGTTTATGATGT-3' R: 5'-AATCAAAGGCATCATTTTAGGGATA-3'
IFNA	F: 5'-GACTCCATCTTGGCTGTGA-3' R: 5'-TGATTTCTGCTCTGACAACCT-3'
IFNB	F: 5'-TTGACATCCCTGAGGAGATTAAGC-3' R: 5'-TTGACATCCCTGAGGAGATTAAGC-3'
PTGES2	F: 5'-AGACGGACCACCTCATTCTC-3' R: 5'-GCCTAAGGATGGCAAAGACC-3'
IDO1	F: 5'-CAAAGGTCATGGAGATGTCC-3' R: 5'-CCACCAATAGAGAGACCAGG-3'
HLA-G	F: 5'-TGGAGCAGGAGGGGCCGGAG-3' R: 5'-CCGCGCAGGGTCTGCAGGTT-3'
ARG1	F: 5'-GGCAATTGGAAGCATCTCTGGC-3' R: 5'-AGTGTTCCCCAGGGTCC-3'
iNOS	F: 5'-GAGATCAACATTGCTGTGATCCATAG-3' R: 5'-CACGGGACCGGTATTCATTC-3'
SDHA	F: 5'-TGGGAACAAGAGGGCATCTG-3' R: 5'-CCACCACTGCATCAAATTCATG-3'
GAPDH	F: 5'- TGCACCACCAACTGCTTAGC-3' R: 5'- GGCATGGACTGTGGTCATGAG-3'
CXCL10	F: 5'-GGTGAGAAGAGATGTCTGAATCC-3' R: 5'-GTCCATCCTTGGAAGCACTGCA-3'
VISTA	F: 5'-AGATGCACCATCCAACTGTGTGG-3' R: 5'-AGGCAGAGGATTCCTACGATGC-3'
Ox40L *CD252*	F: 5'-CCTACATCTGCCTGCACTTCTC-3' R: 5'-TGATGACTGAGTTGTTCTGCACC-3'
YWHAZ	F: 5'-ACTTTTGGTACATTGTGGCTTCAA-3' R: 5'-CCGCCAGGACAAACCAGTAT-3'
STING	F: 5'-CCTGAGTCTCAGAACAACTGCC-3' R: 5'-GGTCTTCAAGCTGCCCACAGTA-3'
IL10	F: 5'-CCCTGGGTGAGAAGCTGAAG-3' R: 5'-CACTGCCTTGCTCTTATTTTCACA-3'

Supplementary Table 4. Cross-correlation CD169^{+} tumor associated macrophages (CD169 PT) and tertiary lymphoid like structures (TLLS PT) or NK cells in small test breast cancer cohort.

		CD169 PT				Total	*P*
		0	**1**	**2**	**3**		
TLLS PT	***0***	1	12	7	0	20	
	1	0	2	1	0	3	
	2	0	0	0	1	1	
Total		1	14	8	1	24[a]	*P*=0.048
NK	***0***	1	7	4	0	12	
	1	0	7	3	1	11	
Total		1	14	7	1	23[a]	*P*=0.449

[a] Pearson Chi-Square, Linear by Linear association

Supplementary Table 5. Cross-correlations for spatial association between CD169^{+} tumor associated macrophages (CD169 PT) and T cells (CD3) in primary breast tumors using the large breast cancer cohort (Kimbung et al., 2016).

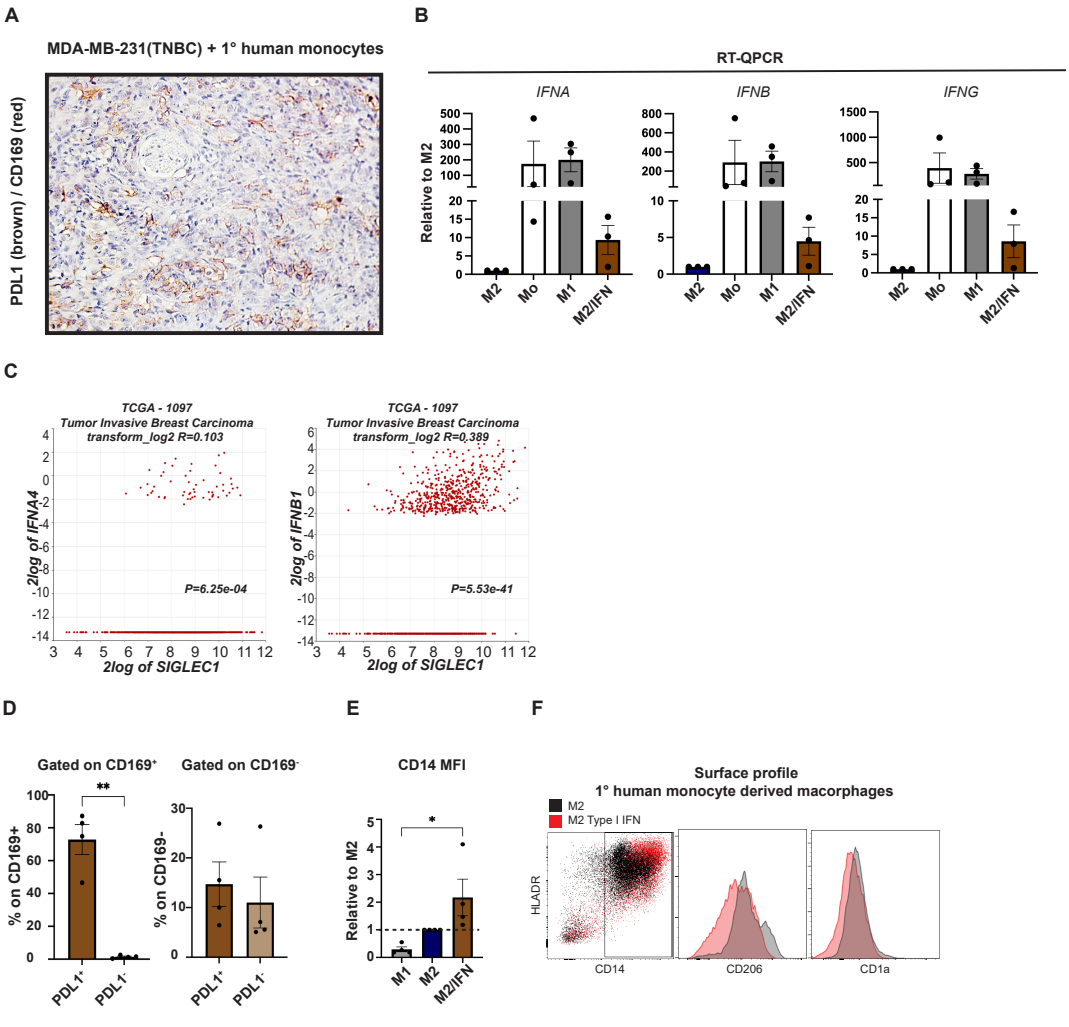
		CD169 PT		Total	*P*
		Neg	**Pos**		
CD3 PT	***low (0-1)***	85	22	107	
	high (2-3)	43	25	68	
Total		128	47	175[a]	*P= 0.018*

[a] Pearson Chi-Square, Linear by Linear association

*****Fisher's exact test was used when fewer observations than 20 were seen in at least 1 category

Supplementary Figures

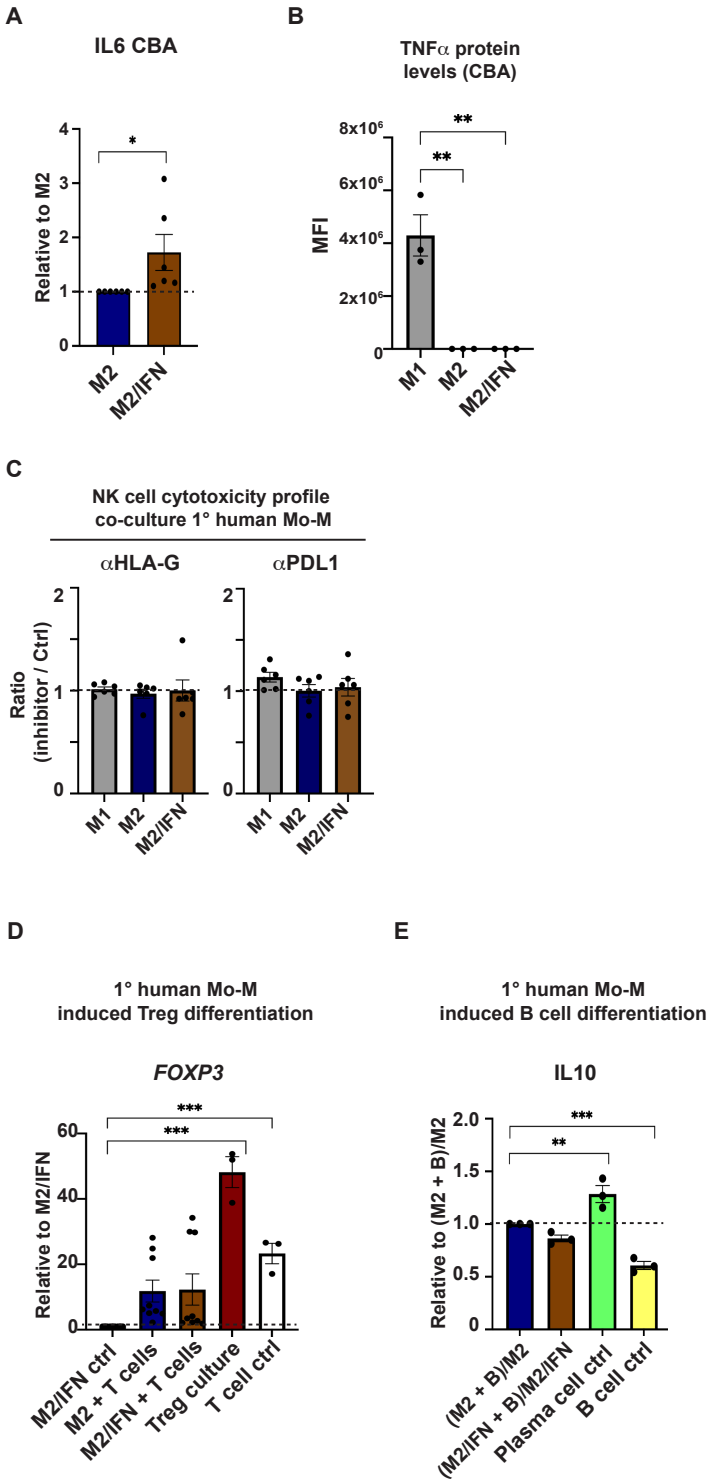
Figure S1



Supplementary Figure 1.

Phenotype of primary human monocyte-derived macrophages. (A) Tumor xenografts in NSG mice. Primary human monocytes were co-transplanted with MDA-MB-231 breast cancer cell lines in NSG mice for 21 d. Controls were transplanted with MDA-MB-231 cells alone. Upregulation of CD169 (red) was only seen in the SUM159 + monocyte xenografts (Fig. 2A-B) but not MDA-MB-231 + monocytes (this Figure S1A), while PDL1 (brown) was seen in all xenografts SUM159 (Fig. 2A-B), SUM159 + monocyte (Fig. 2A-B), MDA-MB-231 (this Figure S1A), MDA-MB-231 + monocytes (this Figure S1A) xenografts. (B) Relative mRNA levels of *IFNA*, *IFNB* and *IFNG* in primary human monocytes (Mo) and Mo-M as measured by RT-qPCR, N = 3, one-way ANOVA multiple comparison Dunnett's test. Note, the donors for Mo are not the same as for macrophages (M1, M2, M2/IFN) in this experiment. (C) *SIGLEC1* association to *IFNA4* (left) and *INFB1* (right) mRNA expression in primary tumors of breast cancer patients using the TCGA data base in R2 (r2.amc.nl) in R2: Genomics Analysis and Visualization platform (www.hgserver1.amc.nl). (D) PDL1 surface expression of primary human Mo-M (left), N = 4, Paired t-test. PDL1 expression on $CD169^{+}$ cells (middle) and $CD169^{-}$ cells (right), N = 4. (E) Ratio of median MFI of CD14 surface expression, N = 3, one-way ANOVA multiple comparison Dunnett's test. (F) Flow cytometry dot plots for HLA-DR and CD14 and MFI histograms for CD206 and CD1a to show gating strategy.

Figure S2


A
IL6 CBA
Relative to M2
M2
M2/IFN
B
TNFα protein levels (CBA)
MFI
M1
M2
M2/IFN
C
NK cell cytotoxicity profile co-culture 1° human Mo-M
αHLA-G
αPDL1
Ratio (inhibitor / Ctrl)
M1
M2
M2/IFN
D
1° human Mo-M induced Treg differentiation
FOXP3
Relative to M2/IFN
M2/IFN ctrl
M2 + T cells
M2/IFN + T cells
Treg culture
T cell ctrl
E
1° human Mo-M induced B cell differentiation
IL10
Relative to (M2 + B)/M2
(M2 + B)/M2
(M2/IFN + B)/M2/IFN
Plasma cell ctrl
B cell ctrl

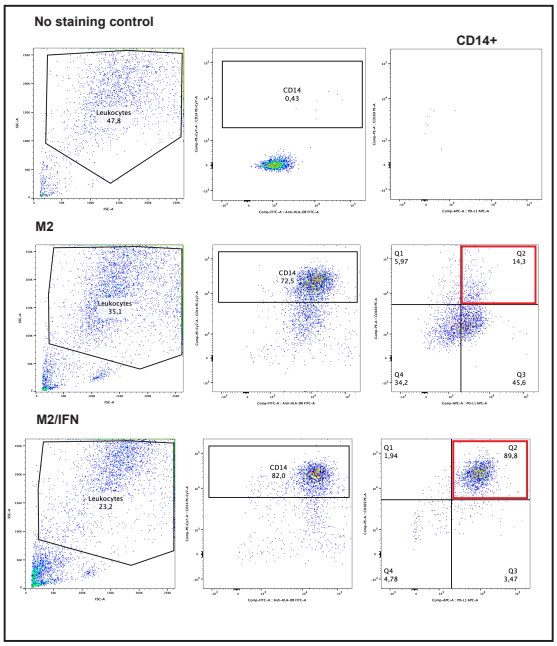
Supplementary Figure 2

Functional phenotype of $CD169^+$ Mo-M. (A) Cytokine secretion of IL6 as measured by CBA, ratio of concentration with M2 as control, N = 6, Ratio paired t-test. (B) TNFα cytokine secretion measured with CBA, N = 3. Student's t-test. (C) Allogeneic co-culture of primary human NK cells, primary human monocyte-derived macrophages and MDA-MB-231 breast cancer cell line with inhibitors for HLA-G (10 μg/ml) and PDL1 (Atezolizumab, 10 μg/ml), N = 6. (D) T_{reg} cell differentiation cultures of primary human monocyte-derived macrophages and primary human $CD4^+$ T cells as measured by RT-QPCR of *FOXP3* expression N=9 and N=3 ctrl. One-way ANOVA multiple comparison Dunnett's test. (E) IL10 secretion measured in B cell differentiation cultures of primary human monocyte-derived macrophages and primary human B cells as measured by CBA, N=6. Ratio paired t-test. For all figures: error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Data File 1

Representative gateing strategies for flow cytometric analyses

A



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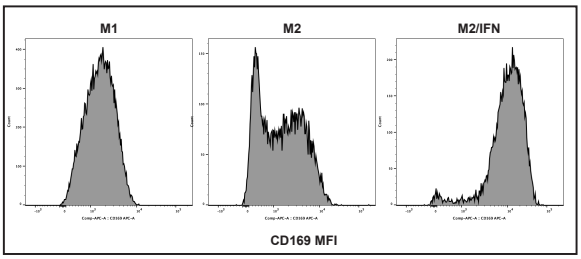
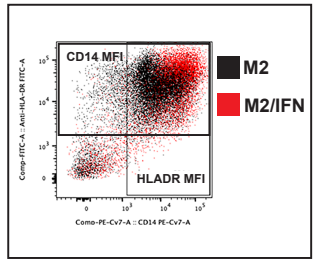
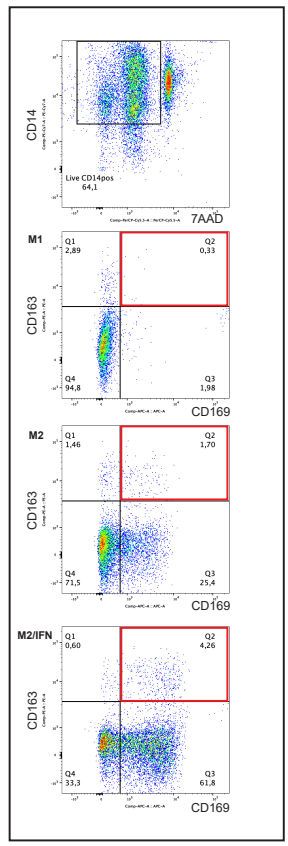


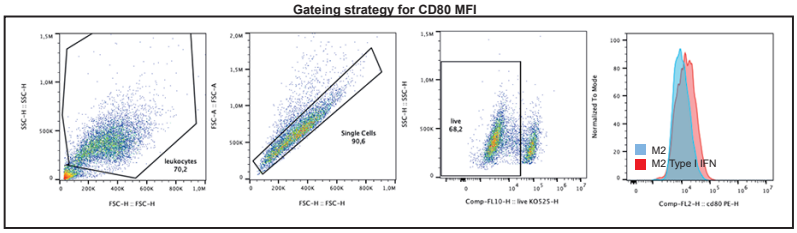
C



D



E



Supplementary Data File 1

The flow cytometry gating strategies are shown for (A) CD14 gated CD169+PDL1+ cells (B) CD169 MFI on CD14 gated M1, M2 and M2/IFN Mo-Ms (C) HLADR and CD14 MFI (D) CD14 gated CD163+CD169+ Mo-M (E) live gated CD80 MFI.

Paper III

Journal of Translational Medicine

RESEARCH Open Access

Altered immune signatures in breast cancer lymph nodes with metastases revealed by spatial proteome analyses

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Abstract

Background Metastasis to lymph nodes is strongly associated with reduced survival in breast cancer patients. To increase the understanding on how lymph node metastasis impairs the local immune response in affected lymph nodes, we here studied spatial proteomic changes of critical lymph node immune populations in uninvolved lymph nodes (UnLN) and paired lymph nodes with metastases (LNM) from five breast cancer patients.

Methods The proteome was analyzed for cortical lymphocyte compartments, subcapsular sinus (SCS) and medullary sinus (MS) CD169$^+$ macrophages, using the Digital Spatial Profiling (DSP) platform from NanoString.

Results Our results identified a stable proteome of SCS CD169$^+$ macrophages in LNM, with the exception for downregulation of the anti-apoptotic protein Bcl-xL and FAPα, but a clear reduction in numbers of SCS CD169$^+$ macrophages in LNM. In contrast, the proteome of MS CD169$^+$ macrophages, B-cell compartments and interfollicular T-cells showed altered immune signatures in LNM, indicating that the decline in SCS CD169$^+$ macrophages coincide with a malfunction in the local, anti-tumor immune responses.

Conclusions The findings from our study support the notion that metastasis to lymph nodes in breast cancer patients modifies local immune responses. These changes may contribute to explain unsuccessful therapeutic responses, and thereby worsened prognosis, for breast cancer patients with LNM.

Keywords Breast cancer, Lymph node metastasis, CD169$^+$ macrophages, B-cell follicles, Germinal centers, Interfollicular T-cells

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Background

A major prognostic variable in breast cancer is whether the tumor has metastasized to sentinel lymph nodes (SLNs) or not [1]. SLN are typically the first site of metastasis, and patients with lymph node metastases (LNM), have a decreased overall and disease-free survival, irrespective of breast cancer subtype [1–3]. The spread of tumor cells to the draining lymph node leads to lymphangiogenesis, cytokine and chemokine trafficking, immune evasion, alterations in the tumor microenvironment and cancer cell expansion [4]. Which of these factors that play a pivotal role in tumor progression remains to be revealed. It has been debated whether this is mainly the result or measurement of a disseminated disease [5–7], or whether the lymph node metastasis actually modulates the local lymph node immune response, hence contributing to a worsened prognosis [5, 8, 9]. To be able to discuss clinical routines, therapeutical responses and future treatment options linked to lymph nodes of breast cancer patients, further research is needed to fully understand the details and impact of LNM on local lymph node immune responses. During disease progression, when compared to an uninvolved lymph node (UnLN i.e. non-metastatic), the anatomical structure of the LNM becomes disrupted [10]. Recent studies show that several features of the LNM microenvironment, including fibroblasts, amount of B-cell germinal center (GC) reactions and presence of certain immune cells, is linked to prognosis [11–14]. One such immune cell is the resident lymph node subcapsular sinus (SCS) CD169$^+$ macrophage, which has been linked to a better patient prognosis when present in regional lymph nodes and LNM of various solid tumors [15–20], including tumor draining lymph nodes and LNM from breast cancer patients [21–24].

In lymph nodes there are various macrophages, but only two types are associated with residency and CD169 expression; the SCS CD169$^+$ macrophages, located at the interface between the subcapsular sinus and B-cell follicles, and the medullary sinus (MS) CD169$^+$ macrophages, located around the medullary cords [25]. Both SCS and MS macrophages reside at sites where they interact with the lymph, indicating their role in antigen processing and presentation. The location of SCS macrophages suggests a role as initiators of adaptive immunity. From studies in the mouse, it has been proposed that SCS macrophages may be critical for tumor antigen presentation in the draining lymph nodes, since they capture antigens from lymph fluid and present these to follicular dendritic cells or naïve B-cells, cross-present and finally activate CD8 T-cells [25, 26]. CD169$^+$ SCS macrophages are also important for activation of T-cells in the cortical interfollicular regions (IFR) of lymph nodes [27, 28]. Although less is known about MS CD169$^+$ macrophages, they exhibit higher lysosomal activity compared to SCS macrophages [29] and may help in clearance, or survival, of short-lived plasma cells [30, 31].

As mentioned above, SCS CD169$^+$ macrophages, B-cells and interfollicular T-cells are connected both by spatial localization in lymph nodes and by initiation of an adaptive anti-tumor immune response [23, 26–28]. Development and maintenance of SCS macrophages is highly regulated by lymphotoxin-α1β2 which is expressed by B-cells [32] and B-cells are dependent on presence of SCS CD169$^+$ macrophages for their expansion [23], partly explaining the importance of their co-localization. In the cortex of lymph nodes, in structures called B-cell follicles, naïve B-cells can be activated in either germinal center (GC)-dependent or -independent pathways [33]. Recently, a study showed that anti-tumor reactive B-cells can be derived from GC-independent B-cell follicles [34]. Interestingly, SLNs from breast cancer patients have distorted GC morphologies, with reduced number of GC in LNM compared to UnLN [13], indicating an effect on B-cell activation yet to be elucidated.

Since presence of SCS CD169$^+$ macrophages in lymph nodes of cancer patients are associated with a better prognosis, it is important to understand their precise involvement in local anti-tumor immune reactions, to understand why they disappear from LNM and what impact this has on local lymph node immune reactivity. We previously studied CD169$^+$ macrophages present in both primary breast tumors (PT) and LNM and found that they co-localize with B-cell containing tertiary lymphoid like structures in both locations [21, 24]. We further found that human monocyte derived CD169$^+$ macrophages can enhance antibody production by activated B-cells, indicating an immune stimulating function with regards to B-cells also in humans [21, 24]. With an aim to progress the understanding of the role of these cells in the ongoing anti-tumor immune reactions occurring in lymph nodes of breast cancer patients, we here investigated the CD169$^+$ macrophage- and B-cell compartments using spatial proteomics analysis in paired UnLN and LNM lymph nodes from five breast cancer patients.

Methods

Clinical samples and tissue processing

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained by the Swedish Ethical Review Authority (Dnr 2021–04869). This study included whole sections of paired paraffinized uninvolved lymph nodes (UnLN) and lymph nodes with metastases (LNM) from five patients with invasive breast cancer. Only LNM with metastatic masses, rather than just diffuse cancer cells, were included. LNM in which metastatic masses comprised the majority of LNM were

excluded to allow for proper visualization of lymph node structures. A clinical pathologist (B.T.) was responsible for evaluation and determination of inclusion/exclusion criteria. All primary tumors were estrogen (ER) and progesterone (PR) positive, with one patient also positive for human epidermal receptor-2 (HER2). NHG ranged from 1 (one case), 2 (three cases) to 3 (one case). Ki67 expression was high in 4 out of 5 primary tumors. All patients were untreated at the time for surgery. Samples were preserved in formalin-fixed paraffin-embedded (FFPE) blocks, sectioned at 4 µm thickness, and mounted on SuperFrost Plus IHC slides for subsequent analysis.

Immunostaining

Presence of $CD169^+$ macrophages and B-cells in all samples was first confirmed with immunohistochemistry (IHC) using anti-CD169 (clone SP216, Invitrogen, 1:100), Hematoxylin/Eosin (HE), or anti-CD20 (clone L-26, ThermoFisher Scientific, 1:200). Antigen retrieval and staining were conducted using a PT-link system (pH 9, K8010, DAKO/Agilent (Santa Clara, CA, US)) before staining with an Autostainer Link 48 system (DAKO/Agilent). Slides were scanned at 20X magnification using an Aperio Scanscope CS scanner (Leica Biosystems (Nussloch, Germany)) and further analyzed with QuPath 2.0 (see Data visualization and statistics). For fluorescent immunostaining of CD169, overnight staining was performed with rabbit monoclonal antibody (mAb) Anti-CD169 (clone SP216; Abcam 183356, 1:500) directly conjugated with AF647 (Invitrogen (Carlsbad, CA, US), A20186).

GeoMx DSP technology

The NanoString Digital Spatial Profiler (DSP) GeoMx platform (Seattle, WA, US) was used to pre-process and collect data to investigate tissue heterogeneity and complexity. The GeoMx Protein Slide Preparation protocol was applied according to manufacturer's description. GeoMx DSP uses morphological markers tagged with fluorophores to visualize the spatial localization of cells of interest. Primary antibodies against morphological and profiling markers (Supplementary Table 1) were applied overnight and nuclei was stained with SYTO13 prior to mounting slides in the NanoString DSP GeoMx instrument. Each slide was scanned, and regions of interest (ROIs) were selected. The scanned paired whole lymph node sections are shown in Supplementary Fig. 1 side by side. Using UV light, the UV-cleavable link attached to the profiling antibodies was disrupted, causing oligo barcodes from the ROIs to be collected in a 96-well plate, hybridized, and counted using an nCounter (NanoString). Our study included 96 ROIs representing three different tissue region types: abundance of CD45-positive cells; CD169-positive cells; and Pan-CK-positive cells, respectively. Subsequent data processing followed GeoMx standard workflow, including quality control based on field of view registration, binding intensity, positive control probe normalization, minimum nuclei count, and minimum surface area for a ROI.

ROI strategy and optimal normalization of data

After quality control, normalization of the data was carefully evaluated to find the most suitable linear scaling normalization based on the geometric mean with either housekeeping proteins, negative isotype control antibodies, ROI area or nuclei count. All ROIs passed quality control and were included in the downstream analysis (Supplementary Fig. 2). The normalization method best suited for our data was determined to be scaling by Housekeeping proteins Histone 3 (H3) and ribosomal protein S6 which showed high correlation (Supplementary Fig. 2A-C). The geometric mean of these two markers was consequently used to scale the data. Signal-to-background ratios of each probe were consistently < 1 for three antibodies: Lag-3, CD80 and GITR. These markers were excluded from all further analysis (Supplementary Fig. 2D).

Data visualization and statistics

QuPath was used for image analysis of IHC, where the output was the number of positive CD169 cell segments or CD20 cell segments in UnLN and LNM and image type was set to brightfield (H-DAB). IHC of paired whole lymph node sections are shown in Supplementary Fig. 3 (CD169) and Supplementary Fig. 4 (CD20 and CD169) side by side. Classification for CD169 positive cell segments was set with intensity threshold 0.8 (cell: DAB OD max), causing segmentation into cells either positive or negative for CD169. The percentage of positive CD169 cells were calculated from all cells present in UnLN and LNM, thereafter all $CD169^+$ cells were divided into SCS or MS macrophages. Differences between UnLN and LNM were calculated with a paired student´s t-test using Graph Pad Prism 10. All spatial proteomics analysis were performed within the GeoMx DSP data analysis suite (V.3.1.0.194) from NanoString, including quality control assessments, data normalization and statistical analysis. Plots for data visualization were made with NanoString-validated R-scripts (GeoScript Hub, NanoString, available at https://nanostring.com/products/geomx-digital-spatial-profiler/geoscript-hub// (accessed 10 June 2023)), used to generate volcano plots, Principal Component Analysis (PCA) and hierarchical clustering analysis. For statistical analyses, Linear mixed model (LMM) adjusted for matched donors was used to compare protein expression between ROIs from UnLN to LNM ROIs, Paired t-test with Benjamini & Hochberg correction was used for SCS CD169 macrophages (where a ROI from each

tissue was available) and Pearson correlation for supervised and unsupervised clustering of all ROIs in this study. Protein expression comparison was illustrated through Volcano plots, showing significantly ($P<0.05$) differently expressed proteins. Forest plots indicating the distribution in ratio values between SCS and MS macrophages for macrophage markers were calculated from the relative expression of target probes in all macrophage ROI.

Results

Fewer SCS CD169$^+$ macrophages in LNM compared to UnLN

We included five paired lymph nodes from breast cancer patients and compared the LNM and UnLN. The criteria for LNM were that the metastasis should not cover a major part of the lymph node to be able to compare compartments and draw conclusions regarding immune cells. We initially investigated presence of CD169$^+$ macrophages in the paired LNM/UnLN samples with the help of IHC and QuPath, to confirm previous knowledge regarding regression of CD169$^+$ macrophages in involved lymph nodes from cancer patients [16, 21, 23, 29, 35, 36], but also to investigate their location as being SCS CD169$^+$ macrophages or MS CD169$^+$ macrophages. Representative IHC staining for SCS and MS macrophages are presented in Fig. 1A-B and whole sections of the paired lymph nodes are shown in Supplementary Fig. 3–4. Cells were visualized and classified using QuPath software, allowing positive cell detection for CD169$^+$ macrophages (Fig. 1A-B). The effect of metastasis was investigated in the three subsets: All CD169$^+$, SCS CD169$^+$, and MS CD169$^+$ macrophages. As shown in Fig. 1C, there was a trend towards fewer CD169$^+$ macrophages in LNM in general. When dividing into SCS CD169$^+$ and MS CD169$^+$ macrophages based on spatial localization, a reduction specifically in SCS CD169$^+$ macrophages was observed, whereas MS CD169$^+$ macrophages were unchanged, or possibly slightly increased, in numbers in LNM compared to UnLN (Fig. 1D-E).

Hence, in LNM, the landscape of lymph nodes is disrupted with a reduction of SCS CD169$^+$ macrophages in the subcapsular cortex, which eventually could lead to reduced interactions with underlying B-cell follicles and a weakened anti-tumor immunity. MS CD169$^+$ macrophages were not affected.

Clear data separation of ROIs reflected in GeoMX biomarker profiles

Using the GeoMx DSP platform, we next investigated proteome changes in CD169$^+$ macrophage regions and cortical CD45$^+$ lymph node follicle regions, in UnLN compared to paired LNM. This was done by staining for two specific biomarkers, CD169 for macrophages and CD45 for leukocytes in general, followed by spatial visualization, selecting CD45$^+$ lymph node follicle regions localized in the cortex near the capsule of the lymph node and if possible, in close contact to SCS CD169$^+$ macrophages. We also analyzed MS CD169$^+$ macrophages as a separate population based on spatial localization in the MS. ROIs were selected to represent regions within the tissue containing the same type of immune cells (either CD45 alone, or CD45 and CD169), to enable comparison of the same types of cells in UnLN and LNM. The different types of ROIs represented SCS CD169$^+$ macrophages, MS CD169$^+$ macrophages, cortical CD45$^+$ lymph node follicle regions and breast cancer metastatic cells stained with Pan-CK. Because SCS macrophages were reduced in LNM, ROIs representing MS macrophages were numerous in each sample. A schematic picture representing our ROI strategy is shown in Fig. 2. Whole sections of the stained paired lymph nodes are shown in Supplementary Fig. 1.

A heatmap of all ROIs illustrated the differential protein expression of CD169, CD45, tumor regions (Pan-CK) (Fig. 3A) and showed that ROIs representing CD45$^+$ lymph node follicle regions, CD169$^+$ macrophages and tumor cells, respectively, were accurately separated in the data. Concordantly, the CD169 ROIs had a high expression of typical macrophage markers such as CD14, CD68 and CD163, while CD45$^+$ lymph node follicle ROIs had high expression of lymphoid markers. The tumor regions expressed Pan-CK, representing our control of metastatic breast cancer cells. The differences between each ROI type were also visualized with PCA, showing clear cluster separation (Fig. 3B). 4 ROIs from CD169 regions were considered as outliers based on the combined assessment from heatmap and PCA interpretation (indicated in grey in Fig. 3A-B) and excluded from the study.

SCS and MS CD169$^+$ macrophages show opposite trends in LNM

As presence of SCS CD169$^+$ macrophages in LNM are associated with a beneficial prognosis, our aim was next to investigate their proteome in paired lymph nodes, but also to compare with the relatively undiscovered MS CD169$^+$ macrophages. The cohort consisted of 46 CD169 ROIs, including four outliers which were excluded from the analysis. This resulted in 24 ROIs from LNM and 18 ROIs from UnLN. Our analysis was based on the localization of the CD169$^+$ macrophage ROIs, i.e., SCS CD169$^+$ macrophages versus MS CD169$^+$ macrophages. Forest plots showing differential protein expression for SCS compared to MS macrophages in UnLN (Supplementary Fig. 5A) and LNM (Supplementary Fig. 5B) are shown to visualize potential macrophage polarization profiles. The proteome differences between SCS and MS CD169$^+$macrophages, in LNM and UnLN

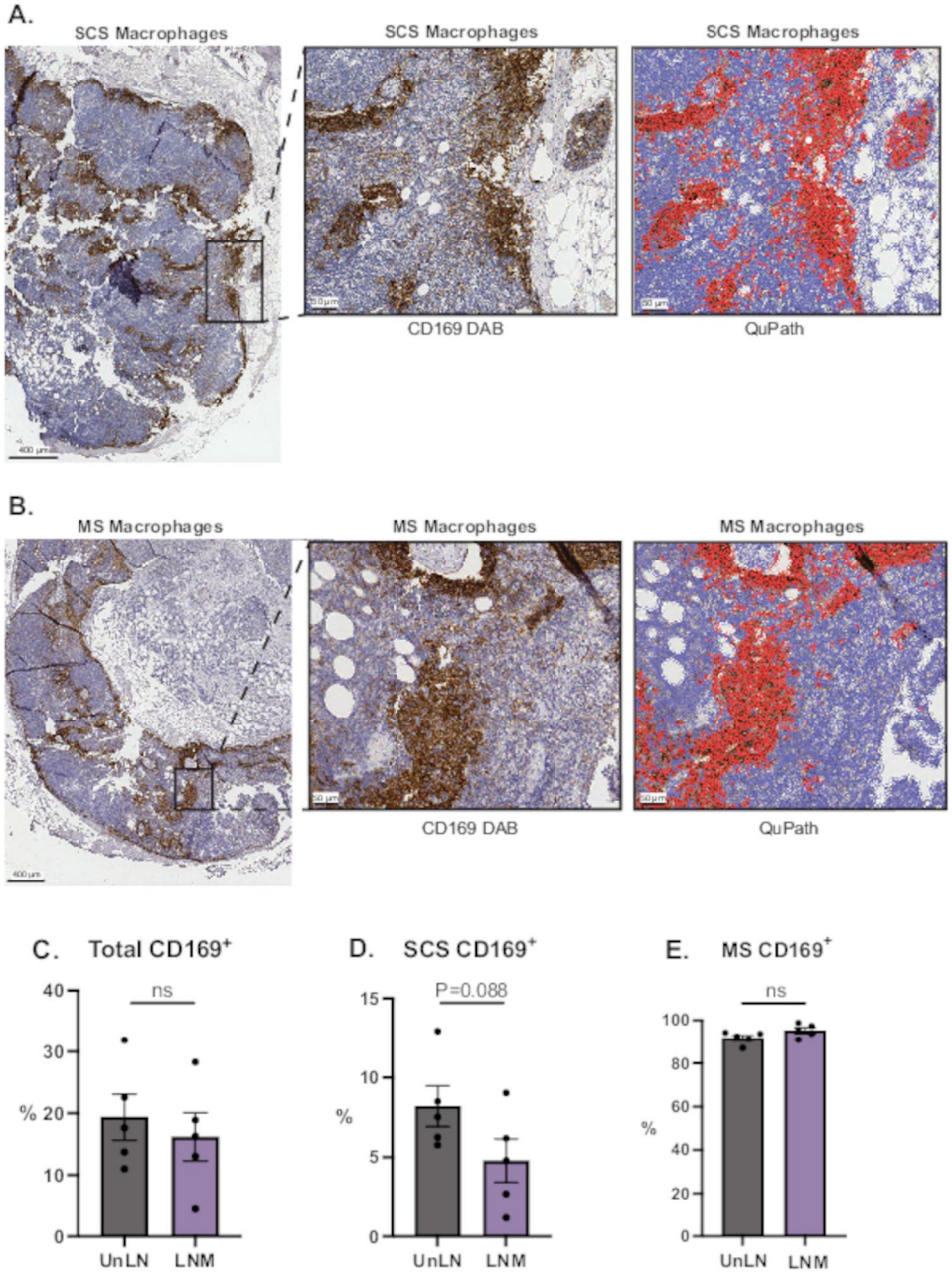
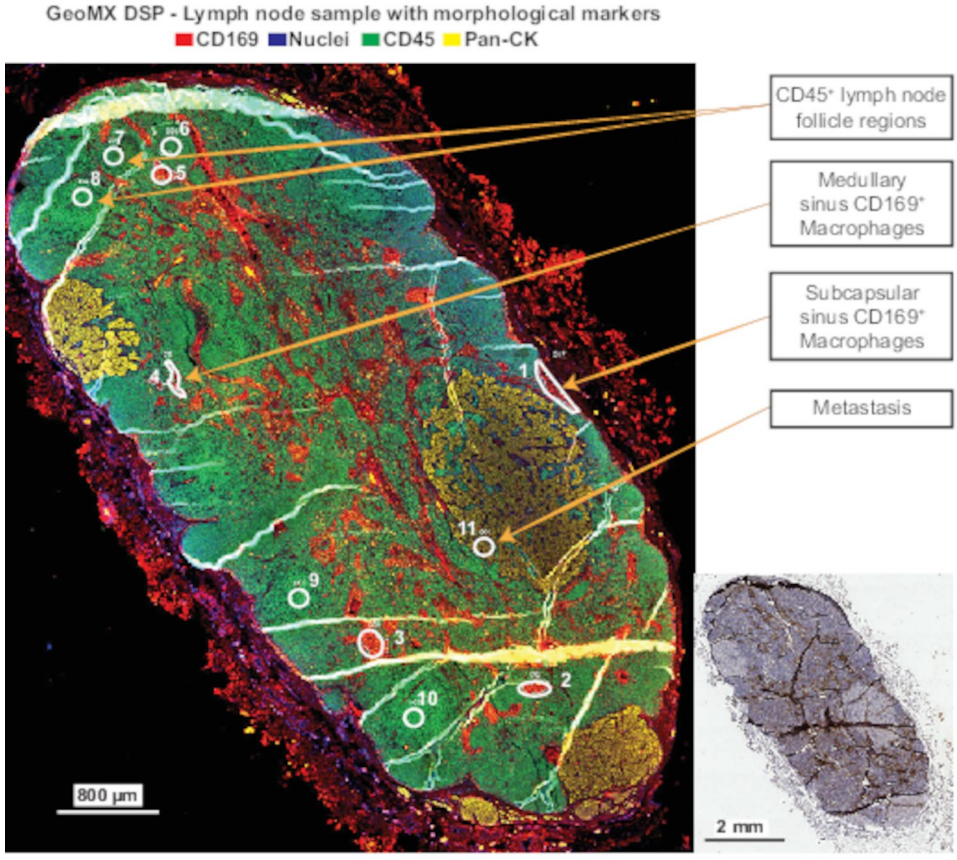


Fig. 1 (**A-B**) Representative of breast cancer lymph node tissue stained with H&E and IHC (CD169) (**A**) SCS CD169$^+$ macrophages and (**B**) MS CD169$^+$ macrophages of whole lymph node sections from LNM together with zoomed visualizations of areas (DAB and segmentation in QuPath) (**C**) Quantification of bulk CD169$^+$ cells (**D**) SCS CD169$^+$ macrophages (**E**) MS CD169$^+$ macrophages in UnLN compared to LNM using QuPath Software. Error bars indicate SEM, Statistics were performed with paired students T-test, * $P<0.01$, ** $P<0.05$, *** $P<0.001$. $N=5$

respectively (Supplementary Figs. 5 and 6 A-B), showed that MS macrophages expressed more CD163, CD14, Cleaved Caspase 9 and CD68 in general. In LNM, MS macrophages showed an anti-inflammatory proteome polarization state or environment compared to SCS macrophages (CD163, OX40L, CTLA4, PD-L1/2, BAD, Bcl-xL, FoxP3, FAPα, SMA) (Supplementary Figs. 5 and 6 A-B). SCS macrophages expressed slightly more HLA-DR compared to MS macrophages in UnLN, although not significant, but not in LNM (Supplementary Fig. 5). SCS macrophages were in close contact with CD20$^+$ cells representing B-cells, both in UnLN and LNM. This was in agreement with previous studies [37] and hence ROIs of SCS CD169$^+$ macrophages versus MS CD169$^+$ macrophages were confirmed.

Although SCS CD169$^+$ macrophages decline in LNM, the remaining SCS CD169$^+$ macrophages could obviously still have a functional role, or an altered function



Patient	ID - 1		ID - 2		ID - 3		ID - 4		ID - 5		Total	
	LNM	UnLN	LNM	UnLN	LNM	UnLN	LNM	UnLN	LNM	UnLN	LNM	UnLN
Pan-CK ROI	1	0	1	0	1	0	1	0	1	0	5	0
CD169 ROIs	5	4	6	4	5	4	5	4	5	4	26	20
CD45 ROIs	5	4	5	4	5	4	5	4	5	4	25	20
Total ROIs	11	8	12	8	11	8	11	8	11	8	56	40

Fig. 2 Visualization of ROI strategy applied to all samples. Four regions of cells were investigated with GeoMX DSP: Subcapsular CD169$^+$ macrophages (ROI #1), Medullary sinus CD169$^+$ macrophages (ROI # 2–5), CD45$^+$ lymph node follicle regions (ROI #6–10), and the metastasis (ROI #11). ROIs from each lymph node are represented in the table below the figure

and hence impact on prognosis. Due to the fewer SCS CD169$^+$ macrophages, our analysis consisted of 4 matched ROIs of SCS macrophages in each tissue type. Only two proteins showed a significant difference in expression comparing SCS CD169$^+$ macrophages in UnLN and LNM: B-cell lymphoma-extra-large (Bcl-xL) and Fibroblast activation protein α (FAPα), with an overrepresentation in UnLN, or a downregulation in LNM of both proteins (Fig. 3C). The log2 fold change for both proteins of interest were below 0.5, indicating only minor changes in protein expression. Bcl-xL is an anti-apoptotic marker that regulates apoptosis. Its downregulation in LNM may therefore allude a potential mechanism explaining the disappearance of SCS macrophages in LNM. Importantly, apart from these two proteins, the rest of the proteome of the remaining SCS CD169$^+$ macrophages remained unchanged, indicating similar functions for SCS CD169$^+$ macrophages in UnLN as compared to LNM.

Next, we investigated the MS CD169$^+$ macrophages in UnLN versus LNM. Due to their higher presence in all samples, a comprehensive analysis with 33 ROIs, 19 from LNM and 14 from UnLN was possible. For MS CD169$^+$ macrophages ROIs, five proteins showed a significantly altered expression (Inducible costimulatory T-cell receptor (ICOS), Cytotoxic T-lymphocyte associated protein 4 (CTLA4), Fibronectin, Granzyme A (GZMA) and Arginase 1 (Arg1)) (Fig. 3D). While GZMA and Arg1 increased in expression, ICOS, CTLA4 and Fibronectin were downregulated in LNM as compared to UnLN.

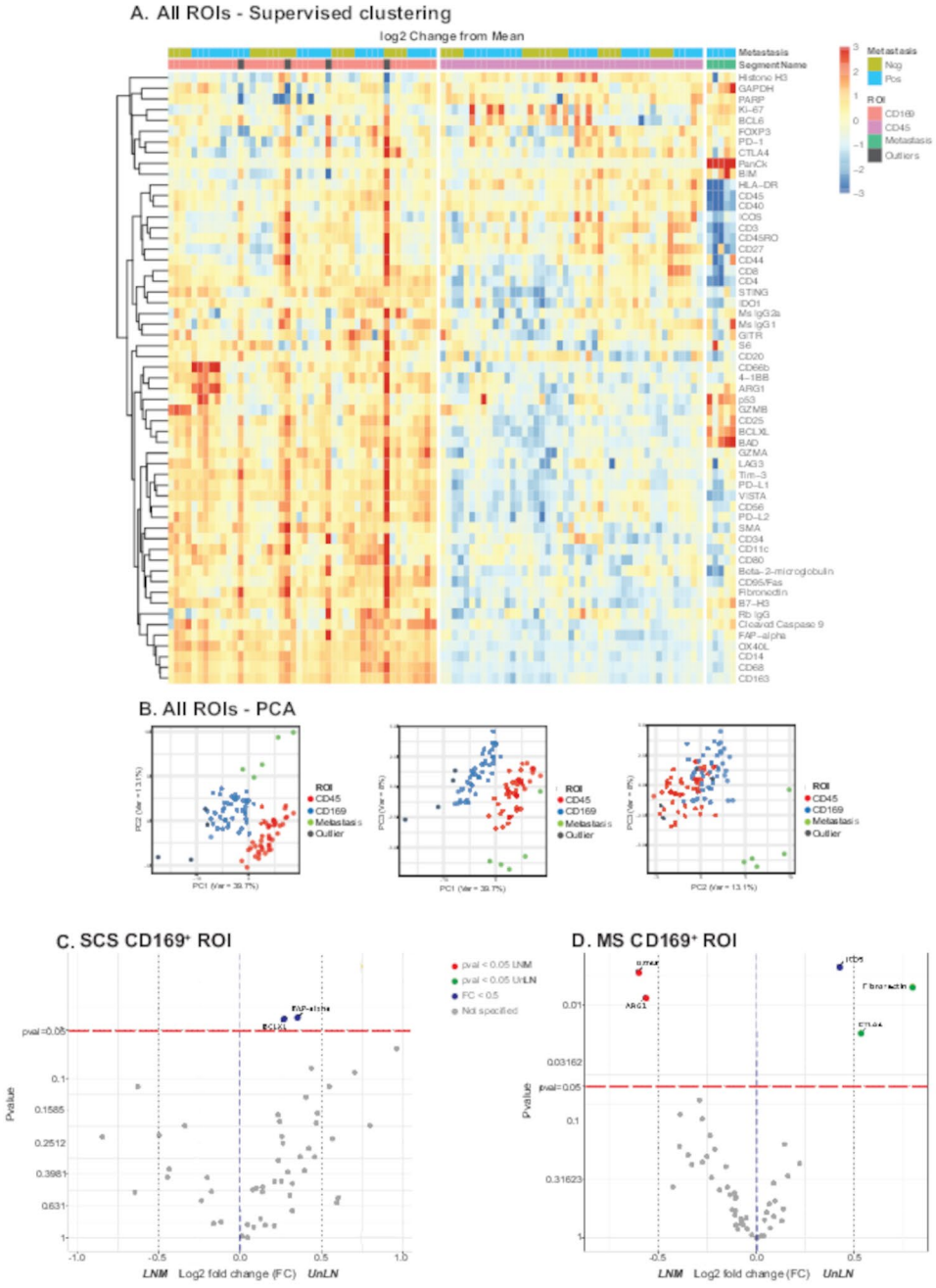


Fig. 3 (**A**) General heat map with supervised clustering performed based on ROI. The color scale represents the log2 change from the geometric mean off all probes in the analysis, 4 outliers were excluded from CD169 ROI. Supervised clustering was performed based on ROI. (**B**) PCA plot representing all samples ROI illustrated with the first three principal components. Annotations: CD169 (macrophages), CD45 (lymphoid follicles), Metastasis (malignant cells metastasis), Outliers. (**C-D**) Volcano plots showing the statistical significance versus the magnitude of change in protein expression between five paired LNM (left) versus UnLN (right). Linear mixed model (LMM) adjusted for matched donors or paired T-test adjusted with Benjamini & Hochberg method was applied as statistical tests. Proteins with significant *P*-value < 0.05 are shown in blue, with proteins showing a log2 fold change above 0.5 plotted in red or green depending on the direction. (**C**) SCS CD169^{+} macrophage associated proteins with significant difference in expression between paired LNM and UnLN. (**D**) MS CD169^{+} macrophage associated proteins with significant difference in expression between paired LNM and UnLN

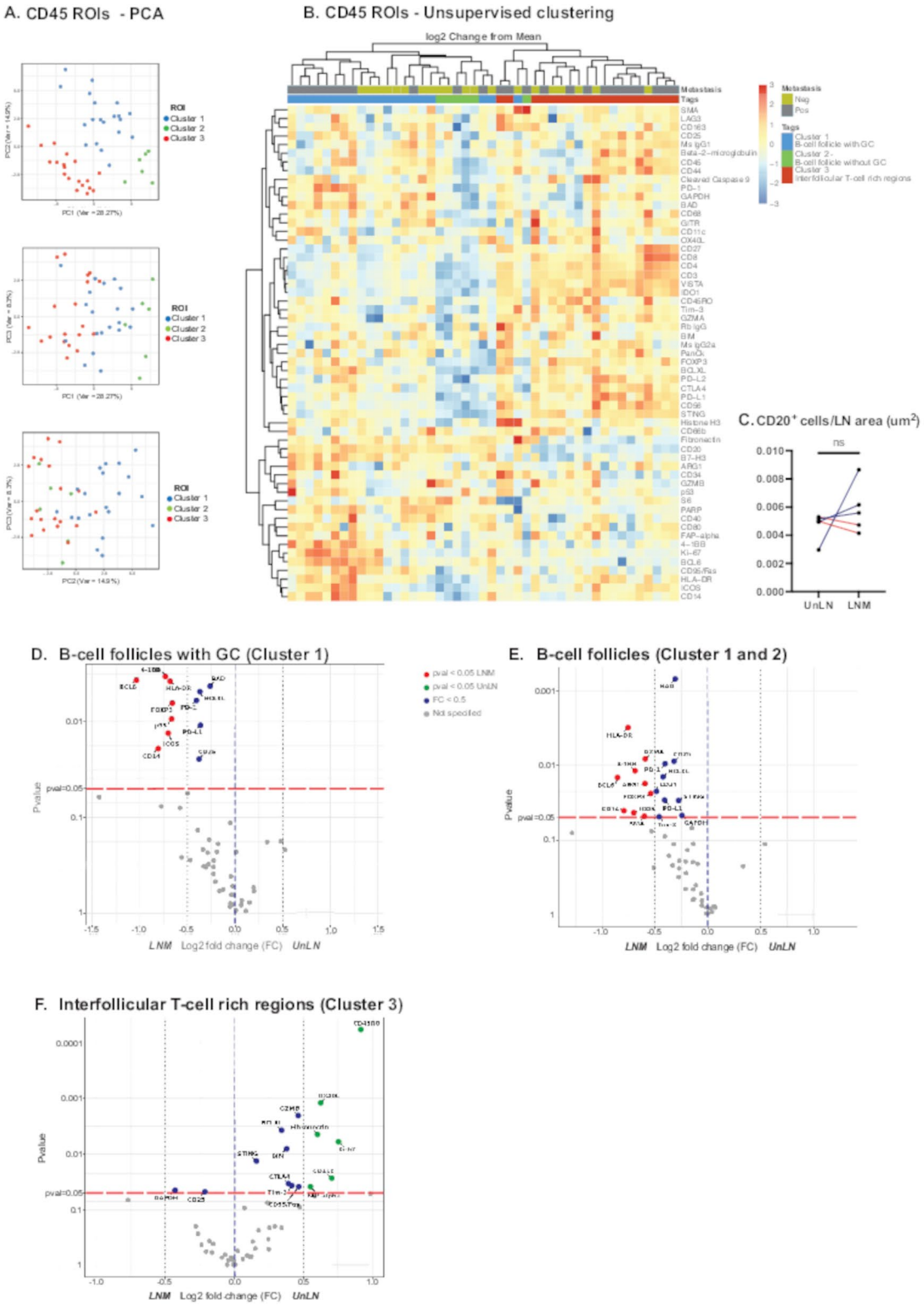


Fig. 4 (See legend on next page.)

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Fig. 4 (**A**) PCA plot representing all CD45 ROIs illustrated with the first three principal components. Three clusters are shown. (**B**) General heat map with unsupervised clustering performed based on CD45 ROIs. The color scale represents the log2 change from the geometric mean off all probes in the analysis. Annotations: Neg (UnLN); Pos (LNM); B-cell follicles with GC (Cluster 1); B-cell follicles without GC (Cluster 2); Interfollicular T-cell rich region (Cluster 3). (**C**) Quantification of bulk $CD20^+$ B-cells in UnLN compared to LNM using QuPath software. Statistics were performed with paired students T-test. (D-F) Volcano plots showing the statistical significance versus the magnitude of change in protein expression between five paired LNM (left) versus UnLN (right). Linear mixed model (LMM) adjusted for matched donors was applied as statistical tests. Proteins with significant *P*-value < 0.05 are shown in blue, with proteins showing a log2 fold change above 0.5 plotted in red or green depending on the direction (**D**) B-cell follicles with GC (Cluster 1) associated proteins with significant difference in expression between paired LNM and UnLN. (**E**) B-cell follicles associated proteins (Cluster 1 and 2) with significant difference in expression between paired LNM and UnLN. (**F**) Interfollicular T-cell rich region (Cluster 3) associated proteins with significant difference in expression between paired LNM and UnLN

In summary, our CD169 ROIs represent two populations of macrophages in lymph nodes; the declining SCS $CD169^+$ macrophages with a stable proteome except for lower expression of the anti-apoptotic protein Bcl-xL and the enduring MS $CD169^+$ macrophages with altered proteome in LNM.

CD45 lymph node follicles separate into three distinct clusters

As the SCS $CD169^+$ macrophages are in spatial contact with B-cell follicles and MS $CD169^+$ macrophages affect plasma cell survival and clearance, we next investigated how LNM impacted the cortical $CD45^+$ lymph node follicle proteomes. ROIs with histology representing CD45 lymph node follicles were selected from the cortex near the SCS. Our cohort consisted of 45 ROIs with CD45 regions located in the cortex of lymph nodes, with typical B-cell follicle structures and morphology. 25 ROIs from LNM were compared to 20 ROIs from UnLN. With a PCA, data dimensionality revealed three distinct clusters (Fig. 4A), indicating that datapoints representing ROIs within one cluster are different from those in another cluster. To investigate the difference between these clusters, differential protein expression was visualized in an unsupervised cluster heat map (Fig. 4B). Cluster 1 represented B-cell follicles with an active GC as deducted by high expression of CD20, Ki-67 and B-cell lymphoma 6 (BCL6), Cluster 2 represented B-cell follicles rich in B-cells yet lacking a GC as deducted by expression of CD20, CD40 and lack of BCL6 and Ki-67; Cluster 3 represented a capsular proximal T-cell rich region, as deducted by higher expression of T-cells markers CD3, CD4 and CD8 and lower expression of CD40, CD20, BCL6 and Ki-67, possibly exemplifying interfollicular regions (IFR) of T-cells [27]. The location of ROIs representing Cluster 3 (ROI #8–10 in Fig. 2) were confirmed by consecutive sections and IHC, showing spatial localization of Cluster 3 in interfollicular areas (17 out of 19 ROIs representing Cluster 3; Supplementary Fig. 6C).

These clusters were respectively annotated as B-cell follicles with GCs (Cluster 1), B-cell follicles without GC (Cluster 2), and interfollicular T-cell rich regions (Cluster 3), all three which were present in both UnLN and LNM.

Cortical lymphocyte regions have an altered immune signature in LNM

We next investigated how LNM impact the B-cell compartment. Hence, the B-cell follicle cluster proteomes were ultimately compared between LNM and UnLN. We initially analyzed the number of B-cells in LNM as compared to UnLN, by quantifying $CD20^+$ cells by IHC and QuPath. As shown in Fig. 4C, no significant difference in B-cell numbers was found between LNM as compared to UnLN, indicating that the interdependence or synergistic decay in SCS $CD169^+$ macrophages and B-cells seen in mice [23, 26], is not reflected in human breast cancer LNM.

For B-cells follicles with GCs (Cluster 1), 10 ROIs from LNM and 10 ROIs from paired UnLN were compared. Results showed a clear upregulation of several proteins involved in active GC reactions in LNM compared to UnLN (Tumor necrosis factor ligand superfamily member 9 (TNFSF9; 4-1BB), p53, BCL6, Bcl-xL, Bcl-2 associated agonist of cell death (BAD), Human Leukocyte Antigen DR isotype (HLA-DR), CD14, ICOS) but also proteins related to immune regulation or GC contraction (Forkhead box P3 (FoxP3), Programmed Cell Death Protein 1 (PD-1), Programmed Cell Death Ligand 1 (PD-L1) and CD25) (Fig. 4C).

ROIs for B-cell follicles without GC (Cluster 2) were scarce in our analyses (2 ROIs from LNM and 4 ROIs from UnLN) and therefore not possible to statistically analyze alone. Instead, B-cell follicles with and without GCs (Cluster 1 and 2) were combined, with 12 ROIs from LNM and 14 ROIs from UnLN. Cluster 1 and 2, showed a similar protein profile as B-cell follicles with GC, but with an additional upregulation of the proteins T-cell Ig- and mucin domain containing molecule-3 (TIM-3), Arg1, GZMA, Stimulator of interferon genes (STING), CD34, α-smooth muscle actin (SMA), GAPDH and loss of p53 in LNM (Cluster 1 & 2; Fig. 4E).

We finally analyzed the interfollicular T-cell rich regions (Cluster 3; Fig. 4F), comprising 13 ROI from LNM and 6 ROI from UnLN. Here, downregulation of several proteins in LNM were observed (CD45RO, Tumor necrosis factor receptor superfamily, member 4 (TNFRSF4; OX40L), Fibronectin, Ki-67, CD11c, FAPα, Bcl-2 Interacting Mediator of cell death (BIM),

Bcl-xL, Granzyme B (GZMB), CTLA4, TIM-3, STING and CD95/Fas) and only two were upregulated in LNM (CD25 and GAPDH) (Fig. 4F). This indicates that critical immunoregulatory changes occur in interfollicular T-cell rich areas in human breast cancer LNM, indicating suppressed immune reactivity in interfollicular T-cells that previously have been shown to depend on CD169$^+$ SCS macrophages [27].

In summary, in LNM of breast cancer patients, critical microenvironmental changes occur in B-cell follicles and interfollicular T-cell rich regions, a finding that possibly could be a consequence of the regression of SCS CD169$^+$ macrophages and immunosuppressive MS CD169$^+$ macrophages found in LNM.

Discussion

Lymph node metastasis (LNM) is a critical prognostic indicator in various types of cancer [10]. However, the precise involvement of lymph nodes in tumor progression remains unclear. Specifically, it is uncertain whether lymph nodes facilitate metastatic invasion and spread of the tumor due to its structure and anatomical location, or if the immune response is suppressed, thereby permitting metastatic invasion [5–9]. To investigate the latter, we here performed spatial proteomics analyzing CD169$^+$ macrophages and cortical lymphocytes in paired lymph nodes from breast cancer patients. Given the relatively limited knowledge regarding MS macrophages in LNM, we here focused on both SCS and MS CD169$^+$ macrophages.

The lymph nodes included in this study were derived from patients with ER$^+$ primary tumors. This is the tumor type that spreads most easily to SLN [38]. However, one of the tumors also had a HER2$^+$ phenotype, representing a more aggressive breast cancer subtype also associated with SLN metastasis and worse prognosis [39]. We have previously shown that HER2-expression in primary breast tumors is significantly associated with CD169$^+$PD-L1$^+$ expression in the primary tumor, but not in LNM [15]. In this study we could also show that HER2 status was one of the variables affecting CD169$^+$ LNM macrophages as prognostic marker in multivariable analyses [15], a finding that was not supported using a different breast cancer cohort [21]. Although the influence of the HER2 breast cancer subtype specifically on lymph node macrophages and immune responses remains unclear, different breast cancer subtypes indeed do affect macrophage polarization and function differently [40, 41]. However, evidence also suggests that macrophages may drive clinical subtype shifts [42, 43], also during LNM [44], a finding that warrants further investigation for understanding the relation between lymph node macrophages and breast cancer subtypes. In the study presented here, although we compare UnLN with LNM, a weakness is that both lymph nodes may still be affected to some extent by the primary tumor.

Consistent with previous research [16, 23, 29, 35, 36] our data show a decline in lymph node SCS CD169$^+$ macrophages in LNM. We observed that this was linked to downregulation of the anti-apoptotic protein Bcl-xL and FAPα in LNM, while the rest of the proteome of SCS macrophage regions was unchanged. SCS CD169$^+$ macrophages proposedly prevents metastatic niche development, as depletion of these macrophages in in vivo breast cancer models significantly increases metastatic burden [23]. The fact that the anti-apoptotic protein Bcl-xL was downregulated in the LNM SCS CD169$^+$ macrophage regions, possibly reflects a cell death associated decline [45] induced by tumor cells in LNM. Alternatively, the decline may involve loss of interaction with fibroblast reticular cells expressing FAPα [46]. The specific loss of SCS CD169$^+$ macrophages in LNM could nevertheless explain their prognostic impact. The reduced numbers of SCS CD169$^+$ macrophages in LNM may also impair acquisition and presentation of tumor-antigens to follicular dendritic cells (FDCs), B-cells, or interfollicular T-cells, thus leading to a worse prognosis, as demonstrated in several studies [15, 18–21, 27, 47, 48]. However, as suggested by our results, apart from Bcl-xL and FAPα, the remaining SCS CD169$^+$ macrophages in LNM do not have an altered phenotype, hence indicating an unaltered function per se compared to UnLN, a finding that needs further investigation.

In contrast, an altered immune signature including increased activity of immunosuppressive Arg1, was found in LNM areas with MS CD169$^+$ macrophages. Also, the numbers of MS CD169$^+$ macrophages in LNM were preserved or even slightly increased. It has previously been shown that MS CD169$^+$ macrophages may help in clearance or survival of short-lived plasma cells [30, 31], but also to regulate tolerance by phagocytosis of antigen specific T-cells [49]. Our results point in the direction that MS CD169$^+$ macrophages likely interact closely with T-cells, evidenced by detection of T-cell related proteins (ICOS, CTLA4 and GZMA). We show that in LNM MS CD169$^+$ macrophage areas, CTLA4 and ICOS were downregulated, while GZMA and Arg1 were upregulated. Downregulation of CTLA4 and ICOS in the LNM MS CD169$^+$ macrophage areas could indicate both immunogenic and immunosuppressive events, possibly affecting anti-tumor T-cell activity in LNM [50, 51]. The higher expression of Arg1, a potent inhibitor of T-cell responses [52], in LNM MS CD169$^+$ macrophage areas would support the latter. Furthermore, GZMA expressed by T-cells [53, 54], can in its extracellular form be internalized by macrophages leading to the secretion of pro-inflammatory mediators, thereby amplifying the inflammatory milieu in the MS macrophage regions

[55, 56]. This has been shown to contribute to extracellular matrix (ECM) remodeling by degrading fibronectin [57], consistent with the observed reduced expression of fibronectin in LNM in this study. In summary, for lymph node CD169$^+$ macrophages in breast cancer patients, our data indicate that LNM enables a metastatic niche inducing loss of SCS CD169$^+$ macrophages and change of MS CD169$^+$ macrophage phenotype into more immunosuppressive cells, hence modulating the local lymph node anti-tumor immune milieu. Indeed, although not significant, immune activating HLA-DR was expressed at higher levels on SCS CD169$^+$ macrophages compared to MS CD169$^+$ macrophages only in UnLN, indicating severe changes in LNM. Comparing the proteome of SCS and MS CD169$^+$ macrophages, also enable evaluation of our previous findings regarding tumor infiltrating CD169$^+$ macrophages in primary breast tumors [24], indicating that these could be more similar to MS CD169$^+$ macrophages than to SCS CD169$^+$ macrophages.

Anti-tumor B-cell responses is an emerging research area and the impact of LNM on B-cell activation is essential for understanding adaptive immune responses against metastasis. Our cohort analysis identified two B-cell clusters: B-cell follicles with active GCs and B-cell follicles without active GCs, each cluster present in UnLN as well as LNM. Expression of the proteins ICOS, BCL6, BAD, p53, 4-1BB, CD14, HLA-DR, PD-1 and CD25 in B-cell follicles with active GCs, shows that GCs are clearly activated in LNM compared to UnLN [58–68]. However, the higher expression of FoxP3, CD25, PD-1, PD-L1 and BCL6, may also indicate functional changes, including premature GC shutdown with reduced generation of long-lived plasma B-cells in LNM [69], or possibly a microenvironment submitted to T_{regs} and high immunosuppressive signals [70–72]. Indeed, initiation of GC shutdown has previously been shown to involve both T_{FH} cells expressing FoxP3, PD-1 and BCL6 [70–72] and ICOS-expressing T_{regs} that inhibit anti-tumor reactions [73, 74]. When adding B-cell follicles without GCs to the analyses, STING was upregulated in LNM, congruent with recent findings that interferon response promotes metastasis [8], but also additional immunoregulatory proteins (e.g. Arg1 and Tim-3) had increased expression. Taken together, this indicates that LNM promotes an immunoregulatory microenvironment in active B-cell follicles.

Finally, we also identified a capsular proximal T-cell rich cluster in the follicle areas, possibly representing interfollicular T-cells. Our data implied that LNM affecting the decay of SCS CD169$^+$ macrophages occurred in parallel with a reduced interfollicular T-cell-expansion, as evidenced by significantly lower Ki-67 expression and T-cell markers in Cluster 3 (e.g. CD45RO, OX40L, GZMB, CTLA4, Tim-3) in LNM compared to UnLN. These data support previous literature where interfollicular T-cells are dependent on presence of SCS CD169$^+$ macrophages for their expansion [27]. In contrast to previous studies on an interdependence or synergistic decay in SCS CD169$^+$ macrophages and B-cells seen in mice [23, 26, 32], we did not find a reduced B-cell compartment in human breast cancer LNM. These data is in line with a recent study [36]. Furthermore, the data point in the direction that T-cell activation is affected in local breast cancer lymph node immune responses, also supporting the recent finding in other cancer forms [75]. Lastly, our analysis showed proteome changes representing other cell populations like fibroblasts and fibroblast reticular cells (FRCs), which could contribute to the formation of the LNM niche. FRCs regression facilitates structural lymph nodes changes, promoting metastasis progression and warrants further investigation.

Conclusions

In conclusion, our study shows that breast cancer patients with lymph node metastasis have a compromised local immune environment in LNM as compared to UnLN. These changes most likely cause a dysregulated local anti-tumor immune response in LNM. Our findings underscore the critical role for CD169$^+$ macrophages in maintaining lymph node integrity and immune responses during metastasis, affecting both the T-cell and B-cell compartment. Our data emphasize the need for further research to understand and mitigate the immune suppression observed in LNM, to predict treatment responses to immunotherapy and clinical strategies regarding lymph node surgery.

Abbreviations

4-1BB	Tumor necrosis factor ligand superfamily member 9
APC	Antigen presenting cell
Arg1	Arginase 1
BCL6	B-cell lymphoma 6
Bcl-xL	B-cell lymphoma-extra-large
BIM	Bcl-2 Interacting Mediator of cell death
CTLA4	Cytotoxic T-lymphocyte associated protein 4
DC	Dendritic cell
ECM	Extracellular matrix
ER	Estrogen receptor
FAPα	Fibroblast activation protein α
FFPE	Formalin-fixed paraffin embedded
GC	Germinal Center
GZMA	Granzyme A
GZMB	Granzyme B
H3	Histone 3
HE	Hematoxylin-eosin
HER2	Human epidermal receptor-2
HK	Housekeeping proteins
HLA-DR	Human leukocyte Antigen DR isotype
ICOS	Inducible costimulatory T-cell receptor
IFR	Interfollicular region
IHC	Immunohistochemistry
LMM	Linear mixed model
LNM	Lymph node metastasis
MS	Medullary sinus
OX40L	Tumor necrosis factor receptor superfamily, member 4

Pan-ck	Pan-cytokeratin
PCA	Principal Component Analysis
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Cell Death Ligand 1
PR	Progesterone receptor
ROI	Region of interest
SCS	Subcapsular sinus
SEM	Standard error of the mean
SLN	Sentinel lymph node
SMA	α-smooth muscle actin
STING	Stimulator of interferon genes
T_{FH}	Follicular helper T-cell
TIM-3	T-cell Ig- and mucin domain containing molecule-3
Tpex	T-cell progenitor exhausted cell
T_{reg}	Regulatory T-cell
UnLN	Uninvolved Lymph Nodes

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06415-4.

Supplementary Figure 1–6 and Supplementary Table 1 (Suppl files Briem et al. JTM.pdf)

Author contributions

The work reported in the paper has been performed by the authors. OB was responsible for managing the data analyses in GeoMx data analysis suite and QuPath with assistance from ASG and EK, for interpretation of results and drafting of the manuscript. BT was responsible for selection of breast cancer slides, collection of clinicopathological traits and for pathology and clinical expertise. OB and EK were responsible for performing the labelling, annotation and interpretation of CD169 and CD20 in the IHC. AMF, ASG and LO provided expertise on the GeoMx NanoString technique and AMF together with OB performed the GeoMx labelling and readout. KL was responsible for designing this study, interpretation of results together with OB and EK and drafting of the manuscript. All authors read, revised and approved the final manuscript.

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Data availability

Available from the authors upon request.

Declarations

Ethical approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained by the Swedish Ethical Review Authority (Dnr 2021–04869).

Consent for publication

All authors read, revised and approved the final manuscript.

Competing interests

Authors declare no competing interest.

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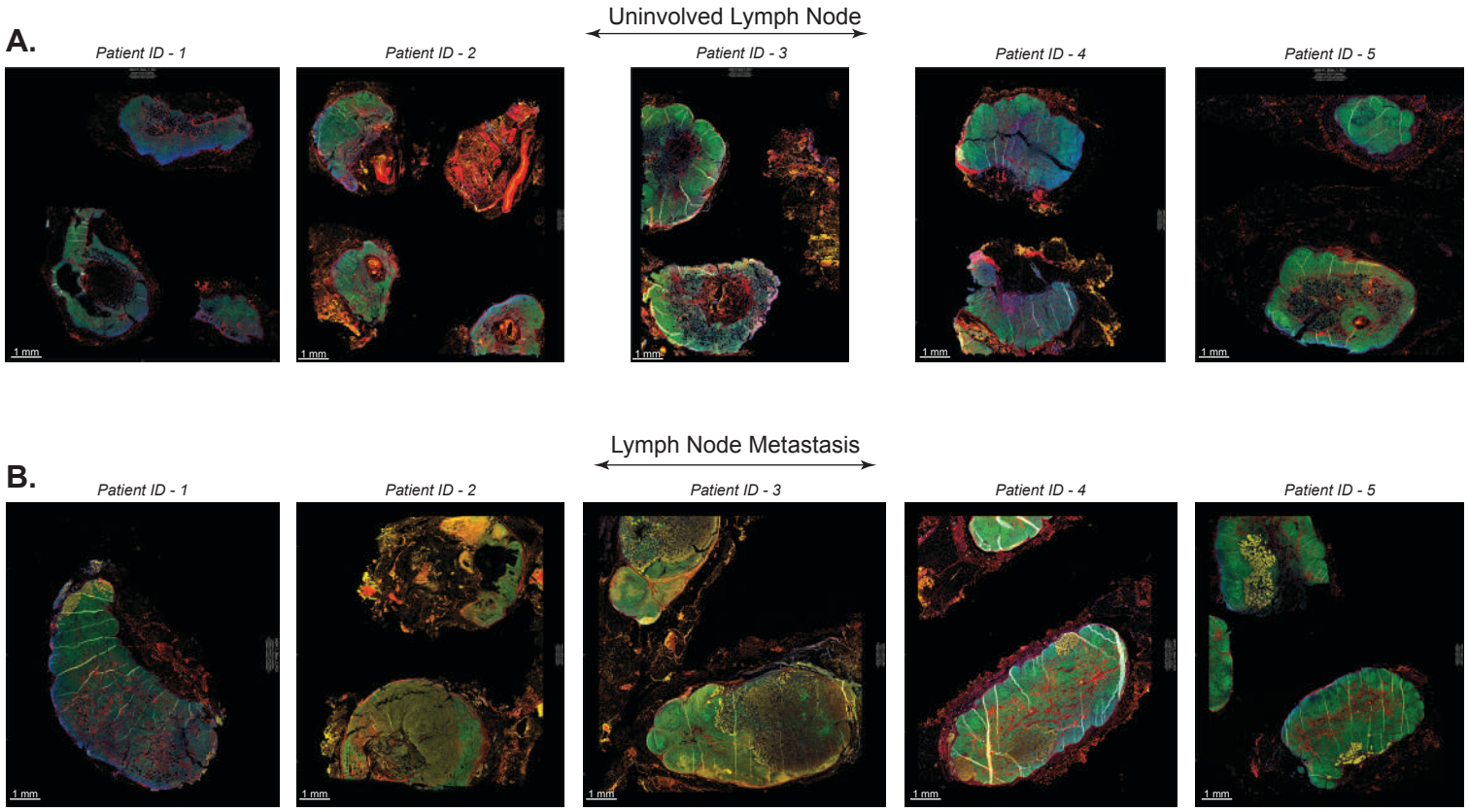
Publisher's note

Whole lymph node sections DSP GeoMx analysis

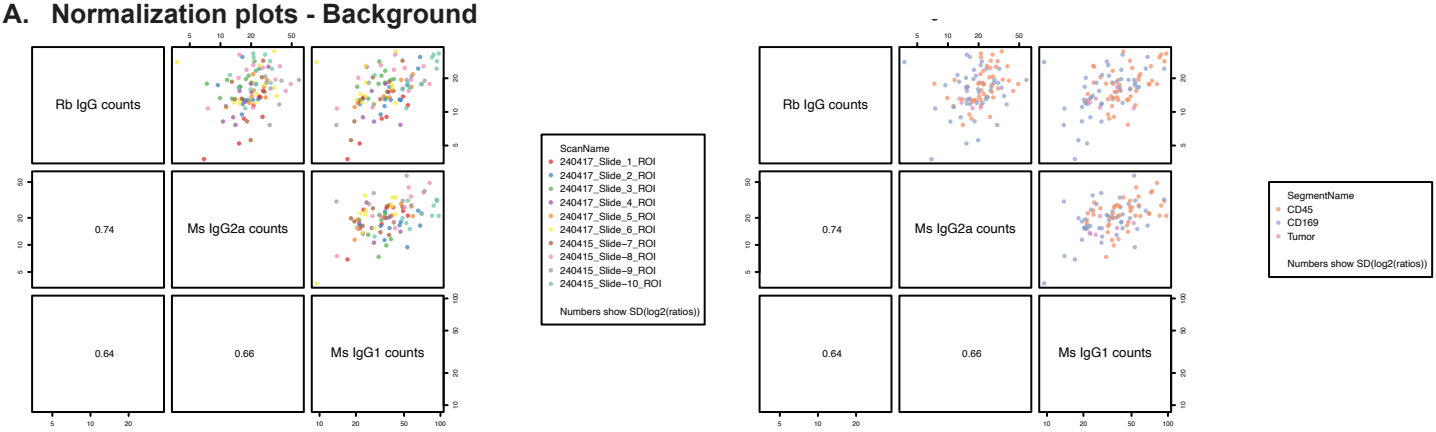


Supplementary Figure 1

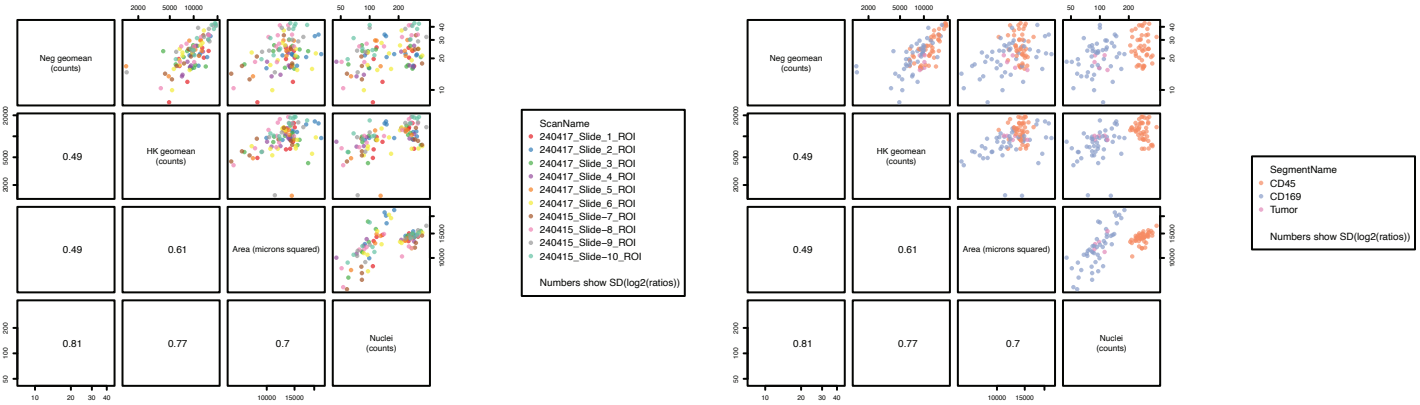
Paired whole lymph node sections without (UnLN) and with (LNM) metastasis from five breast cancer patients stained with pan-leukocyte CD45 (green), Pan-CK for malignant cells (yellow) and CD169 (red) in the GeoMX DSP platform.

Supplementary Fig. 2

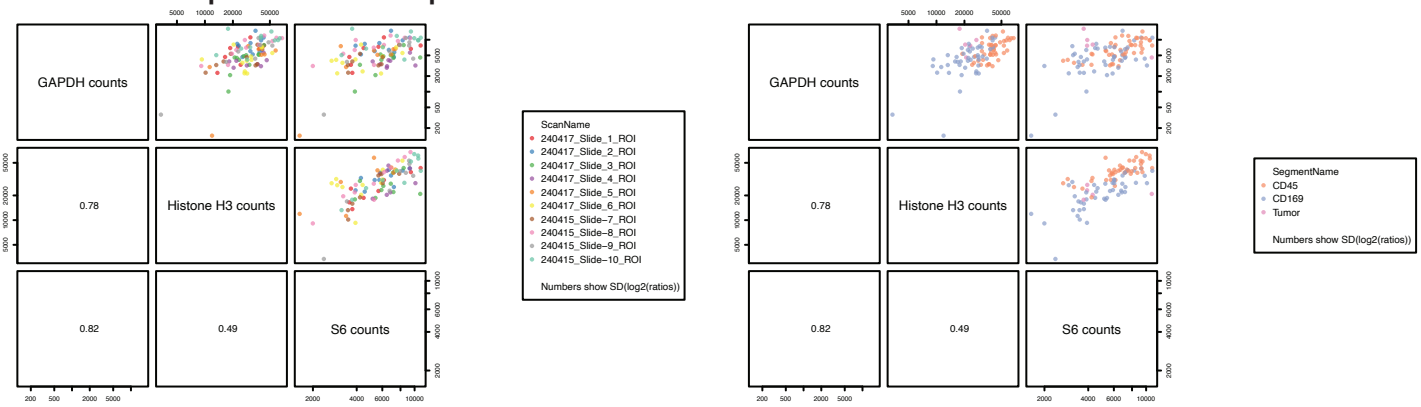
A. Normalization plots - Background



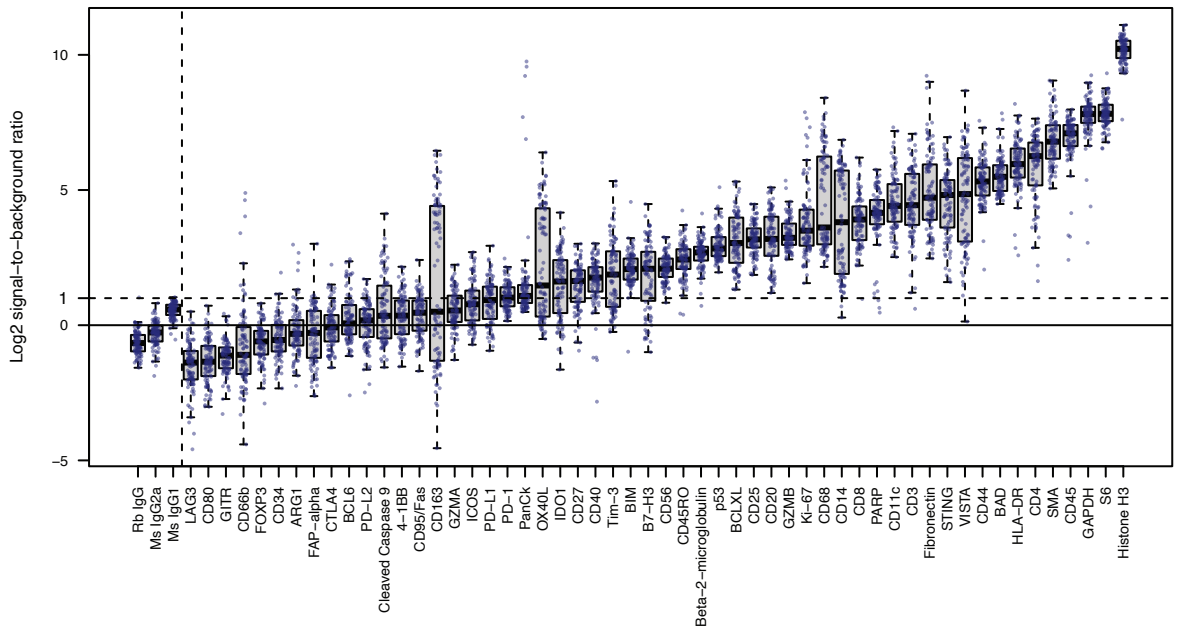
B. Normalization plots - Area and nuclei scaling



C. Normalization plots - House keepers



D. Signal background for each probe



Supplementary Figure 2

Normalization strategy used after quality control (QC), concordance between each probe is shown by standard deviation of the log ratios between the geometric means. (A) Pairwise correlation plots of background probes from the dataset. Background isotype controls revealed weak correlations and low geometric mean counts, thus indicating statistical instability and not suitable as a normalization factor (B) Pairwise correlation plots of background, housekeeper, area scaling or nuclei scaling from the dataset. Due to variation in cell size and cell density between macrophages, lymphocytes and tumor cells, nuclei counts and ROI area displayed high variability between ROIs and low correlation with housekeeping probes and background signaling, thus not best suited for normalization (C) Pairwise correlation plots of housekeeper probes from the dataset. Housekeeping probes showed strong correlation and high statistical stability, with the best suited correlation observed between housekeeping proteins Histone 3 (H3) and ribosomal protein S6. (D) Signal background from each probe in the dataset, CD80, LAG-3 and GITR were excluded from the analysis due to lower signal as compared to background probes (left).

Supplementary Figure 3

Whole slide H&E/CD169 DAB IHC

Uninvolved Lymph Node

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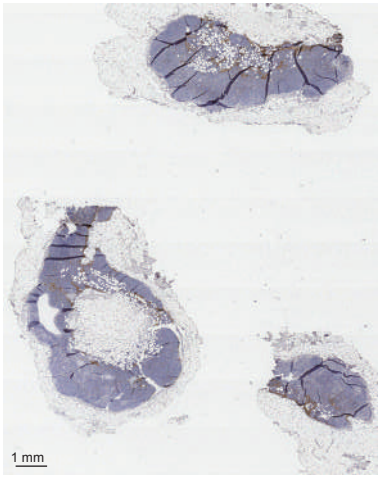

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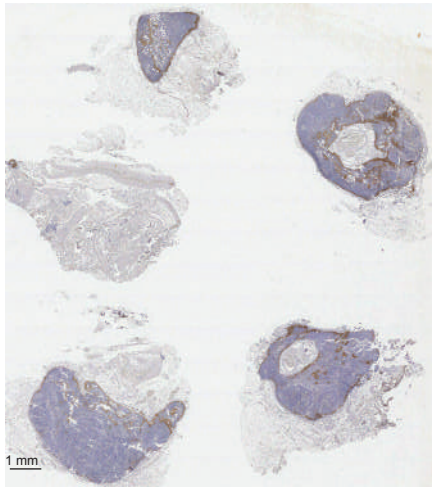

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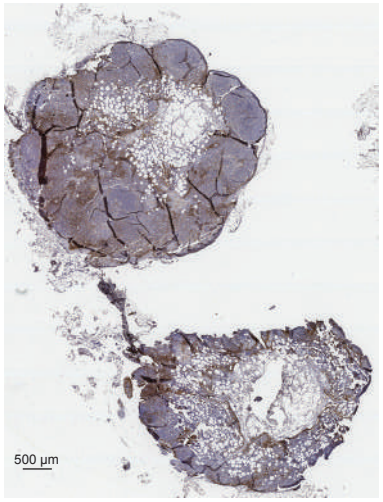

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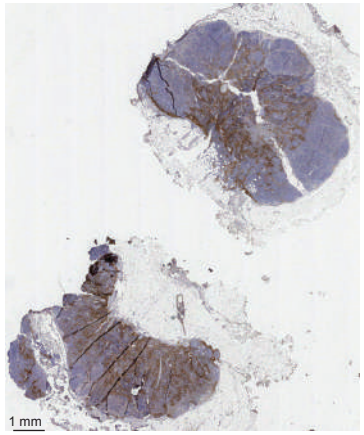

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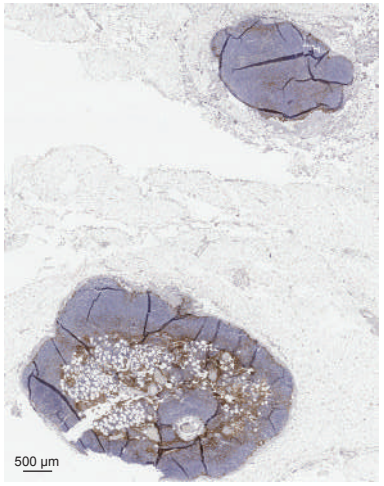

Lymph Node Metastasis

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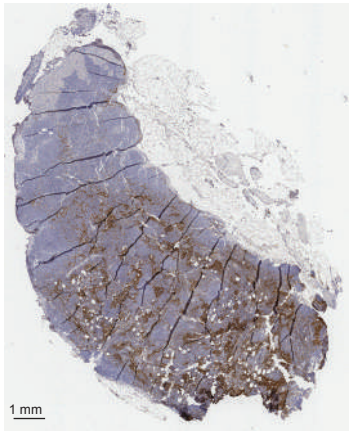

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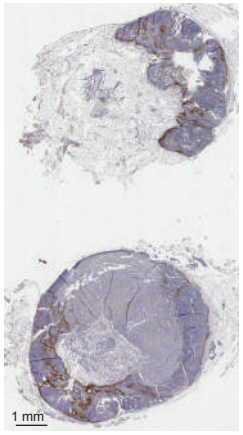

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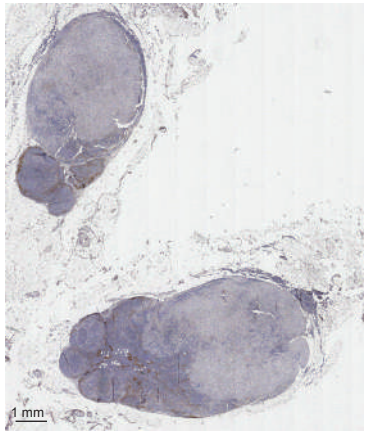

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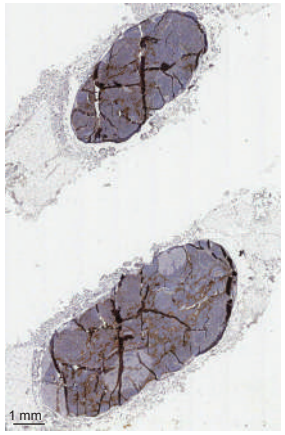

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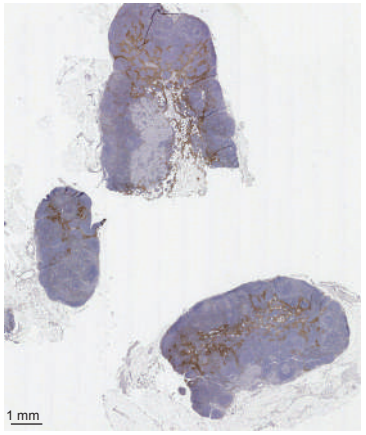


Supplementary Figure 3

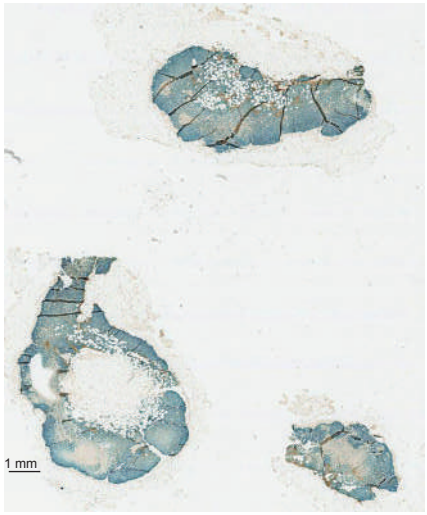
Paired whole lymph node sections without (UnLN) and with (LNM) metastasis from five breast cancer patients stained with H&E and IHC (CD169 brown).

Supplementary Figure 4

Whole slide CD169/CD20 IHC

Uninvolved Lymph Node

Patient ID - 1



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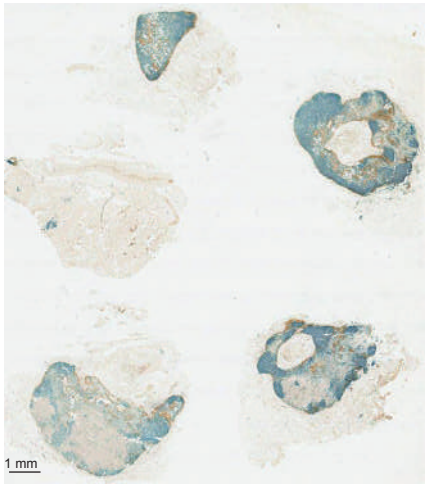

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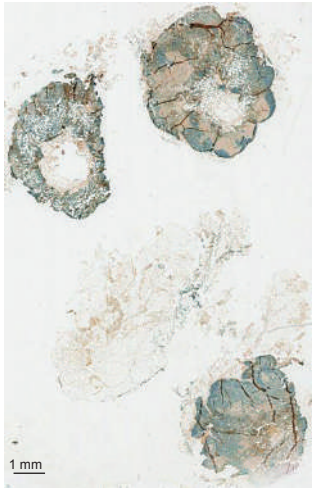

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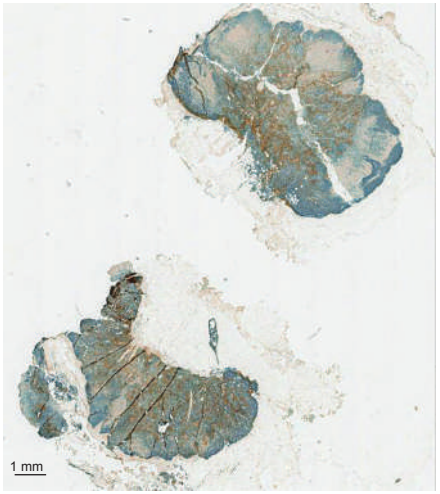

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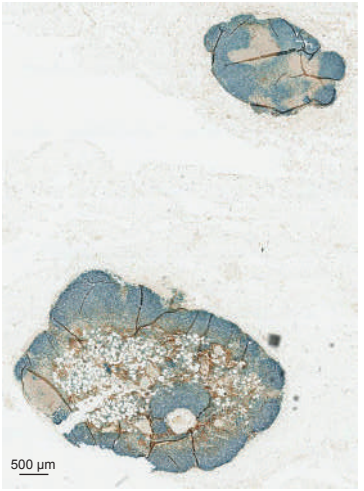
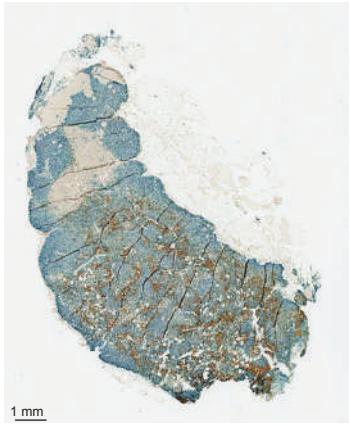


Lymph Node Metastasis

Patient ID - 1



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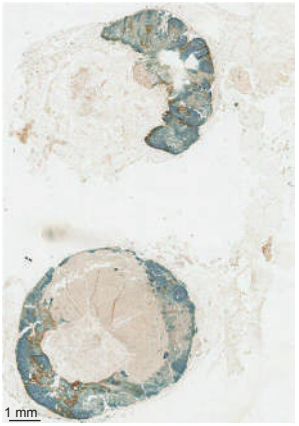
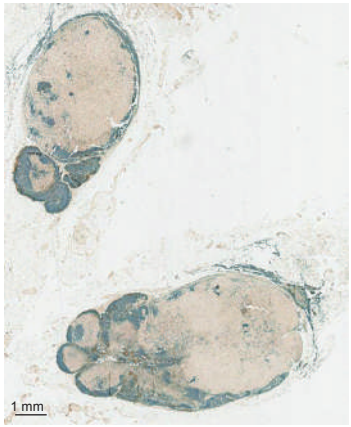


Patient ID - 3



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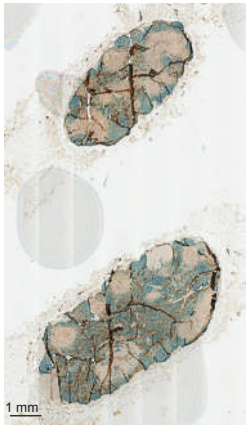

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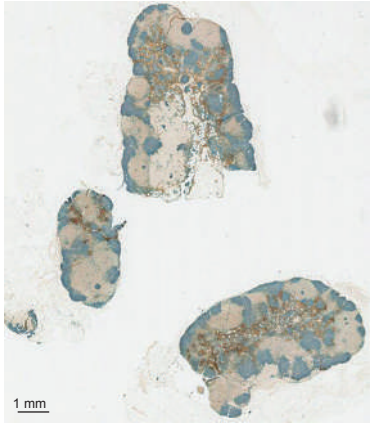
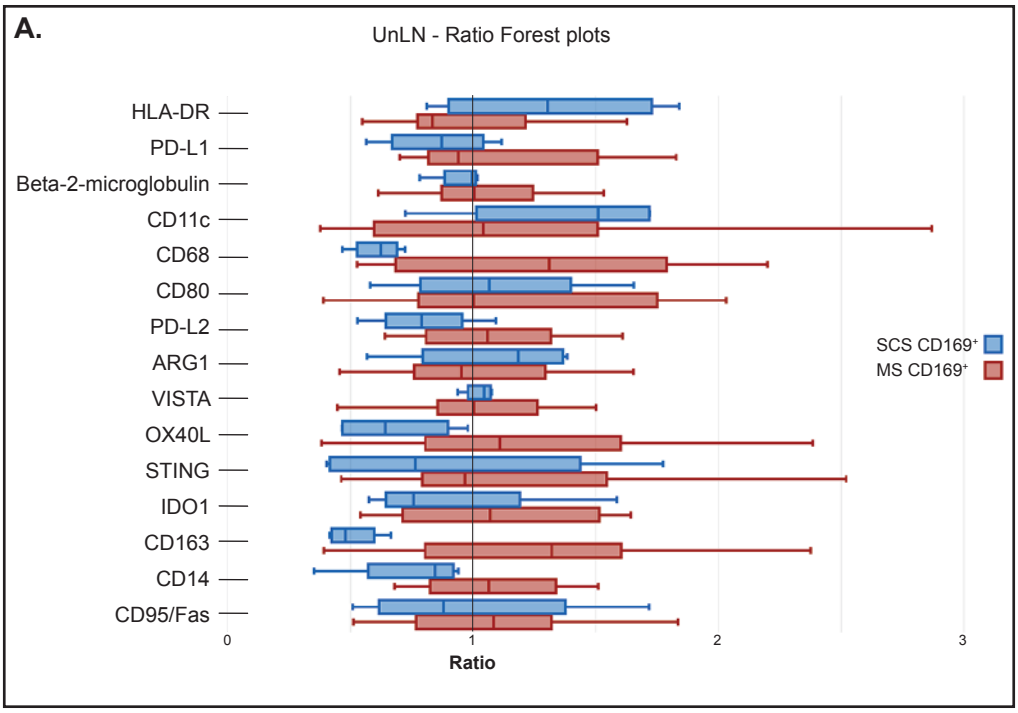


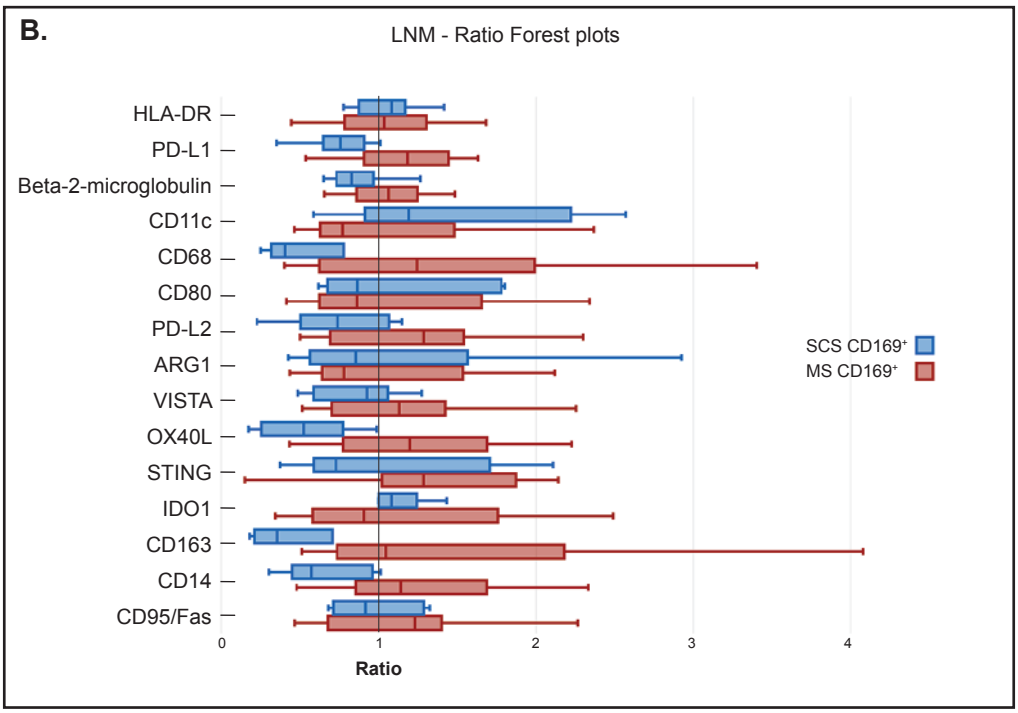
Supplementary Figure 4

Paired whole lymph node sections without (UnLN) and with (LNM) metastasis from five breast cancer patients stained with H&E and IHC (CD20 (blue) and CD169 (brown)).

Supplementary Figure 5

Differences in expression levels for selected proteins SCS CD169⁺ vs MS CD169⁺ macrophages in UnLN and LNM

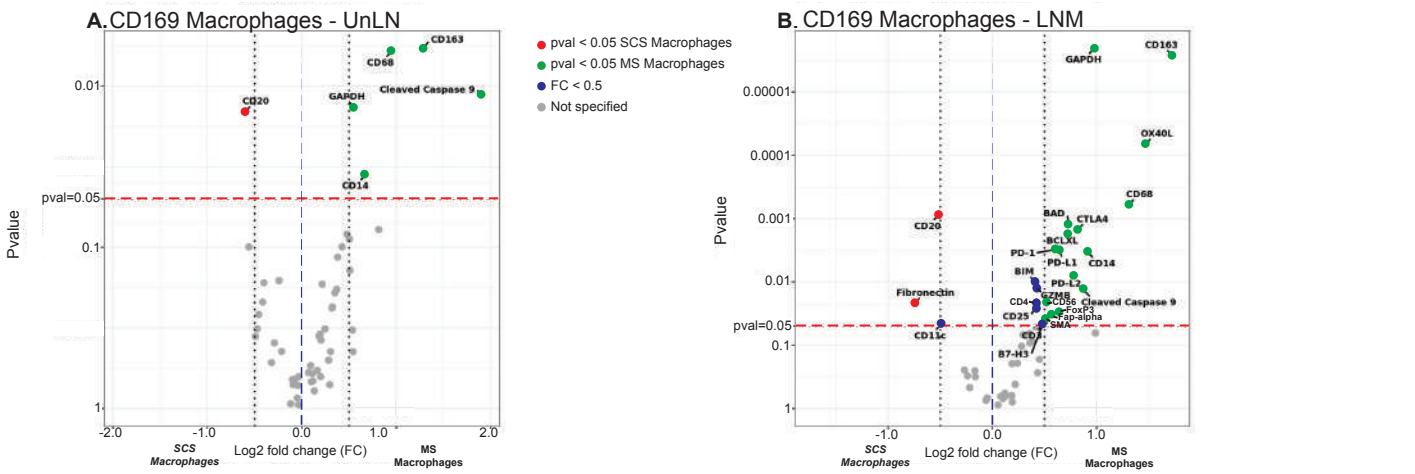




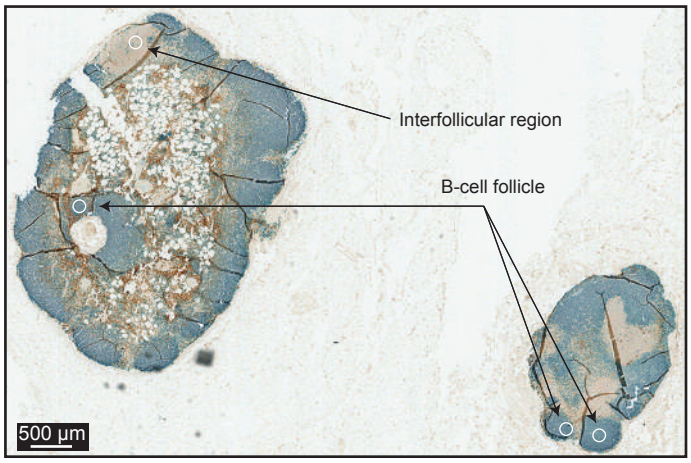
Supplementary Figure 5

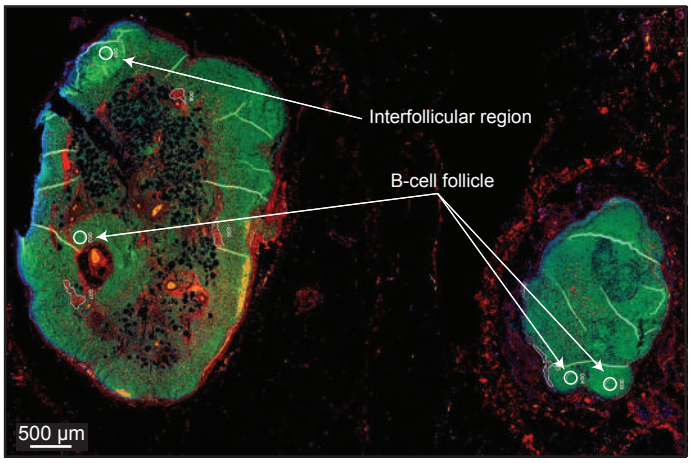
Forest plots presenting selected proteins with differences in protein expression levels for SCS $CD169^+$ macrophages (blue) versus MS $CD169^+$ macrophage (red) in UnLN (A) and LNM (B).

Supplementary Figure 6



C. Consecutive sections - IHC and GeoMx





Supplementary Figure 6

(A-B) Volcano plots representing statistical significance versus the magnitude of change in protein expression for SCS CD169^{+} macrophages (left) versus MS CD169^{+} macrophage (right) in UnLN (A) and LNM (B). (C) anti-CD20 IHC of a consecutive section from one representative patient UnLN. IHC (left) shows that the location of cortical CD45^{+} lymph node follicle ROIs used in GeoMX (right) are present in both cortical CD20^{+} regions representing B-cells follicles and in CD20^{-} regions representing interfollicular region (IFR) T-cell areas.

Supplementary Table 1: Antibodies used for GeoMX DSP analysis

Abs/reagent	Type	Clone	Ab Dilution	DSP channel - EM max
Anti-CD169	Morphological marker	SP216	1:50	Cy5 – 666 nm
Anti-CD45	Morphological marker	NanoString	1:200	Cy3 – 568 nm
Anti-PanCK	Morphological marker	NanoString	1:1000	Texas Red – 615 nm
SYTO13	Morphological marker	NanoString	500 nM	FITC – 525 nm
Core Abs – Cell profiling	Profiling marker	NanoString	8 µl in 200 µl	None
Module Abs – Drug Target	Profiling marker	NanoString	8 µl in 200 µl	None
Module Abs - Immune activation	Profiling marker	NanoString	8 µl in 200 µl	None
Module Abs – Cell Death	Profiling marker	NanoString	8 µl in 200 µl	None
Module Abs - Cell typing	Profiling marker	NanoString	8 µl in 200 µl	None