Function of Innate Immune Cells in Breast Cancer

Gunnarsdóttir, Fríða Björk

2021

Document Version:
Publisher's PDF, also known as Version of record

Function of Innate Immune Cells in Breast Cancer

Function of Innate Immune Cells in Breast Cancer

Fríða Björk Gunnarsdóttir

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Department of Translational Medicine, Lund University, Sweden.

To be defended at Clinical Research Center Aula, Jan Waldenströms gata 35, Malmö on December 3rd 2021 at 9:15 am.

Faculty opponent
Professor Charlotta Dabrosin
Department of Biomedical and Clinical Sciences,
Linköping University, Linköping, Sweden

Organization: LUND UNIVERSITY Faculty of Medicine Department of Translational Medicine Malmö	Document name DOCTORAL DISSERTATION
	Date of issue 2021-12-03
Author: **Fríða Björk Gunnarsdóttir**	Sponsoring organization

Title and subtitle: **Function of Innate Immune Cells in Breast Cancer**

Abstract:

Tumor associated macrophages (TAMs) are key cells in creating an immunosuppressive tumor microenvironment (TME). In general, presence of TAMs is associated with worse outcome in cancer patients. Macrophages with anti-tumor effect can be found in the TME but are usually in minority. This thesis focuses on the role of innate immune cells, and especially macrophages, in breast cancer.

In the first project we showed that pro-inflammatory macrophages downregulate estrogen receptor alpha (ERα) on breast cancer cells. We unveiled the molecular mechanism behind this, showing that TNF-α derived from macrophages inactivates transcription factor FOXO3a. Moreover, presence of TAMs in breast cancer tumors associated with ER negativity and worse prognosis in ERα$^+$ patients.

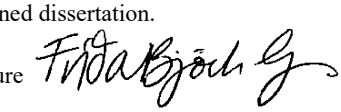
In projects two and three we shifted our focus towards CD169$^+$ macrophages in lymph nodes (LN) and primary tumors (PT) of breast cancer patients. In project II we showed that presence of CD169$^+$ macrophages in metastatic LN correlated with better prognosis, while presence of CD169$^+$ macrophages in PT did not. Association with PD-L1 expression was found in both locations. In project III we saw that CD169$^+$ TAMs are most likely monocyte derived in a type I IFN environment and display a unique pro-inflammatory phenotype and cytokine profile, but with immunosuppressive function. In a patient cohort they were associated with tertiary lymphoid structures and regulatory T cells, and therefore with worse prognosis.

In conclusion, TAMs represent a broad spectrum of macrophages with unique origin, phenotype, and function. In this thesis we have added to the growing knowledge of these cells and their role in breast cancer. Not only does the type of cancer matter for their function, but further their location and surrounding environment within breast cancer.

Key words: Tumor associated macrophages, breast cancer, ERα, FOXO3a, CD169, PD-L1, metastatic lymph node

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language**:** English
ISSN and key title: 1652-8220		ISBN**:** 978-91-8021-147-5
Recipient's notes	Number of pages: 77	Price
	Security classification	

Signature  Date 2021-10-28

Function of Innate Immune Cells in Breast Cancer

Fríða Björk Gunnarsdóttir

LUND
UNIVERSITY

Cover photo by the Author

All illustrations (Fig. 1-10) are created with BioRender.

Lund University
Faculty of Medicine
Department of Translational Medicine, Malmö

ISBN 978-91-8021-147-5
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund 2021

Deyr fé,
deyja frændur,
deyr sjálfur ið sama.
En orðstír
deyr aldregi
hveim er sér góðan getur.

Cattle die,
kindred die,
Every man is mortal:
But the good name
never dies
of one who has done well.

Hávamál – 76

Table of Contents

List of papers:

I. Inflammatory macrophage derived TNF-α downregulates estrogen receptor α via FOXO3a inactivation in human breast cancer cells.

Frida Björk Gunnarsdottir, Catharina Hagerling, Caroline Bergenfelz, Meliha Mehmeti, Eva Källberg, Roni Allaoui, Sofie Mohlin, Sven Påhlman, Christer Larsson, Karin Jirström, Daniel Bexell, and Karin Leandersson

Exp Cell Res. 2020 May 1;390(1):111932

II. Co-localization of $CD169^+$ macrophages and cancer cells in lymph node metastases of breast cancer patients is linked to improved prognosis and PD-L1 expression.

Frida Björk Gunnarsdottir, Nathalie Auoja, Pär-Ola Bendahl, Lisa Rydén, Mårten Fernö, and Karin Leandersson

Oncoimmunology. 2020 Nov 22;9(1):1848067

III. $CD169^+$ macrophages present in primary tumors are monocyte derived type I IFN producers that possess broad immunosuppressing functions

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

Manuscript. [#] and † Equal contribution.

Papers not included in this thesis:

I. Human G-MDSCs are neutrophils at distinct maturation stages promoting tumor growth in breast cancer.

Meliha Mehmeti-Ajradini, Caroline Bergenfelz, Anna-Maria Larsson, Robert Carlsson, Kristian Riesbeck, Jonas Ahl, Helena Janols, Marlene Wullt, Anders Bredberg, Eva Källberg, **Frida Björk Gunnarsdottir**, Camilla Rydberg Millrud, Lisa Rydén, Gesine Paul, Niklas Loman, Jörgen Adolfsson, Ana Carneiro, Karin Jirström, Fredrika Killaner, Daniel Bexell and Karin Leandersson

Life sci Alliance. 2020 Sep 21;3(11):e20200893

II. Establishment of Melanoma Tumor Xenograft Using Single Cell Line Suspension of Co-injection of Patient-Derived T Cells in Immune-Deficient NSG Mice – Chapter 15, *Immune Checkpoint Blockade, Methods and Protocols.*

Fríða Björk Gunnarsdóttir, Rolf Kiessling and Yago Pico de Coaña

Methods Mol Biol. 2019;1913:207-215

List of Abbreviations

ACT	adoptive cell therapy
ADCC	antibody-dependent cellular cytotoxicity
AIRE	autoimmune regulator
APC	antigen presenting cell
Arg-1	Arginase-1
BCR	B cell receptor
BRCA	breast cancer gene
CAF	cancer associated fibroblast
CAR	chimeric antigen receptor
CD	cluster of differentiation
cDC	conventional DC
CTL	cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
ECM	extracellular matrix
EMT	Endothelial to mesenchymal transition
ER	estrogen receptor
G-CSF	granulocyte stimulating factor
G-MDSC	granulocytic-MDSCs
GM-CSF	granulocyte-macrophage colony-stimulating factor
HER2	human epidermal growth factor receptor 2
HLA	human leukocyte antigen
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
iNOS	nitric oxide synthases
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
M1	pro-inflammatory macrophage
M2	anti-inflammatory macrophage
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
Mo-MDSC	monocytic MDSC
moDC	monocyte derived DCs
NETs	neutrophil extracellular traps
NK	natural killer
OS	overall survival
PD-1	programmed cell death protein 1
PD-L1	programmed death-ligand 1
pDC	plasmacytoid DC
PGE2	prostaglandin E2
PR	progesterone receptor
PRR	pattern recognition receptors
ROS	reactive oxygen species
SCS	subcapsular sinus
SSM	subcapsular sinus macrophage
TA	tumor antigen
TAM	tumor associated macrophage
TCR	T cell receptor
TDLU	terminal duct lobular unit
Tfh	T follicular helper cell
TGF	transforming growth factor
Th	T helper cell
TIL	tumor infiltrating lymphocyte
TME	tumor microenvironment
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
Treg	regulatory T cell
VEGF	vascular endothelial growth factor

Popular Science Summary

With around 9000 new cases diagnosed in Sweden every year, breast cancer is a burden on the healthcare system and greater society. In most cases the prognosis is good, but still around 15% of patients diagnosed will die because of the disease. When breast cancer is diagnosed it is categorized into subtypes according to expression of hormone receptor on the breast cancer cells. These receptors are Estrogen receptor, Progesterone receptor and HER2 receptor. If the tumors are receptor positive that means that the tumor depends on hormones or growth factors and uses the receptors to capture and make use of these growth factors to grow. The cancer can be treated by blocking these receptors, but some breast cancers do not express any of these receptors. They are called triple negative breast cancers and have worse prognosis due to lack of treatment options and faster growing cancers. If the tumor cells have spread to nearby lymph nodes the cancer has metastasized which means worse prognosis for the patient.

The body's immune system is designed to protect from disease. It detects and responds to a wide variety of invading pathogens such as bacteria, viruses, parasites, but also to cancer cells. Cancer is a group of diseases that all involve abnormal growth of cells that can spread to other parts of the body. Cancer results from damages in DNA and loss of normal function of the cell. Under normal circumstances cell division is tightly regulated but mutations that spontaneously occur can sometimes affect genes that control cell growth and division. This can lead to tumor formation. Some immune cells can recognize tumor cells, since they do have features that distinguish them from normal cells even though they are not invading pathogens. After recognition of tumor cells, the immune system initiates a process to eliminate the cancer cells. In most cases this eradication is successful, and the body has been protected, but in some cases the tumor can evade immune surveillance and continue to grow and spread. It does so by creating a microenvironment in the tumor which benefits the cancer cells and dampens further attacks from the immune system. This can be because of proteins that cancer cells secrete, receptors on their surface that inhibit function of immune cells, or by recruiting anti-inflammatory immune cells and hijacking mechanisms from the immune system for its own benefit.

Macrophages are cells of the immune system that are professional eating cells or phagocytes. They are found in all types of tissue in the body, patrol the environment for pathogens, and recruit other immune cells to the site of infection or damage.

They also have an anti-inflammatory role and can limit immune responses. Inflammatory macrophages have been known as M1-like macrophages and anti-inflammatory macrophages as M2-like macrophages. Macrophages have been shown to infiltrate tumors, but tumor associated macrophages are usually anti-inflammatory and lead to tumor growth and worse prognosis for cancer patients. In this thesis we have looked at different types of macrophages in breast cancer and what role they have within the tumor environment.

In the first project we looked at downregulation of the hormone receptor estrogen receptor alpha (ERα) and what could possibly be responsible for it. Patient with ERα positive breast cancer can respond to hormone treatment, but treatment resistance is common and is associated with downregulation of ERα on the breast cancer cells. Without ERα on the breast cancer cells the patient cannot be treated with hormone therapy, limiting the treatment options and worsening prognosis. We saw that when we injected mice with breast cancer cells that formed tumors in combination with monocytes from humans, the breast cancer cells lost their expression of ERα. The injected monocytes develop into macrophages in the mice, so we wanted to know if these macrophages were responsible for the loss of ERα. We saw that the pro-inflammatory M1-like macrophages secrete a protein called tumor necrosis factor alpha (TNF-α), which sends a message to other immune cells to start inflammation that destroys tumor cells. TNF-α in this setting was also the reason for ERα downregulation. TNF-α downregulates ERα by inactivating a regulatory protein in the tumor cells named FOXO3a. Inactivation of FOXO3a is often seen in cancer and is known as a tumor suppressor, but here we saw that it also regulated ERα expression on breast cancer cells. This would mean that the pro-inflammatory and anti-tumor M1-like macrophages also have the less desirable effect of downregulating ERα on the tumor cells, and consequently worsening prognosis for breast cancer patients.

In our second project we shifted our focus to macrophages in breast cancer that express a surface marker known as CD169. It is found in high amounts on macrophages that are located in lymph nodes and are called subcapsular sinus (SCS) macrophages. There they are the first layer of cells in draining lymph nodes where they capture proteins derived from pathogens and cancer cells and present them to other immune cells for recognition. We had also observed macrophages expressing CD169 in primary breast cancer tumors and wanted to see if they were related to the $CD169^+$ SCS macrophages in lymph nodes. We used a breast cancer patient cohort and saw that patients with $CD169^+$ macrophages in lymph nodes with breast cancer metastases had better prognosis than patients without. Interestingly, presence of $CD169^+$ macrophages in the tumor ($CD169^+$ TAM) did not correlate with better prognosis but showed the opposite trend. We also saw that presence of CD169 on macrophages was correlated with presence of another surface protein, PD-L1. PD-L1 plays a major role in suppressing immune cells in cancer that would normally

attack tumor cells. Binding of this protein on cancer cells to their ligand on immune cells put a brake on the immune response.

In project three we wanted to investigate these $CD169^+$ tumor associated macrophages ($CD169^+$ TAMs) better and see if they were related to the $CD169^+$ macrophages found in lymph nodes with regards to origin, surface proteins, and function. Using a mouse model like in project one, was saw that these $CD169^+$ TAMs were most likely derived from monocytes, just like the other tumor macrophages are. In our experiments we saw that they have a similar surface protein expression as pro-inflammatory M1-like macrophages and secrete proteins that are linked to activation and recruitment of other immune cells. Surprisingly, they showed and anti-inflammatory and pro-tumor function. In another patient cohort, we saw that they were positioned together with clusters of immune cells (tertiary lymphoid structures; TLS) that has in many cancer types been correlated to better survival of patients. Here we saw that the presence of $CD169^+$ TAMs in this location correlated with worse survival and it also correlated with presence of highly anti-inflammatory immune cells, regulatory T cells. Our conclusion here was that $CD169^+$ TAMs and $CD169^+$ SCS macrophages in lymph nodes are not of the same origin or function and should be considered as different targets in breast cancer studies.

The main conclusion of this thesis is that we have uncovered more pieces in the tumor macrophage puzzle. They are very diverse cells with different function, and their subtype, origin, protein production, and interaction with other cells must be assessed individually for each subset of macrophage, to know more about their function in breast cancer. Not only does that matter, but their location and the tumor environment surrounding them plays perhaps the biggest role in their activity.

Introduction to the immune system

Every day the human body is exposed to a variety of threats and invaders. To protect the normal functions of the body, the immune system has evolved to be finely tuned and ready to shield from and eliminate intruders such as bacteria, virus, fungi, and parasites. Not only is the protection from external threats an important role of the immune system, but also protection from internal threats. These can be tissue damage, cell death and cancer cells. The immune system is therefore involved in several processes that are linked to internal stress of the body, like wound healing processes, tissue remodeling and elimination of cancer cells. The key here is the ability to distinguish the body's own tissue from foreign invaders – self from non-self. The immune system consists of a complex network of various immune cells, their mediators, and organs of the immune system. It can be divided into two arms, the innate immune system and the adaptive immune system (**Fig. 1**).

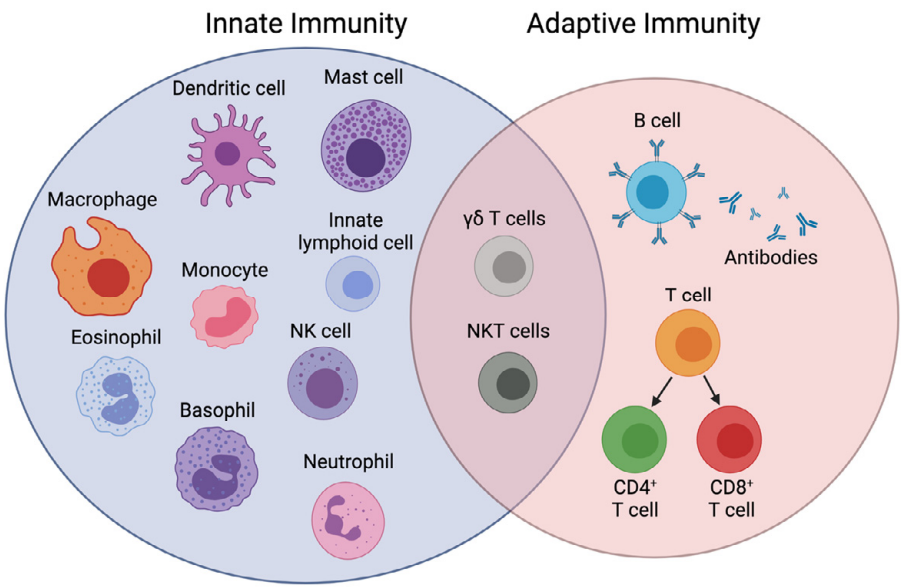


Figure 1. Overview of immune cells of innate and adaptive immunity. The left side shows cells mainly operating within the innate immune response. On the right are B cells and T cells, which belong to the adaptive arm of the immune system. Overlapping are cells that possess both innate and adaptive immune characters. Even though they are of the T cell lineage, they recognize antigens in an invariable and often semi-specific manner.

Leukocytes, also known commonly as white blood cells, appear white when isolated from red blood cells and plasma. In Greek, *leuk-* means white and *cyt-* means cells. Leukocyte is therefore an umbrella term for cells of the immune system. Leukocytes are divided into subgroups based on their progenitor cells: myeloid cells, which includes granulocytes, monocytes, macrophages, and dendritic cells; and lymphoid cells, which are B cells, T cells and Natural killer cells.

Innate immune system

When a pathogen enters the body, the first line of defense is the innate immune system. Immediate innate immune mechanisms include features that are constantly present in the body such as epithelial barriers, mucus, defensins, certain innate receptors and soluble mediators, such as cytokines, expressed by cells already present in all tissue [1]. Cytokines are small proteins that are important in cell signaling. They are produced by a broad range of cells, immune cells as well as endothelial cells, fibroblasts and stromal cells [2]. Receptors in or within these cells, known as pattern recognition receptors (PRRs), recognize molecules from pathogens, pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs) which are endogenous cell derived molecules released from damaged or dying cells [3]. This initiates the process of acute inflammation, activating innate immune cells and releasing inflammatory mediators and secreting cytokines, which leads to clinical signs of inflammation [4,5]. The later induced innate immune mechanisms includes innate immune cells recruited to the site of infection like granulocytes (neutrophils, eosinophils, basophils, mast cells), monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells [6]. Even though this first line of defense detects and destroys pathogens that enter the body within hours, due to low-specificity recognition receptors, an innate immune response is less effective than an adaptive immune response and holds no immunological memory of previous pathogen encounters.

For efficient protection, the adaptive immune system needs to be activated. Certain cells of the innate immune system called professional antigen presenting cells (APCs) are responsible for this activation. The main APCs are DCs and macrophages. Adaptive immune cells consist of B and T cells and are primarily located in secondary lymphoid organs in the body, which means that they are generally not present as naïve cells at the main entry points of pathogens. They therefore rely on innate immune APCs to capture products from pathogens, process them and present them as peptides, also known as antigens, to adaptive immune cells to be able to activate them. Antigens are presented on either Major Histocompatibility complex (MHC) class I or MHC class II molecules on the cell surface. The human version of MHC is also known as human leukocyte antigen (HLA). MHC class I is expressed on all nucleated cells and presents antigens from intracellular pathogens, like viruses [7]. There are three major (HLA-A, B, C) and 3 minor (HLA-E, F, G) MHC class I genes in humans. MHC class II on the other hand

are mainly found on professional APCs and presents antigens from extracellular sources, like bacteria [8]. There are 3 major (HLA-DP, DQ, DR) and 2 minor (HLA-DM, DO) MHC class II genes in humans. Activation of the adaptive immune cells by the innate immune cells leads to immune mechanisms capable of destroying the invading pathogens, as well as to an immunological memory, immunity. In this way, the co-operation between innate and adaptive immune cells results in elimination of most invading pathogens.

Neutrophils

Granulocytes are a subtype of innate immune cells that are characterized by presence of granules in their cytoplasm. This category includes Eosinophils, Basophils, Mast cells and Neutrophils. Neutrophils are the most abundant of the granulocytes, as well as being the predominant circulating immune cell population in humans, making up between 40 and 70% of leukocytes in blood [9]. They are rather short-lived cells, with an average lifespan of 8 hours in circulation in humans [10]. Due to their numbers, neutrophils are among the first cells to encounter pathogens within the body. They have high motility and are attracted to site of infection through cytokines secreted by endothelial cells and activated macrophages. They in return secrete cytokines that amplify the immune reaction. They have three main methods for pathogen attack: phagocytosis and internal killing of microbes; degranulation where they release an assortment of proteins with antimicrobial properties; and lastly release of neutrophil extracellular traps (NETs) [11]. NETs are web-like structures made up of fibers composed of chromatin and serine proteases, trapping, and killing microbes [12]. Recent studies have shown that neutrophils are complex cells that are capable of many specialized functions. There are several subsets, some with anti-inflammatory roles, and recent studies have linked neutrophils to myeloid derived suppressor cells (MDSCs) involved in cancer [13], which will be discussed in more detail later in this thesis.

Monocytes

Monocytes are versatile cells, comprising around 10% of circulating leukocytes in human blood. They have a role in early inflammation, clearing of pathogens and dead cells, tissue repair, homeostasis, activation of the adaptive immune system and controlling inflammation. This wide range of effect shows how important they are in both health and disease. Monocytes further provide a progenitor pool of cells that can differentiate into DCs, macrophages and MDSCs [14]. Monocytes are generated from a common myeloid progenitor in the bone marrow, and can be characterized

by high cytoplasm to nucleus ratio, kidney shaped nucleus, and certain surface markers [15]. They have been grouped into subtypes based on the expression of surface markers cluster of differentiation (CD), CD14 and CD16: Classical monocytes ($CD14^{++}CD16^{-}$) which correspond to up to 90% of blood monocytes and are more likely to differentiate into monocyte-derived DCs (moDC); non-classical ($CD14^{dim}CD16^{++}$); and intermediate monocytes ($CD14^{++}CD16^{+}$), both of which are more likely to differentiate into macrophages [16]. Recent research has expanded the number of surface markers for monocyte phenotyping [17].

Classical and intermediate subsets have high phagocytic capacity and respond better to bacterial stimuli than non-classical monocytes, which respond better to viral stimuli and have a lower phagocytic capacity. Classical monocytes are actively recruited to sites of inflammation and can produce both pro- and anti-inflammatory mediators [17]. The frequency of monocyte subsets seems to be tightly regulated, which reinforces the idea that functional differences between the subsets merit strict regulation [16]. Different subsets of monocytes have context-dependent functions, which has generated controversy in the literature, where a function of a specific monocyte subset has often been generalized for all monocytes responding to the same stimuli [18]. Furthermore, most of the knowledge we have on monocytes comes from extensive studies in murine models, where the surface marker Ly6c is used to divide monocytes into subtypes [19]. This means that a clearer definition of human monocytes subsets and functions is needed.

Macrophages

Macrophages originate either as recruited macrophages from circulating monocytes, or as resident macrophages derived from the yolk sac erythro-myeloid precursors or liver before birth, that are then maintained in their tissue location throughout life [20,21]. Macrophages are found in essentially all tissues throughout the body, and during an infection the majority of macrophages derive from recruited, circulating monocytes that have relocated to the site of infection. Macrophages got their name from the Greek words *makrós* meaning large and *phagein* which means to eat, being aptly named as the main recyclers of the body. They phagocytose cells and recycle cell components, removing cell debris, clearing dead cells and are involved in healing wounds [22]. Like monocytes, they are highly plastic cells, and in humans are usually identified by the pan-macrophage marker CD68 [23]. During infection, macrophages respond to stimuli that can originate from pathogens, innate immune cells, antigen-specific T cells or even to autocrine signals. Depending on signals from the microenvironment they can polarize towards a spectrum of subtypes. Modern fate-mapping techniques have been used to explore the origin of macrophages, proving that macrophages can also be established in the embryo as mentioned before. The tissue specific niche can highly impact the phenotype of

tissue resident macrophage as well as the ratio between embryonically derived and monocyte derived macrophages. The various subtypes of tissue resident macrophages throughout the body have different names based on their location, such as Kupffer cells in the liver, alveolar macrophages found in pulmonary alveoli of the lungs, and microglia in the brain and spinal cord. These cells are all classified as macrophages and are therefore a part of the mononuclear phagocyte system [24,25].

Macrophage subtypes

In the past, macrophage subtypes have been classified along a linear scale, with the two ends of the scale represented by M1 as classically activated macrophages, and M2 as alternatively activated macrophages. This division of macrophages into classically or alternatively activated, was first described in the early 1990s [26]. Just under a decade later, the M1 and M2 nomenclature was introduced to reflect the nomenclature for T helper cells [27]. M1 macrophages are defined as pro-inflammatory macrophages that are induced by bacterial lipopolysaccharide (LPS), Interferon (IFN)-γ and tumor necrosis factor (TNF). They secrete high levels of Interleukin (IL)-12 as well as IL-6, TNF, IL-1β, inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS). M2 on the other hand are defined as anti-inflammatory or tissue-remodeling. They are induced by IL-4, IL-10 and IL-13 and lack TNF-α secretion, but secrete IL-10, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) [28,29].

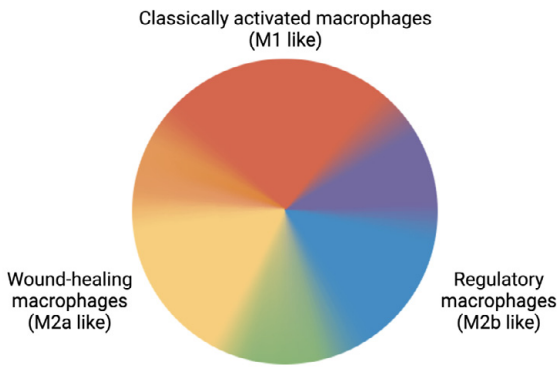


Figure 2. Color wheel of macrophage activation. The three main populations of macrophages proposed by Mosser and Edwards, arranged on a primary color wheel. Here the three primary colors represent sub-populations of macrophages, red as classically activated, blue as regulatory and yellow as wound healing. Shown in parenthesis is alternative nomenclature. Adapted from Mosser and Edwards, 2008 [22].

This classification system has its flaws, since essentially, M2 macrophages covered all macrophages that did not fall into the M1 classification [22]. M2 macrophages have been expanded into even more subtypes (M2a, M2b, M2c and M2d) due to the vast difference in biochemistry and function of these immune cells [28]. In 2008, Mosser

and Edwards proposed that macrophages be classified according to their different activities: host defense, wound healing, and immune regulation. This would, just like the three primary colors, represent a wheel of macrophage spectrum, where these three basic macrophage populations would blend into different shades of activation [22]. This is illustrated in **Fig. 2**. Secondary colors, such as green, would represent populations with a mixture of functions, such as tumor associated macrophages (TAMs) which have both wound healing and regulatory functions. TAMs will be discussed in more detail later in this thesis. In 2013 a group of researchers, including Mosser, met at the International Congress of Immunology and drafted a macrophage-activation nomenclature and reporting standard for *in vitro* experiments, which was published a year later [30]. They recommend among other things that scientist should note whether macrophages were differentiated using Macrophage colony-stimulating factor (M-CSF) or Granulocyte-macrophage colony-stimulating factor (GM-CSF). This would be followed with post-differentiation stimulation using IFN-γ or IL-4 respectively to give rise to different subtypes. To describe how macrophages are activated, scientists should adopt nomenclature that mirrors the activation standards. This can for example be M(IL-4) or M(IFN-γ) instead of M2a, M2b etc. In the end, using a combination of markers or lack of markers to describe macrophage activation is recommended [30]. An overview including surface markers and this nomenclature can be seen in **Fig. 3**.

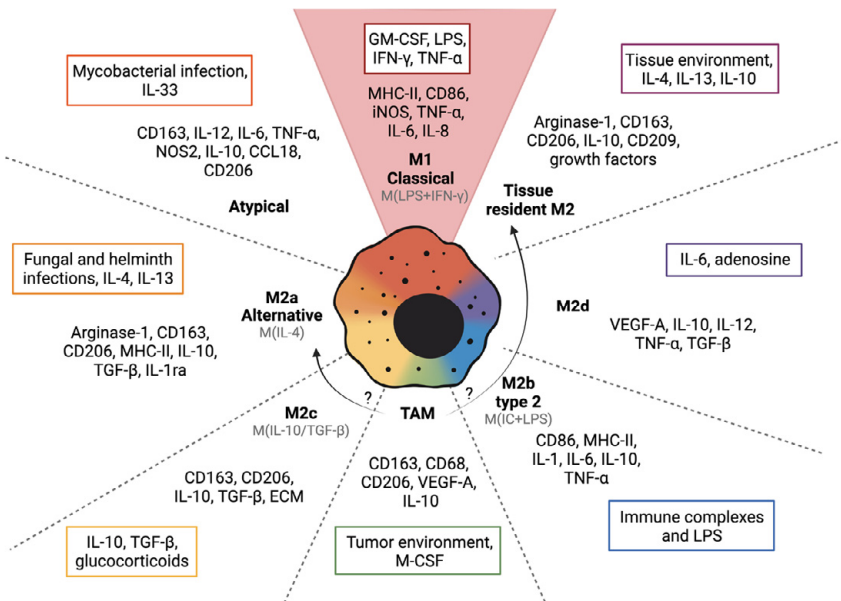


Figure 3. Overview of macrophage activation states. Summary macrophage subtypes with different nomenclature, main function, and surface marker expression. The diagram shows most prevalent examples of macrophage activation and lists markers associated with distinct activation subtypes. Alternative nomenclature mentioned in text is shown in grey. Upstream signals are shown in boxes. This figure illustrates the problem with M1 and M2 division of macrophages, where M2 macrophages are heterogeneous and functionally distinct subtypes of macrophages, illustrated in all sections in white. Arrows show how TAMs can have many different phenotypes. Adapted from Rőszer, 2015 [31].

For remainder of this thesis, macrophage subtypes will be referred to as M1 or M2 like, with reference to their culture conditions, surface markers or function where applicable.

Secondary lymphoid organs and resident macrophages

Lymph nodes are kidney shaped, secondary lymphoid organs of the lymphatic system of the body. They are enclosed in fibrous capsule with outer cortex and inner medulla. Lymphatic vessels link together a large number of individual lymph nodes, serving as major sites for immune cells. They act as filters for foreign particles, filtering lymph for identification of ongoing infections. Lymph enters the capsule of the lymph node and passes into the narrow subcapsular sinus (SCS) that overlays the cortex, rich with lymphocytes. After passing through the cortex, lymph collects in medullary sinus that then drains into efferent lymphatic vessels and exits the lymph node [32]. It is to the secondary lymphoid organs that professional APCs travel to present antigen to the adaptive immune system. Just like secondary lymphoid, so called tertiary lymphoid structures (TLS) are lymph node-like cell follicles that can form at sites of inflammation. They share structural and functional characteristics with conventional lymph nodes in that they can contain B-cell follicles and germinal centers surrounded by T cells. They are prominent peripheral centers of antigen presentation. TLSs exist also at different maturation stages in tumors, culminating in germinal center formation [33-35]. Schematic overview of a lymph node is shown in **Fig. 4**, highlighting the various compartments and cell types found in each location. Just like most tissues of the body, lymph nodes contain specialized macrophages, with both lymph node specific and general immune functions. They are found both in the SCS and the medulla and are named accordingly; Subcapsular Sinus macrophages (SSMs) and Medullary Sinus macrophages or Medullary Cord macrophages [36]. This thesis work will focus on SSMs. SSMs form a dense cellular sheet that lines the SCS above the B cell follicle (**Fig. 4**).

Early mouse work showed that SSMs have great ability to acquire various soluble antigens, but have a low rate of internalization and degradation [37]. They are instead able to catch these antigens on their surface and present them to follicular B cells, serving as a fly trap for lymph entering the lymph node [38-40]. They have also been shown to be specialized in bringing distant tumor cell antigens to lymph nodes in mice [41]. In the mouse, these SSMs are $CD11b^{+}CD18^{+}CD169^{+}$ while having a low expression of F4/80, a murine macrophage marker. Despite low expression of F4/80, SSMs are still defined as macrophages due to their differentiation depending on M-CSF [42,43]. In humans, SSMs are $CD68^{+}CD169^{+}$ [44]. CD169, also known as Siglec-1 or sialoadhesin, binds sialylated glycans and facilitates interactions between cells [45]. CD169 interaction with sialic acid on the surface of microbes is believed to be involved directly in the before mentioned lymph filtration. When exposed to type I IFN, which is common in viral infections, SSMs upregulate CD169 on their surface. This upregulation can also be seen on macrophages in the

periphery, which normally don't express high levels of CD169 [46]. The role of SSMs and other CD169^{+} macrophages in cancer is discussed in Paper II and III of this thesis.

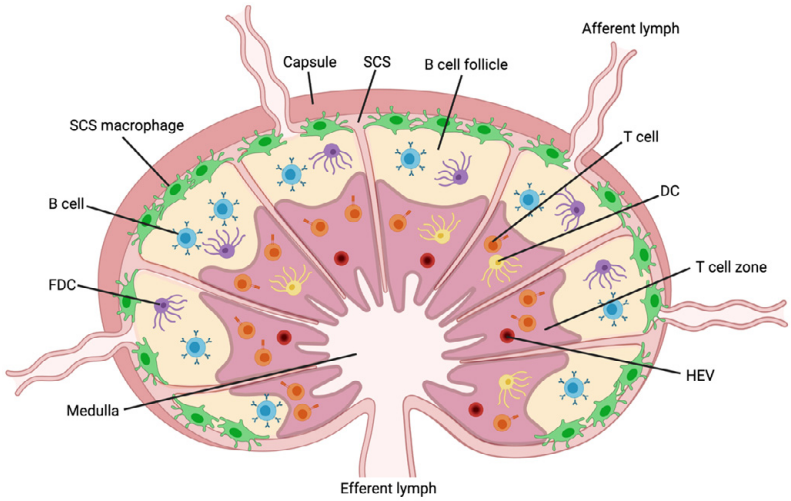


Figure 4. Schematic overview of lymph node. Shown here are the different compartments and cell types found in each location. Lymph nodes are encased in a capsule. Lymph enters lymph nodes through afferent lymphatic vessels and enter the subcapsular sinus (SCS). SCS macrophages line the SCS overlying follicles containing B cells and follicular dendritic cells (FDCs). Deeper in the lymph node lies the T cell zone, containing naïve T cells, DCs and high endothelial venules (HEVs). The medulla is the innermost layer of lymph nodes and medullary sinus drains lymph into efferent lymphatic vessels. Note that not all cells found in lymph nodes are shown in the figure.

Dendritic cells

Dendritic cells (DCs) are the most efficient professional APCs and play the main role in connecting the innate and adaptive immune system. DCs main function is to recognize and process extracellular and intracellular pathogens and present them as peptide antigens to naïve T cells. Immature DCs circulate in the blood and tissue, sampling possible antigens. Upon stimulation by innate immune receptors, DCs upregulate expression of co-stimulatory signals for other immune cells and increase their own cytokine secretion. They migrate towards lymph nodes in order to get in contact with and stimulate T cells [47]. There is no single cell marker that identifies DCs in humans, so a combination of markers either present or absent is usually used for identification. DCs originate from the bone marrow like monocytes, arising from precursor cells but with distinct progenitor cell populations that split the monocytes and macrophages from the DC lineage.

Once the cells have committed to the DC lineage, they are further divided into subtypes based on pattern recognition receptors, surface markers and function: plasmacytoid DCs (pDC) which respond to viral and intracellular pathogens and produce large amounts of type I IFN; conventional DC1 (cDC1) which also respond to viral and intracellular pathogens and are specially adapted to perform cross-presentation and prime $CD8^+$ T cells; and cDC2 which along with intracellular stimuli also respond to extracellular bacteria, fungi, and parasites. Lastly, as previously discussed, there are moDCs that originate from monocytes that differentiate into DC like cells during infection and inflammation. They can cross-present antigens to adaptive immune cells and activate $CD8^+$ T cells and secrete IL-12 [48]. Cross presentation is when innate immune cells, mostly DCs, pick up extracellular antigens and present them on MHC class I. These can be antigens from virus infected cells, bacteria and from tumors [49,50]. They process the antigens, reload them intracellularly and present them to adaptive immune cells on their own MHC class I. This is of particular importance, since it facilitates the presentation of exogenous antigens, normally presented on MHC class II and activating helper T cells, to be presented on MHC class I molecules, activating cytotoxic T cells. This is important for presentation of tumor antigens, since DCs can present antigen derived from tumor cells without expressing it themselves, as will be discussed later [51,52].

NK cells

Natural killer (NK) cells make up around 5-15% of peripheral blood lymphocytes and are a part of the innate immune system, even though they differentiate from the common lymphoid progenitor like B and T cells, and not the common myeloid progenitor like monocytes and macrophages. NK cells are classified as innate immune cells even though their function overlaps with both innate and adaptive immune cell functions. They express both activating and inhibitory receptors, but unlike B and T cells lack antigen-specific receptors. Since they fall on the border between innate and adaptive immune functions, they have sometimes been classified with a recently discovered group of immune cells named innate lymphoid cells (ILCs) [53]. ILCs do not carry antigen-specific receptors but rather regulate the immune system through cytokines resembling T cell generated cytokines. NK cells do differ from ILCs in some crucial ways. ILCs either have very low or no cytotoxicity and are tissue resident, while NK cells have high cytotoxicity and circulate [54,55].

The name natural killer cells refers to their ability to kill tumor cells and cells that are missing MHC class I self-markers, in an inherent or natural way without requiring prior activation, as described when they were discovered in the 1975 [56,57]. This ability to recognize and kill stressed or virus infected cells without immune

sensitization makes the immune reaction very fast. Human NK cells are traditionally defined as $CD3^{-}CD56^{+}$ and have been divided into subtypes based on the expression level of CD56, into $CD56^{bright}$ and $CD56^{dim}$ NK cells [58]. $CD56^{dim}$ NK cells are able to kill target cells that lack self MHC class I, by releasing cytotoxic granules that contain granzymes, perforin and other lytic proteins, resulting in apoptosis of the target cell. The apoptosis of the target cell can also be death receptor mediated, e.g. FasL or TRAIL mediated [59]. $CD56^{dim}$ NK cells also express high levels of CD16 and can initiate antibody-dependent cellular cytotoxicity (ADCC), where the CD16 receptors recognize antibodies bound on the target cell resulting in cell lyses [58,60]. $CD56^{bright}$ cells are less cytotoxic and release high levels of cytokines such as IFN-γ and TNF-α as well as other immunoregulating cytokines and chemokines [61].

NK cell activity needs to be tightly regulated which is accomplished through integration of signals from inhibitory and activating receptors. The strength of the NK cell response is regulated by interaction of receptors with ligands during NK cell development. Binding to HLA-A, B, C and E inhibits NK cell killing of normal cells [62,63]. Inhibitory signals dominate over activating signals to maintain self-tolerance. The activating receptors bind IgG antibodies, MHC class I related chain A and B, DNAX accessory molecule 1 and Natural cytotoxicity receptors [64]. NK cell activation receptors can be inhibited, even during lack of MHC class I, through binding with HLA-G. NK cells are not solely stimulated by receptors but also by secreted cytokines, the most prominent being IL-2, IL-12, IL-15, IL-18 and type I IFNs. NK cell activity is further negatively regulated by cytokines such as TGF-β and IL-10 [65].

The adaptive immune system

As stated before, innate immunity serves as a broad first line defense of the body against invading pathogens. However, it is limited in adaptability against the diversity of pathogens [66]. If an infection persists and cannot be cleared by the innate immune response, the adaptive immune response is activated. An adaptive immune response is way more diverse and mediates a finely tuned antigen-specific attack. This attack is mediated by lymphocytes, which are broadly divided into two major populations: T cells, which provide cell-mediated immunity; and B cells, which provide humoral or secreted antibody immunity (immunoglobulins; Ig). Ig are the secreted form of a plasma membrane bound B cell receptor (BCR) that can recognize and bind to antigens directly in their natural form. T cells are responsible for the cell-mediated arm of adaptive immunity. They carry T cell receptors (TCRs) on their surface, which just like BCRs are highly specific for antigens. Both BCR and TCR are formed with somatic rearrangements of their DNA in order to develop a broad repertoire of receptors with unique binding specificity [67-69].

During development, B and T cells undergo a selection process. BCRs and TCRs need to recognize and bind potential non-self-antigens strongly enough without binding to self-antigens [67,70,71]. This selection process, known as central tolerance, results in a broad but fine-tuned adaptive immune response that can distinguish self from non-self. Without this fine-tuning, adaptive immune cells responding to self-antigens would result in autoimmune disease and complications [72]. Despite this selection system, some self-reactive T and B cells do end up in circulation, so peripheral tolerance needs to be maintained. Peripheral tolerance includes for T cells: continued peripheral deletion of self-reactive lymphocytes; anergy induction; regulation by other immune cells; and an anti-inflammatory cytokine environment [73]. For central B cell tolerance there are three mechanisms: clonal deletion, where after recognition of self-antigens in the bone marrow and cross-linking of BCRs they undergo apoptosis; receptor editing, which re-activates genes associated with BCR rearrangement and recombination, creating a new BCR; and anergy, after low affinity recognition of self-antigens and downregulation of the BCR [74].

B cells

B cells develop and mature in bone marrow and then migrate to secondary lymphoid organs such as spleen and lymph nodes. There they are activated upon binding of antigen to BCR. The BCR is formed by the same genes that encode for antibodies or Immunoglobulins (Ig), so the BCR is also known as membrane immunoglobulin or surface immunoglobulin [75]. After antigen binding to BCR, helper T cells recognize the peptide fragments presented on MHC class II molecules on B cell surface and stimulate B cells, by binding of CD40 on T cell surface to CD40 ligand on B cell surface, as well as with cytokines. This happens on the border of B cell and T cell areas of secondary lymphoid organs [75]. This interaction continues after migration of the activated cells to follicles and formation of germinal center where somatic hypermutation and isotype switch occurs (**Fig. 5**) [76].

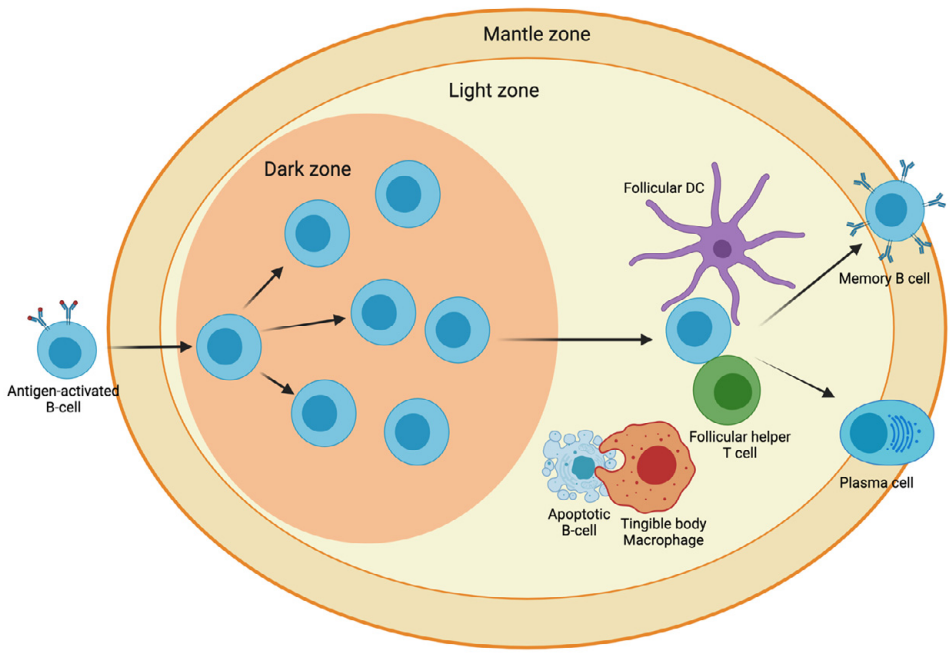


Figure 5. Overview of Germinal center (GC) reaction. Naïve B cells respond to antigens and get help from $CD4^+$ T cells and the B cell : T cell border. They then proliferate within the dark zone of GCs. This displaces resting B cells towards the periphery, forming the mantle zone. In the dark zone, B cells go through a process called somatic hypermutation, altering Ig genes which results in affinity maturation and selection of mutated B cells with high affinity for antigen. Additionally, class switching allows selected B cells to produce Igs with various effector functions. In the light zone, B cells with high affinity BCR capture antigens presented by follicular DCs, receive help from follicular helper T cells, and differentiate into either plasma cells or memory B cells. Those with no antigen bound to BCR receive no help, eventually die, and are cleared by tingible body macrophages.

B cells can also be activated without help from T cells, through T cell-independent antigens [77]. Activated B cells then proliferate and differentiate into plasma B cells or memory B cells. Plasma cells are the effector form of B cells that secrete antibodies with the same specificity for antigens as the BCR. [78,79]. Secreted antibodies have many functions: Neutralizing antibodies that bind to surface of pathogens to render its attack ineffective; glue together foreign cells and antigens, forming targets for phagocytosis; and activating the complement system, resulting in lysis of foreign cells and inflammation [5]. Memory B cells are long lived and circulate in the blood in quiescent state, sometimes for decades [80]. When memory B cells bind to their target antigen, they process it and present it to T cells as peptide MHC class II complex [81]. B cells can secrete both pro- and anti-inflammatory cytokines depending on their activation conditions, but do not secrete cytokines to the same degree as T cells [82].

T cells

T cells unlike B cells do not secrete their surface TCR. TCRs further differ by the fact that they are not able to bind directly to antigens in their natural form. They require antigen to be processed and presented by APCs as a ligand bound to MHC molecules on the APC surface to be activated. As stated before, MHC class I is found on all nucleated cells and MHC class II on professional APCs. APCs carrying antigen on their MHC molecule usually encounter T cells in secondary lymphoid organs such as lymph nodes. Reacting T cells will have TCRs with specificity to that particular antigen, carried by the APC. The TCR is constructed with two protein chains, around 95% of T cells in humans carry TCR composed of one alpha and one beta chain ($\alpha\beta$ T cells) and about 1-5% of T cells carry TCR composed of one gamma and one delta chain ($\gamma\delta$ T cells) [83]. The TCR further contains a receptor complex named CD3 that is the protein complex responsible for TCR signaling. T cells are generally divided into two major types; $CD4^+$ T-helper (T_h) cells and $CD8^+$ cytotoxic T cells (CTL) [84], as explained below. Activation of T cells requires two signals: from engagement of the TCR, through binding of the TCR to peptide presented on MHC molecules on surface of another cells; and from co-stimulation, where surface protein CD28 on T cells binds to co-stimulatory ligands CD80 or CD86, also known as B7 proteins 1 and 2 respectively, expressed mainly on professional APCs. TCR signaling alone, without co-stimulatory signals results in anergy of the T cell [85-87].

$CD4^+$ T_h cell are further divided into subtypes based on their transcription factor profile, cytokine secretion or location, the main ones being T_h1, T_h2, T_h17, follicular helper T cells (T_{fh}) and regulatory T cells (T_{regs}) [88]. T_h cells assist other lymphocytes, like activating B cells or cytotoxic $CD8^+$ T cells, hence their name. $CD4^+$ T_h cells become activated when binding to its peptide in a complex with MHC class II

molecules [89]. T_h1 cells secrete pro-inflammatory cytokines and are mainly involved in activating macrophages but can also stimulate B cells to produce antibodies and activate CTLs. T_h2 cells are important for B cell stimulation and antibody production while T_h17 cells are characterized by their production of IL-17, which results in recruiting of neutrophils among other things. T_h17 cells are heavily involved in defense against gut pathogens [88]. T_{fh} are found in lymph node follicles, and provide help to B cells there [90]. T_{regs} are important for maintaining immunological tolerance. Their main function is to counteract T cell mediated immunity when no longer needed, and to suppress autoreactive T cells. The most specific T_{regs} marker is the transcription factor FoxP3. They produce inhibitory cytokines such as TGF-β and IL-10 and can induce apoptosis of effector cells among other mechanisms [90,91].

Cytotoxic $CD8^+$ effector T cells (CTLs), also known as killer T cells, can destroy virus infected cells and tumor cells. They recognize their target after binding to peptides presented on MHC class I molecules, which all nucleated cells express. They can then release cytotoxins such as perforin, granzymes and granulysin which eventually leads to apoptosis of the infected or cancerous cells. They can also induce apoptosis through surface protein interaction, which it thought to play a bigger role in eliminating other T cells [60,92]. Memory T cells are long lived and can quickly expand to large number of effector T cells after re-exposure to their specific antigen [93-95]. Innate-like T cells have also been described, which trigger rapid immune response independent of MHC molecules. There are three large populations: Natural killer T cells, which recognize glycolipid antigens presented on CD1d, and can perform functions associated with both $CD4^+$ and $CD8^+$ T cells [96]; Mucosal associated invariant T cells; and Gamma Delta T cells, which have a γδ TCR instead of the more common αβ TCR, and seem to be able to recognize whole proteins rather than peptides [97,98].

Tumor immunology

Overview of cancer

Cancer is a grouping of around 200 pathological diseases, all of which include the abnormal or out of control division of cells. Every day, cells in the human body undergo cell division to maintain normal body function. During the course of normal cell division, DNA replication errors can occur, leading to mutations and genetic aberrations. This risk of mutations can be increased by environmental factors such as UV light and smoking. Under normal conditions most of these mutations are silent or are repaired by the DNA repair mechanisms of the body. However, in some cases this repair mechanism fails, and cells accumulate mutations which can in the end lead to them becoming cancerous. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer shared among all tumor types [99]. In 2011 they added four additional hallmarks, two involving the immune system (**Fig. 6**) [100].

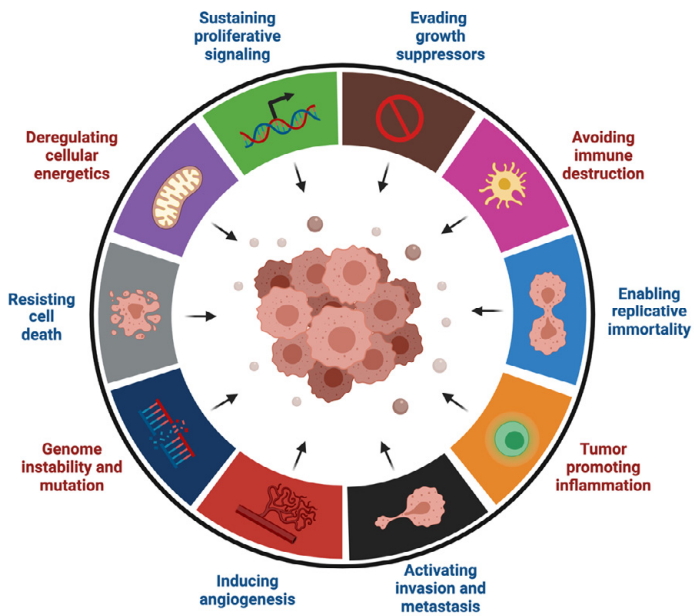


Figure 6. Hallmarks of cancer. Hallmarks introduced in 2000 are shown in blue and hallmarks introduced in 2011 in red. Adapted from Hanahan and Weinberg, 2011 [100].

Immunity and cancer

In 1902 Ehrlich proposed the idea that the immune system is able to suppress cancer development and this idea has been the foundation for the concept of immune surveillance, a hypothesis published in the 1960s [100], although it took decades before being confirmed [101,102]. Immune surveillance is the process by which cells of the immune system detect and destroy premalignant or malignant cells in the body. The immune system can protect the body from developing tumors by eliminating tumor inducing pathogens and by resolving inflammation promptly and preventing establishment of inflammatory environment that can lead to tumorigenesis. Lastly, it can identify and eliminate tumor cells based on their expression of tumor-antigens (TAs) [103]. This immune surveillance concept has been built up over decades using various murine experiments which have shown that both innate and adaptive immunity is involved [102]. Humans with systemic immune suppression have been found to have increased rates of both viral- and non-viral induced cancer [104,105], and presence of both T cells and NK cells in tumors has been correlated with improved prognosis in multiple cancer types [106-109]. Unfortunately, the immune system does not only have a protective role in cancer immunity, but also a detrimental one. One example is the presence of macrophages within the tumor environment being associated with worse prognosis [110]. This interaction between immune cells and cancer cells has been defined as immune editing and is usually divided into three steps, the three E's of immune editing: Elimination, Equilibrium and Evasion (**Fig. 7**)[102].

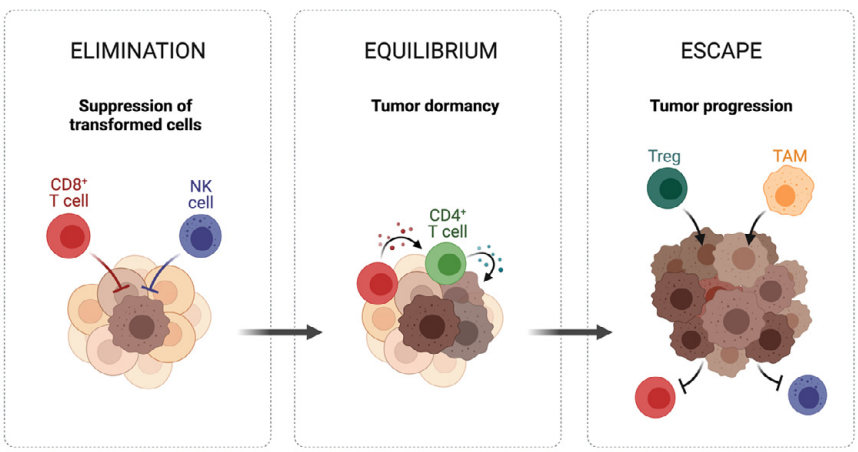


Figure 7. The three E's of cancer immune editing. During the elimination phase, cancer cells are detected and eliminated by the immune system. Over time, tumor cells can escape eradication by the immune system, which leads to an equilibrium or balance between tumor cell death and survival. Lastly, the cancer cells become non-immunogenic and avoid immune eradication and escape immune surveillance [102].

During early tumorigenesis, cells of the innate immune system can respond to DAMPs that are expressed by cells during transformation. This activates innate immune responses, with innate immune cells killing the tumor cells, which releases tumor antigens that can be taken up by phagocytes and presented to adaptive immune cells in draining lymph nodes. T cells activated by this presentation, along with NK cells, eliminate tumor cells and enhance the already present anti-tumor immune responses through pro-inflammatory cytokine secretion. This results in elimination of the tumor cells, and in most cases ends the process of tumor formation [102,111]. In some cases, elimination of all tumor cells is unsuccessful, and the tumor environment moves into equilibrium.

During the equilibrium phase, tumor cell that are less immunogenic can escape elimination by the immune system and through mutagenesis can acquire features that increase their immune resistance. These non-immunogenic cells are selected for further growth but at the same time the immune system is still capable of eradicating tumor cells, just not completely. This phase is the longest of the three and can last for years, with a balance between proliferation and division versus elimination. With prolonged selection of immunotolerant tumor cells, immunogenicity decreases and immune suppressive mechanisms within the tumor environment increase, leading to immune escape of the tumor [102,112].

In the final escape phase, the immune system does no longer recognize cancer cells and can therefore not fully eradicate the tumor. The tumor cells have acquired immune-tolerance through various mechanisms such as down-regulation of MHC class I on their surface that inhibits effective T cell responses, downregulation of costimulatory molecules or up regulation of inhibitory co-receptors, upregulation of non-classical MHC I that inhibit NK cell reactions, resistance to apoptosis, secretion of anti-inflammatory cytokines, and through recruitment of immune-suppressing immune cells. This results in uncontrolled growth and expansion of the tumor [102,113-115].

Tumor microenvironment

The tumor microenvironment (TME) is a complex mixture of cells and structures that provide the tumor with essential support for sustainability. TME includes surrounding blood vessels and endothelial cells, immune cells, cancer-associated fibroblasts (CAFs), extracellular matrix (ECM) and various proteins and cytokines. Tumor ECM differs from normal ECM, with stiffer structure and remodeling. This is due to increased collagen crosslinking and integrin signaling, which supports the finding that women with extensive mammographic breast density have increased risk of developing breast cancer [116]. Since most cancers are formed from epithelial tissues which is not vascularized, angiogenesis or formation of new blood vessels is

upregulated in tumors. The TME is often hypoxic as the tumor mass increases, leading to genetic instability and cancer progression [117]. The stroma surrounding the cancer cells is made up of nonmalignant cells such as CAFs, immune cells and endothelial cells and can comprise up to 90% of the TME, making it an important part of the tumor and its progression (**Fig. 8**) [118].

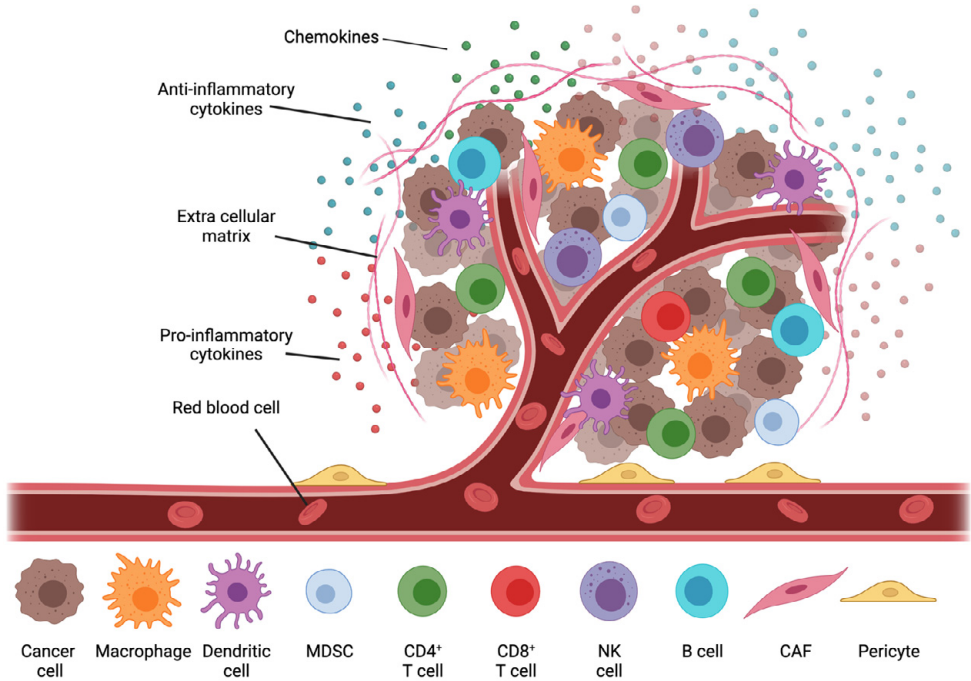


Figure 8. Tumor microenvironment (TME). TME is made up of multiple cell types, which together form an intricate network involved in the tumor environment and progression.

Fibroblasts in healthy tissue are spindle-shaped cells that produce ECM-regulating components such as collagen and fibronectin and play an important role in wound healing. They drive homing of circulating leukocytes, enhance local T cell persistence through TGF-β secretion and reduce T cell apoptosis through secretion of type I IFN [119]. During tumorigenesis their function is pirated by the tumor, supporting the tumor growth by secreting VEGF, fibroblast growth factors and producing pro-angiogenic signals [120]. They further contribute to pro-tumor and anti-inflammatory environment with enhanced TGF-β secretion [121]. CAFs also produce matrix metalloproteinases (MMP) that break down the ECM and facilitate endothelial migration, allowing cancer cells to escape from their location into the blood stream where they can metastasize to other locations [122]. In general, presence of CAFs is associated with worse prognosis for cancer patients [123].

Macrophages in cancer

Tumor associated macrophages (TAMs) are one of the most abundant immune cell types in TME. They usually have an anti-inflammatory phenotype, being M2 like in function, with low capacity to present antigens, impaired phagocytosis, low cytotoxicity, and an immunosuppressive cytokine profile [124]. They further secrete growth factors benefiting the tumor and pro-angiogenic factors such as VEGF and accumulate in hypoxic areas of the tumor [125]. One of the major functions of TAMs in the TME is immune suppression. They suppress T cell mediated immune responses towards tumor cells, through IL-10 and TGF-β secretion, the latter polarizing $CD4^+$ T cells into T_{regs}, as well as through surface expression of programmed death-ligand 1 (PD-L1) and B7-homologs [126,127]. PD-L1 binds to programmed cell death protein (PD-1), and B7 to cytotoxic T-lymphocyte antigen 4 (CTLA-4), which are both immune checkpoints present on the surface of T cells that provide inhibitory signals [128].

Although most TAMs show an M2 like phenotype, pro-inflammatory macrophages can be found within the TME. TAMs can therefore, just like macrophages present in healthy tissue, be described with a spectrum of functional phenotypes, rather than just pro- or anti-tumoral. The same can be said about their origin, where numbers of monocyte derived macrophages versus tissue resident macrophages most likely can depend on the tumor type, stage, size and the location [129]. Several immune- and tumor-cell derived factors have been linked to TAM recruitment and induction, such as CCL2, GM-CSF, M-CSF, VEGF, IL-4, IL-10 and TGF-β [130-132]. Some of these factors promote homodimerization of the inhibitory NFκB family member p50 leading to anti-inflammatory profiles [133]. Using the M1 and M2 nomenclature, studies have found that the M1/M2 ratio is a better prognostic factor, compared to the total amount of TAMs, which correlates with worse outcome in many different cancer types [126,134-137]. Patients with a higher number of M1 like macrophages compared to M2 like macrophages often have better prognosis and TAMs with M2 like phenotype have been associated with more aggressive form of cancer [138-142].

Myeloid derived suppressor cells

During severe infection and cancer, a heterogeneous group of immune cells termed myeloid derived suppressor cells (MDSCs) expand strongly. This term was coined in 2007 to describe both the origin of these cells as well as their distinctive immunosuppressive function [143]. They have been studied in sepsis, chronic infections and autoimmune disease, but MDSC research has mainly been focused on studying them in relation to cancer [144]. They are usually divided into two subpopulations: polymorphonuclear or granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (Mo-MDSCs). In mouse models, MDSCs are defined as $CD11b^+Gr1^+$, with G-MDSCs as $Ly6G^+/Ly6C^-$ and Mo-MDSCs as $LY6G^-/Ly6C^+$ [145]. Characterizing MDSCs solely based on their surface marker phenotype is

limiting in humans, due to overlap with markers common on other myeloid cells. Mo-MDSCs are in fact very similar to monocytes, being defined as $CD14^{+}CD11^{+}HLA\text{-}DR^{-/low}CD15^{-}$, and G-MDSCs are similar to neutrophils being defined as $CD11b^{+}CD33^{+}CD15^{+}HLA\text{-}DR^{-/low}$ and lacking lineage markers. G-MDSCs have also been recorded having CD66b surface expression and collecting in low density in Ficoll gradients [146].

The origin of MDSCs has been debated for years but recent evidence suggests that Mo-MDSCs derive from reprogrammed monocytes and G-MDSCs might be derived from all stages of neutrophil development including as a subset of mature neutrophils [13,147]. MDSCs respond to tumor-derived factors such as Granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-6, IL-10, VEGF, and Prostaglandin E_2 (PGE2), accumulating in peripheral blood, lymphoid organs, and in tumors [145,146,148,149]. MDSCs have a highly immunosuppressive profile, most importantly their ability to inhibit T cell functions. They produce Arginase-1 (Arg-1) and iNOS which depletes L-arginine and hinders T cell proliferation, as well as producing ROS which inhibits T cell activity. This results in enhanced resistance in malignant cells to T cell cytotoxicity and impairs infiltration of T cells to the TME [146,150]. Not only do MDSCs inhibit T cell activity, but also NK cell, DC and macrophage activity [151]. MDSCs also produce anti-inflammatory cytokines such as IL-10 and TGF-β, promote angiogenesis through production of MMP9, and induce T_{regs} [146,151].

Cancer Immunotherapy

As mentioned before, the immune system is equipped to eradicate cancer, and does so in most cases, but sometimes the cancer evades the immune response. Cancer immunotherapy aims to artificially stimulate the immune system's natural ability to fight and eliminate cancer cells. The foundation for cancer immunotherapy can be traced back to the 17th and 18th century. The best known experiments are from 1890s, where heat-inactivated bacteria mixture was used as cancer cure, but cancer immunotherapy still remained in obscurity for decades [152]. Cancer immune treatment now includes a broad range of therapies that have been expanded greatly in the last two decades, and there are now over 2000 immuno-oncology agents in either preclinical or clinical development [153]. Therapies can be divided into either active therapy, which targets tumor cells specifically, or passive therapy, which does not target cancer cells directly but instead enhances anti-tumor immune cell functions. This thesis will discuss and summarize cytokine therapies, cellular immunotherapies, and antibody therapies.

Cytokine therapies

The first immunotherapeutic treatments for cancer to be tested in humans were recombinant cytokines. Both type I and type II IFNs have been tested, but only type I has been shown to be clinically effective, although responses in patients with solid tumors were limited. IFN-α has been approved as treatment for multiple cancer types, while IFN-γ has not been approved for clinical use even though showing promising results in patients with bladder carcinoma, ovarian carcinoma, and melanoma. IFN-λ has shown promising anti-tumor effects but only in animal models [154,155]. IL-2 has been used in treatment of melanoma, with long lasting response but in only a small fraction of patients [156]. High dose of IL-2 has also been effective in patients with renal cell carcinoma, but this high dose IL-2 treatment comes with severe adverse side effects. Lower doses have been used in more recent times, in combination with other therapies as an enhancer [157,158].

Cellular therapies

The principle for adoptive cell therapy (ACT) is to isolate immune cells from cancer patients, usually tumor infiltrating lymphocytes (TILs), and expand cells that react to the tumor before re-injecting them into the patient. The major limitation of ACT is the isolation and culture of cells, as well as limited success in solid tumors other than melanoma which is a highly immunogenic tumor due to mutational load [159]. Scientists have developed strategies to bypass these bottlenecks, most notably using artificially designed chimeric antigen receptors (CAR) that recognize TAs, expressed on the surface of activated T cells after viral transduction [160]. As of March 2019, there are over 350 ongoing clinical trials using CAR-T cells, most targeting blood cancers, with FDA approval for CD19 CAR-T cells, for acute lymphoblastic leukemia and non-Hodgkin lymphoma [161]. Scientists have recently engineered human macrophages with CARs (CAR-M) which activates and enhances their phagocytic effect and shifts their phenotype from anti-inflammatory to pro-inflammatory [162]. There are also studies underway to develop CAR-NK cells, which could provide an "off the shelf" treatment with less toxicity. This could offer the advantage of combining NK cell ability of intrinsic tumor killing with the CAR-dependent killing mechanism, but so far this treatment is still in pre-clinical studies and clinical trials [163].

Dendritic cell therapy has also been developed. DCs can be induced to present TAs by vaccination with either short peptides corresponding to TAs or with autologous tumor lysates. DCs can also be activated *in vivo* by getting tumor cells to produce GM-CSF either with genetic engineering or oncolytic viruses. DCs can like T cells be isolated from patients and stimulated *ex vivo* with TAs [164]. The only approved DC treatment is Sipuleucel-T in prostate cancer, where APCs are removed from blood and grown with a fusion protein, made from GM-CSF and antigen prostatic acid phosphatase that is present in 95% of prostate cancers, that are then re-infused

into the patients [165]. In late stage trials, use of Sipuleucel-T resulted in extended survival compared to placebo, with around 22% overall reduced risk of death in treated patients compared to control group [166].

Antibody therapies

Monoclonal antibody technology is used to engineer and generate antibodies that are specific against tumor antigens, with two types used in cancer treatments; naked monoclonal antibodies and conjugated antibodies that are joined to other molecules, either cytotoxic or radioactive. Approved antibodies can trigger ADCC from NK cells, activation of complement system or bind to proteins, blocking them from interacting with other proteins like growth factors on tumor cells or immune cell receptors [167-169]. This blocking forms the basis of immune checkpoint inhibition therapies. Immune checkpoints are key regulators of the immune system and are critical for dampening immune responses. Immune checkpoint inhibitor therapies have been expanded immensely in the last two decades and most notable are T cell targeted therapies, which have revolutionized cancer treatments [170]. A complete T cell activation is dependent on TCR binding to an antigen-presenting MHC molecule as well as binding of costimulatory molecules CD28 on T cells and B7 on APCs. On the surface of T cells are CD28 homologues, and checkpoint proteins, CTLA-4 and PD-1 that impair T cell activation. CTLA-4 binds to B7 in a competitive manner and PD-1 binds to its own ligand PD-L1, which is a B7 homolog present on APCs (**Fig. 9**) [171].

The first checkpoint antibody approved by the FDA was Ipilimumab in 2011, targeting CTLA-4, which has been approved in multiple cancer types [172]. In patients with advanced melanoma, it induces durable response and significantly prolonged overall survival (OS) [173]. However, severe side effects are associated with Ipilimumab treatment, and only a limited number of patients respond to treatment. Inhibition of PD-1 was approved as treatment in 2014 with Nivolumab, followed by Pembrolizumab, which resulted in higher response rate, less toxic side effects and longer survival compared with Ipilimumab [174,175]. Several PD-L1 antibodies have been approved as well, including Atezolizumab, which in combination with nab-paclitaxel has been approved as treatment for triple negative breast cancers, discussed in more detail later in this thesis [176]. PD-1/PD-L1 is expressed on many types of cells, such as T cells, NK cells, moDCs, epithelial-, and endothelial cells, as well as on cancer cells. It is notably expressed on macrophages and treatment can increase their anti-tumor functions, although macrophages might also play a negative role in anti-PD-1 treatments by preventing CTLs from reaching the tumor [177,178]. While treatment targeting the PD-1/PD-L1 axis has been more successful with regards to response and adverse effects compared to those targeting CTLA-4 alone, combination treatment with agents targeting both pathways has been even more beneficial with improved response rate and OS in melanoma and colorectal cancer, although with the addition of anti-CTLA-4 severity of side effects increases

[174,179,180]. Current immune checkpoint inhibition research focuses mostly on understanding the precise mechanism of action in the CTLA-4/PD-1 combination therapy success, and why some patients don't respond to treatment at all, mapping and exploring resistance mechanisms.

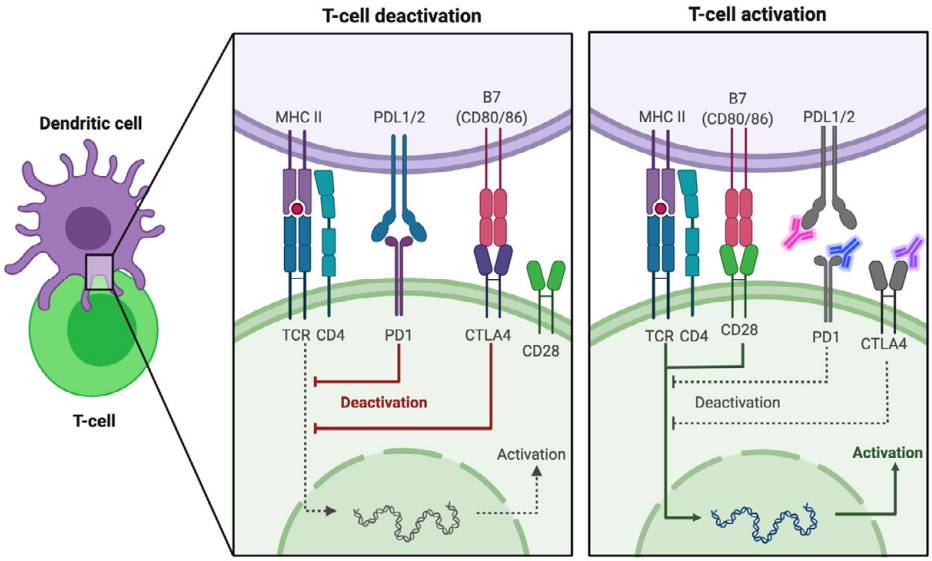


Figure 9. Immune checkpoint inhibitors. Checkpoint proteins CTLA-4 and PD-1 are expressed on T cells. Upon binding to their ligands PD-L1/2 and B7 homologs (CD80/86) on APCs or cancer cells, T cell activation dependent on binding of costimulatory molecules, such as CD28, is inhibited (left). Checkpoint inhibitor antibodies bind to CTLA-4, PD-1 and PD-L1, inhibiting this checkpoint control which leads to T cell activation and increased anti-tumor T cell activity (right).

Breast cancer

In 2019, over 70.000 individuals in Sweden were diagnosed with cancer. Out of those, 8.288 women were diagnosed with breast cancer, making it the most common form of cancer diagnosed in females. One of ten women are diagnosed with breast cancer before they turn 75 years old, with the median age of women diagnosed being 65 years. Even though the number of diagnosed breast cancer cases has been increasing in the last decades, the mortality rate is slowly going down [181]. In general, the prognosis is good for breast cancer patients. As of 2018, the 5- and 10-year survival of breast cancer patients in Sweden was approximately 90% and 85% respectively [182]. About 1% of all breast cancers are diagnosed in males, but this thesis will focus on breast cancer in females.

Etiology

In most cases, it's not clear what exactly has caused the breast cancer to form, but around 10% of cases can be linked to inherited genetic mutations. Breast cancer gene 1 (*BRCA1*) and *BRCA2* were the first two major susceptibility genes that were identified for breast cancer in the 90's [183,184]. They are the best known of a small group of genes that are associated with breast cancer. Others include *TP53* and *PTEN* [185]. The *BRCA* genes function as tumor suppressors, maintaining and repairing DNA double strand breaks. Mutations in *BRCA* genes account for approximately 40% of familial breast cancers [186]. The risk of getting breast cancer increases with age and is way more common in women. Longer exposure to hormones such as estrogen can increase the risk of breast cancer development. A number of things can affect the hormonal exposure and have been correlated to increased risk of breast cancer: increased age of first childbirth and breastfeeding [187]; earlier first menstrual period and late menopause [185]; and hormonal replacement therapy [187]. Other risk factors also include diet and obesity, alcohol consumption, smoking and mammography density [188-190]. Obesity can even lead to elevated estrogen levels in serum and local estrogen production. This can then promote breast cancer development in postmenopausal women, adding obesity to the number of factors that can affect hormonal exposure [191].

Breast cancer development

The human breast is a mammary gland, composed of layers of different tissue with two types being the most prominent, glandular tissue surrounded by supportive tissue. The supportive tissue is made up of adipose- and connective tissue which provides structure for the branching ductal network of the breast, blood vessels and a range of cells such as fibroblasts and immune cells. The ductal network connects the terminal duct lobular units (TDLUs) that secrete milk, with the nipple for release. The lobes and milk ducts are made up of lumen that is lined with inner luminal epithelial and an outer myoepithelial cell layer, with a basement membrane surrounding the structure (**Fig. 10**).

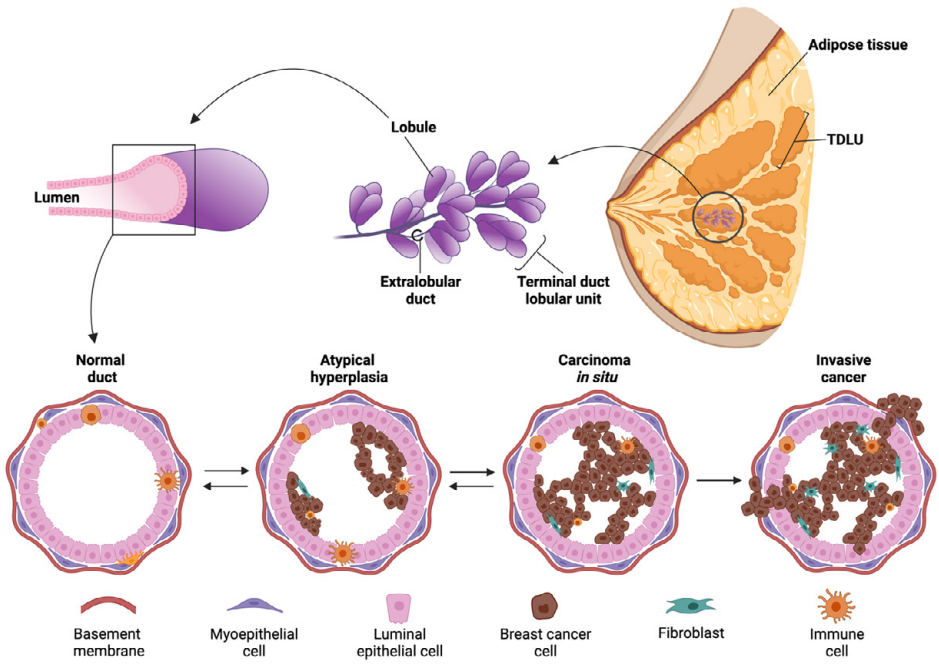


Figure 10. Schematic illustration of a breast and breast cancer development. The terminal duct lobular unit (TDLU) is a branch like complex that extends from the nipple. It is composed of an inner lumen lined with lumen epithelial cells, myoepithelial cells, and basement membrane. Schematic overview of breast cancer progression, from normal intact duct, through atypical hyperplasia with appearance of lesions, carcinoma *in situ* characterized by increased genetic instability and recruitment of stromal and immune cells, leading to invasive cancer with disruption of the basement membrane.

The luminal cells can be divided further into alveolar cells that produce milk and ductal cells that line the ducts [192]. Immune cells are not only located in the surrounding fat tissue, but also within the epithelial component in the TDLUs [193]. Majority of breast cancers originate from TDLUs and start with appearance of

lesions or epithelial atypia. The next step is atypical hyperplasia and development of carcinoma *in situ.* During this transition, increased genetic changes and instability occur. Shift from carcinoma *in situ* to invasive cancer involves recruitment of stromal cells and ECM proteins, resulting in breakdown of basement membrane and spread of malignant cells to surrounding stroma [194].

Diagnosis and histological classification

The most commonly used screening methods for breast cancer are physical examination of breasts, mammography and ultrasound. If abnormalities are detected a biopsy can be taken. Most types of breast cancers are easy to diagnose based on a microscopic evaluation of a biopsy. This is then used to classify the breast cancer. Breast cancers are primarily classified based on their histological appearance and most breast cancers are derived from the epithelium lining the ducts or lobules, classified as ductal or lobular carcinoma. Carcinoma *in situ* is where cancer cells proliferate within the epithelial tissue without invading surrounding tissue while *Invasive carcinoma* has invaded surrounding tissue (**Fig. 10**). Both invasive and non-invasive carcinoma can originate from lobular and ductal unit of the breast [194]. The majority of breast cancers are invasive ductal carcinoma, with the second most common being ductal carcinoma *in situ* [195].

The Nottingham system is a valuable grading tool for breast cancer. It grades breast carcinomas based on morphological characteristics: tubule formation, nuclear polymorphism, and miotic count, with each scored on a scale of 1 to 3 [196]. These are then added together for a final overall score. A lower Nottingham score means a well differentiated carcinoma with better prognosis, and a higher score means a poorly differentiated carcinoma with worse prognosis. Staging is another tool for evaluating breast cancer progression. This is assessed using TMN staging, which takes tumor size (T), lymph node involvement (N), and distant metastases (M) into account [197]. Stages 1-3 are within the breast or regional lymph nodes, while stage 4 is metastatic cancer that has spread beyond the breast and regional lymph nodes and is associated with the worst prognosis for breast cancer patients [204]. Presence of metastases in axillary lymph nodes predicts increased risk of local and distant recurrence, and lymph node metastasis has long been considered the most important prognostic factor for poor prognosis [198].

Receptor status and molecular subtype

Receptor status was traditionally considered by reviewing each individual receptor (Estrogen receptor (ER), Progesterone receptor (PR), and Human epidermal growth factor receptor 2 (HER2)), with recent approaches looking at these together, and in combination with tumor grade. This divides breast cancer into molecular classes with varying prognosis [199]. The most recent molecular classification categorizes breast cancer into five different subtypes: Luminal A and B that in general are ER and PR positive, with a higher expression of Ki67 in Luminal B subtype, which is associated with increased proliferation and worse prognosis for the patients [200]; HER2 positive, where HER2 is overexpressed or amplified, which have higher recurrence rate and a more aggressive disease [201]; Basal-like which have features similar to basal or myoepithelial cells, are aggressive and have poor prognosis [202]; and Claudine-low which display high expression of epithelial to mesenchymal transition (EMT) related genes, have stem cell like gene expression and high levels of immune cell infiltration [199,203]. Basal-like and Claudin-low breast cancers most often lack all expression of hormone receptors and are therefore classified as triple negative breast cancers (TNBC).

Breast cancer treatment

Treatment of breast cancer depends on various factors, such as stage of the cancer, age of patient, spread to lymph nodes, hormone receptor status, and molecular characteristics such as *BRCA* status. The most common treatment is surgical removal of the tumor, which can then be followed by chemotherapy or radiation therapy [205]. Surgery standards can include removal of the whole breast, one quarter of the breast, or a small part, and one or more lymph nodes may be resected during surgery. Adjuvant radiation and/or chemotherapy can be added as a treatment. Endocrine therapy which includes selective estrogen receptor modulators, Tamoxifen and aromatase inhibitors being the most common, is used for ER^+ tumors. Therapy targeting HER2 is used for HER2 amplified tumors, including trastuzumab and lapatinib [206]. Chemotherapy is most commonly used in patients with ER^- and $HER2^-$ tumors and advanced breast cancer of all subtypes. Poly ADP-ribose polymerase inhibitors have been approved for treatment of TNBC, which makes it harder for cancer cells with *BRCA1* and *BRCA2* mutations to survive [207]. Recently Atezolizumab, which blocks PD-L1, has been approved for treatment of TNBC in combination with nab-paclitaxel, a chemotherapeutic medicine [208]. PD-L1 is significantly higher expressed in TNBC patients compared to non-TNBC patients and further research is ongoing into expanding treatment option of PD-1/PD-L1 pathways in TNBC [176,208,209].

The present investigation

Aims

The general aim of this thesis is to investigate the role of innate immune cells, with focus of macrophages of different subtypes and functions, in breast cancer. This includes both *in vitro* and *in vivo* research in mouse models and humans.

The specific aims were:

I. To investigate the effect primary human macrophages have on ERα expression on breast cancer cells and uncover the mechanism involved in the proposed downregulation of ERα by macrophages, previously described.

II. To investigate whether resident subcapsular sinus lymph node $CD169^+$ macrophages located in direct contact with lymph node metastasis, as compared with $CD169^+$ macrophages located in primary tumors, would be a prognostic factor for breast cancer patients. We further wanted to see if these were associated with PD-L1 expression.

III. Based on results from paper II, we wanted to explore the function of $CD169^+$ TAMs in breast cancer further. We wanted to investigate why presence of $CD169^+$ TAMs does not lead to a beneficial prognosis in breast cancer patients, and relate this to $CD169^+$ subcapsular sinus macrophages, with regards to origin, function, and phenotype.

Paper I

Inflammatory macrophage derived TNF-α downregulates estrogen receptor α via FOXO3a inactivation in human breast cancer cells.

Background

Breast cancers are classified and divided into subtypes based on expression of hormone receptors. The luminal A subtype ($ER^+PR^+HER2^-Ki67^{low}$) is the one most often associated with better prognosis for the patients [210], in part because endocrine therapy can be used as a treatment targeting the ERα signaling pathway. For patients with estrogen receptor positive (ER^+) breast cancer, resistance to endocrine therapy is commonly caused by downregulation of ERα in the disseminated cancer cells. Myeloid cells in the tumor microenvironment can be both beneficial and detrimental for the patient [136,137,211]. Macrophages have also been proposed to downregulate ERα in breast cancer cells through an unknown mechanism [212,213].

Methods and materials

In this paper we used xenograft breast cancer models to study the effect of human monocyte derived macrophages on ERα expression *in vivo*. Immunohistochemistry was used to analyze relevant markers in a breast cancer patient cohort. Gene expression analyses were also performed, using the publicly available database R2. For *in vitro* studies, immune cells from healthy donors and breast cancer cell lines were used. Condition media from primary human monocyte derived macrophages was analyzed for cytokines and used for breast cancer cell line cultures. ERα expression of the breast cancer cell lines was analyzed on mRNA level using RT-qPCR, as well as on protein level, using western blots. Expression of various downstream transcription factors was analyzed, in combination with blocking or silencing strategies of signaling mediators.

Results and discussion

We saw that co-transplantation of primary human monocytes with ER^+ breast cancer cells in NSG mice results in long-term significant downregulation of ERα on the breast cancer cells, which was not observed in xenografts with breast cancer cells injected without monocytes. Using primary human monocyte derived macrophages cultured *in vitro,* we further confirmed this downregulation in MCF7 cells cultured

in macrophage condition media (CM) from M1 like macrophages. This was seen on both protein level using western blots, as well as on mRNA level using RT-qPCR.

We showed that M1 like macrophages secrete significantly higher levels of TNF-α compared to M2 like macrophages, and addition of TNF-α to MCF7 and T47D cultures resulted in downregulation of ERα. When TNF-α inhibitor Etanercept was added to the cultures, that hindered the M1 CM induced ER-α downregulation. When looking at the signaling pathways downstream of TNF-α in the breast cancer cells, we confirmed that it was the Akt-pathway that was specifically activated, rather than MAPK/ERK pathway. TNF-α induced activation of Akt/PKB and subsequent downregulation of the tumor suppressor FOXO3a by phosphorylation. We again saw that the use of Akt inhibitor reversed the M1 CM induced downregulation of ERα, and when the mouse xenografts were stained for P-FOXO3a we saw significant increase in the co-transplanted MCF7 and monocyte xenografts, with sequestration of P-FOXO3a in the cytoplasm of the MCF7 cells. We further observed that presence of macrophages and downregulation of ERα may correlate with initiation of epithelial to mesenchymal transition (EMT), which results in increased motility and metastasis of tumor cells.

In conclusion, this study shows that different macrophage subtypes within the tumor microenvironment in breast cancer have various and unique impact on breast cancer progression. In general, pro-inflammatory M1 like macrophages are associated with better prognosis and slower tumor progression compared to anti-inflammatory M2 like macrophages. Here we observe that despite being pro-inflammatory, M1 like macrophages may have unwanted and detrimental effect on endocrine resistance in breast cancer patients, as well as playing a potential role in EMT of the tumor, through TNF-α mediated downregulation of FOXO3a.

Paper II

Co-localization of CD169$^+$ macrophages and cancer cells in lymph node metastases of breast cancer patients is linked to improved prognosis and PD-L1 expression.

Background

Although survival among breast cancer patients has improved, there is still a large group of patients with dismal prognosis. One of the most important prognostic factors for poor prognosis is lymph node metastasis. Increasing knowledge concerning the lymph nodes of breast cancer patients indicates that they are affected by the primary tumor. Tumor associated macrophages (TAMs) have in general been associated with worse prognosis in cancer patients [135,136,211], while the presence of CD169$^+$ macrophages in metastasis-free lymph nodes of cancer patients have in contrast been associated with improved survival [214-217] . This has however not been found in breast cancer so far [218]. High gene expression of CD169 has been associated with worse survival in breast cancer patients though [219]. In viral infections, CD169$^+$ macrophages are responsible for induction of PD-L1 expression, via local type I IFN secretion, which leads to T cell exhaustion [220].

Methods and material

In this study, a patient cohort including 286 patients with primary breast cancer was used for immunohistochemical staining and statistical analysis. All patients had received 2 years of adjuvant tamoxifen and were a part of a representative cohort from two randomized clinical trials that included patients from the South Swedish Health Care Region during 1985-1994 [221-223]. Tissue microarrays were stained for common macrophage markers, as well as the markers investigated in this study, CD68, CD169, PD-L1 and PD-1, using immunohistochemistry (IHC). For statistical analysis, primary tumor and metastatic lymph node samples were scored and evaluated, and previous clinical parameters evaluated by physicians were included in the analysis.

Results and discussion

In this study we used a primary breast cancer tissue cohort from patients that received treatment with adjuvant tamoxifen for 2 years. We analyzed samples from primary tumor as well as from synchronous lymph nodes with metastasis for

expression of CD68, CD169, PD-L1 and PD-1 using IHC. We observed that presence of $CD169^+$ macrophages near lymph node metastases of breast cancer patients was associated with smaller tumor size and, in univariable analyses, to improved prognosis after adjuvant tamoxifen. This is in contrast with $CD68^+$ macrophages in lymph node metastases, which were not associated with prognosis, although these macrophages were associated with more aggressive tumor characteristics of the primary tumor. To our knowledge, we here show for the first time that $CD169^+$ macrophages located in direct vicinity of lymph node metastasis in breast cancer patients, correlate with improved prognosis. The evidence for a prognostic importance in our study was, however, not retained after adjustment for other clinicopathological features.

We further showed that $CD169^+$ macrophages were spatially associated with expression of PD-L1 on nearby cells, both in primary tumors and metastatic lymph node, although PD-L1 expression in metastatic lymph node as such did not have further prognostic impact. The previously described prognostic effect of $CD169^+$ macrophages in metastatic lymph nodes of breast cancer patients was only seen in patients with PD-$L1^+$ primary tumors. This merits further research since to our knowledge, the relationship between CD169 and PD-L1 expression in breast cancer has not been explored. Our data suggests that $CD169^+$ resident lymph node macrophages have a unique function in targeting immune responses against breast cancer and investigating the biological differences between lymph node and primary tumor $CD169^+$ macrophages is of great importance. That includes investigating possible origins, as well as phenotype and function of $CD169^+$ TAMs and if they can be re-programmed or shifted towards a more anti-tumor status.

Paper III

CD169$^+$ macrophages present in primary tumors are monocyte derived type I IFN producers that possess broad immunosuppressing functions

Background

In *Paper II* we showed that CD169$^+$ macrophages in lymph nodes with metastasis were associated with better prognosis in breast cancer patients, while CD169$^+$ macrophages present in primary breast tumor (CD169$^+$ TAMs) were not associated with better prognosis. Interestingly, this effect of CD169$^+$ lymph node macrophages was only seen in patients with PD-L1$^+$ primary tumors. It has been shown that CD169$^+$ subcapsular sinus macrophages in lymph nodes can phagocytose and present lymph-borne tumor cell antigens to other cells in the lymph node, but their exact role is unknown in cancer patients [41]. The role of CD169$^+$ TAMs in primary tumors is so far unknown as well. We wanted to investigate whether CD169$^+$ TAMs and CD169$^+$ lymph node macrophages had any functional relationship or other similarities that would be of importance.

Methods and materials

For *in vitro* studies, monocytes were isolated from healthy donors and differentiated into different macrophage subtypes. Type I IFN was used to upregulate surface expression of CD169. Phenotypical and functional assays included flow cytometry for surface markers and cytokine secretion, ELISA and multiplex for cytokine and chemokine analysis, pinocytosis assay for phagocytic ability evaluation, co-cultures with both T cells and NK cells for immune function assays and RT-qPCR for gene expression analysis. Mouse models were used to investigate the origin of tumor associated CD169$^+$ macrophages, using the syngeneic 4T1-model, Balb/c mice, and by using NSG mice co-injected with SUM-159 breast cancer cell line cells and primary human monocytes isolated from donor blood. Samples were stained and analyzed using immunohistochemistry (IHC) and fluorescence. Two clinical breast cancer cohorts consisting of 23 patients (small) with invasive primary breast cancer with lymph node or distal metastasis, from the South Swedish Health Care Region between 1976-2005, and 304 patients (large) diagnosed with locally advanced inoperable or metastatic breast cancer in Sweden between 2002 and 2007, were used for IHC analysis of cores from primary tumor, lymph node metastasis and/or distal metastasis. The patient cohorts were used to investigate the spatial organization of infiltrating CD169$^+$ TAMs in relation to other immune cells, to guide in understanding their function in primary human breast tumors. Gene expression

analysis were also performed, using the publicly available database R2 and the Michigan Portal for the Analysis of NGS Data (MiPanda).

Results and discussion

In this study we showed that $CD169^+$ TAMs can be derived from monocytes in a type I IFN stimulating tumor microenvironment. We saw this using a xenograft co-transplantation of human TNBC cell line SUM-159, together with primary human monocytes in NSG mice. Co-transplanted monocytes showed co-expression of CD169 and PD-L1. This indicated differentiation into $CD169^+$ TAMs. By evaluating expression of the murine macrophage marker F4/80 in the 4T1-model, we confirmed that tumor associated $CD169^+$ macrophages can be monocyte derived also in the murine setting.

In vitro we saw an upregulation of CD169 on primary human monocyte derived macrophages cultured under M2 culture conditions with the addition of IFN-α, co-expression of PD-L1 on their surface. The $CD169^+$ macrophages had a pro-inflammatory cell surface phenotype and showed secretion of IL-15 and CXCL10, confirmed at the mRNA gene expression level. Using the TCGA database and MiPanda, we also showed that *CXCL10* and *Siglec1* expression in breast cancer specimens were significantly associated. This indicated a unique cytokine profile of the $CD169^+$ macrophages *in vitro*. However, these $CD169^+$ macrophages had low pinocytotic ability, a T cell suppressing effect, and did not induce an increase in NK cell cytotoxic killing of breast cancer cells in co-culture. We further saw upregulation of immunoregulatory genes *PGE2* and *HLAG* in the $CD169^+$ macrophages. Inhibitors for PD-L1 and HLA-G did not affect NK cell cytotoxicity or alleviate T cell suppression. $CD169^+$ macrophages further had significantly lower ability to kill MDA-MB-231 cells, compared to M1 like macrophages.

Using a clinical human breast cancer cohort, we finally showed a spatial association of $CD169^+$ TAMs with tertiary lymphoid structures (TLS), and more importantly with presence of T_{regs}. $CD169^+$ TAMs were also associated with worse prognosis for the breast cancer patients. This showed that the unique beneficial functions that $CD169^+$ subcapsular sinus macrophages have when located in lymph nodes are not the same for monocyte derived $CD169^+$ TAMs in primary tumors of breast cancer patients.

The main conclusion for paper III is that $CD169^+$ TAMs located in primary breast tumors are functionally distinct from lymph node resident subcapsular sinus $CD169^+$ macrophages and are likely to be monocyte derived in a type I IFN environment, rather than tissue resident macrophages. *In vitro* they show a surface phenotype similar to pro-inflammatory M1 like macrophage, but do not exhibit the same pro-inflammatory function. *In vivo,* they are spatially associated with tertiary lymphoid structures (TLS), and more importantly with presence of T_{regs}. $CD169^+$

TAMs are also associated with a worse prognosis in the breast cancer patients. Our findings show that the origin and therefore location of $CD169^+$ macrophages in breast cancer patients, has profound impact on their prognostic, functional and thus therapeutic perspectives, since only lymph node resident $CD169^+$ macrophages have a beneficial effect on survival. We propose that lymph node resident macrophages should be considered as a therapeutic target, while considering the negative side effects of $CD169^+$ TAMs.

Conclusions

I. We showed that M1-like macrophages may have a detrimental effect on endocrine resistance in breast cancer cells *in vitro* through TNF-α mediated downregulation of FOXO3A and hence estrogen receptor alpha. They further seem to be linked to EMT of the breast tumor. This shows that TAMs can have various and unexpected roles in breast cancer.

II. Presence of $CD169^+$ macrophages near lymph node metastases of breast cancer patients was associated with improved prognosis, but only in patients with PD-$L1^+$ primary tumors. This effect was not seen when looking at $CD169^+$ macrophage presence in primary tumor ($CD169^+$ TAMs), giving us a foundation for the hypothesis that cells in these locations, although sharing CD169 surface expression, do have functional differences. We also saw that CD169 expression correlated strongly with PD-L1 expression in both locations.

III. Building on the hypothesis from paper II, we showed that $CD169^+$ TAMs are functionally distinct from lymph node resident subcapsular sinus $CD169^+$ macrophages. They are also likely to be monocyte derived in a type I IFN environment, and even though they show a pro-inflammatory surface phenotype, their function seems to be anti-inflammatory. They further correlate with presence of tertiary lymphoid structures in breast cancer patient's primary tumors and metastasis and with presence of T_{regs}. They are therefore associated with worse prognosis in the breast cancer patients.

Acknowledgements

The work in this thesis was carried out at Clinical Research Center (CRC) at the Department of Translational Medicine, Lund University, Malmö, Sweden. The financial support was provided by Vetenskapsrådet, Cancerfonden, the Governmental Funding of Clinical Research within the National Health Service (ALF), Gyllenstiernska Krapperups foundation, Almänna Sjukhusets i Malmö Stiftelse för bekämpande av cancer, Åke Wibergs foundation, Percy Falks foundation, The Royal Physiographic Society in Lund, and Maja och Hjalmar Leander foundation. Thank you to all the funders for making this project possible, and to all the patients who participated in our studies.

To my supervisor **Karin Leandersson**. I can't believe how incredibly lucky I was to be the right person at the right time to join your group. You take such good care of your students, you care so much, and you make sure everyone in the group knows their worth. I sincerely feel sad to be leaving the group, but as you've made clear, that does not mean that we will lose contact. Thank you for being an inspiration and a role model, as well as a friend and my extra family member during the pandemic.

My co-supervisors; **Karin Jirström** and **Rebecka Hellsten**. It's been very valuable to know that I have two more fantastic scientists in my corner. I want to thank **Karin** for her help with the clinical part of my work, complementing my pre-clinical knowledge, and for her help with the oh so confusing multivariable analysis. And **Rebecka**, I'm so happy I even got to spend non-scientific work time with you, during fika, spring excursions and Christmas celebrations. You have been a fantastic support scientifically as well, always willing to lend a hand or discuss my research with me. You two are also fantastic role models for younger scientists.

To past and present members of the **Cancer Immunology group**. **Melly**! I've missed you dearly. Thank you so much for welcoming me into the group, showing me where everything is and goes, how to claim territory with the clever use of tape and for all the hugs, encouraging words, lunches and fikas. A very merry Christmas from Karin's atheist PhD student to her Muslim PhD! **Eva**, the ying to my yang in the lab. Thank you for everything, all the discussions, knowledge, laughs and the honey. **Oscar**, our most recent addition to the lab. You will be a great continuation of the amazing PhD students in Karin's group. I know you will keep it organized and running smoothly with your attention to detail and mutual love for order. **Camilla**, my original mentor in the group. Thank you for all the patience in the

beginning while showing me the ropes. To the students I have supervised in the group: **Mika**, **Robert**, **Cassandra**, and especially **Aida**. I'm so happy that I gained a true friend when I got to work with you. Keep on knitting! Supervising students has been my favorite part of this journey. You all give such a great variety to the group. You ask all the important questions, and you make sure that the group is young and fresh. Same goes for all our other students in the group; **Marcus**, that even though you were not my student it really felt like it, **Elly**, **Tannas** and **Hannah**.

Anita and **Tommy** and all their group members past and present, and other "residents" at CRC in Malmö: **Diana**, **Elin**, **Farnaz**, **Geriolda**, **Gunilla**, **Janina**, **Kumar**, **Lisa**, **Lubna, Mansi**, **Puja**, **Puru**, **Qing**, **Shakti**, **Souvik**, **Syrina**, **Vikas**, and **Zdenka**. Thank you for all the support, for sharing your knowledge and resources, and for all the wonderful interactions, conversations, and cultural exchange during Friday fika, MCC retreats and Christmas bowling. To **Njainday**, an extra thanks for the scientific and more importantly the emotional support. Everyone needs a strong and fierce queen like you in their life. Also, an extra thank you to **Lena**, for all the help with science and for the neighbor support.

Members of the CMP fika, spring excursion and afterwork group. **Agi**, **Anders**, **Anna**, **Ben**, **Evangelia, Helén**, **Johan**, **Macarena**, **Maite**, **Mathieu**, **Rebecca**, **Susan**, **Tamae** and **Totte**. I wish I had joined Karin's group sooner, only to have gotten to know you all better, before we all moved in different directions. Thank you for the fun excursions and the now deceased Tuesday fika. Extra thank you to **Kiki** for all the IHC work.

To **Carro** for organizing our Thursday seminars and for all the valuable help in the lab, both intellectual and in helping me find stuff. And everyone in the **Wallenberg seminars**, thank you for great scientific exchange.

My best science friends: **Hófí** and **Sólveig**. Having you just across one bridge makes all the difference in the world. You are the best cheerleaders, emotional support network, knitting club, gossip gal pals and friends one can imagine. The fact that you also understand and can give feedback on my research and PhD journey is invaluable. The rest of **Genagengið**, you know who you are! What a fantastic group of people I have in my life. You are all amazing and I know you always have my back.

My "non-science" friends: **Hulda Rós**, my best and oldest friend! Every time I come back home to Iceland, seeing you is one of my favorite things to look forward to. I think you are so amazing and I'm so proud of who we've become. And **Laufey**, the third piece of this Kit-Kat. You are so amazing, and you charge my happiness and life batteries every time we meet. Keep being hilarious. **Anna,** my first and best Swedish friend. Thank you for all the visits, dinners, red sauce pastas, and laughs. My elementary school friends, **Ágústa**, **Guðrún**, **Helen**, and **Jóhanna**. Thank you for always being there and for coming to visit me here in Lund. It means so much when I'm so far away.

My extra family in Sweden, **Sandra**, **Jack**, **Greg**, **Evelyn** and **Nemanja**. Thank you for taking me into your home and hearts. Being away from family can be so hard, but you all make it so much easier. Tack för allt.

My family in Iceland. Ég veit að það er erfitt að vera svona langt í burtu og sérstaklega í heimsfaraldri, en mér þykir svo vænt um ykkur og allan ykkar stuðning. Ég veit að þið eruð svo stolt af mér og ég er svo stolt af sjálfri mér fyrir að gera ykkur stolt. Takk fyrir allt, **Mamma**, **Pabbi**, **Anna Sigrún**, Ömmur mínar, sem eru svo frábærar og sterkar konur, og Afi, Frænkur og Frændur og allir hinir. **Sigrún Tinna** og **Halldóra Birta** eiga sérstakt hrós skilið fyrir stuðning og dýrmæta vináttu sem er svo alls ekki sjálfsögð.

And last but not least, my favorite person in the world, **Douglas**. I know you are always there for me, and you would do anything for me. Thank you so much for all the support, for making me feel smart, dragging me down to earth whenever needed, and making me laugh. Can't wait for the future. You are my home and my happiness.

♥

FBG

References

1. Chaplin DD. Overview of the immune response. *J Allergy Clin Immunol*. Feb 2010;125(2 Suppl 2):S3-23. doi:10.1016/j.jaci.2009.12.980
2. Zhang JM, An J. Cytokines, inflammation, and pain. *Int Anesthesiol Clin*. Spring 2007;45(2):27-37. doi:10.1097/AIA.0b013e318034194e
3. Tang D, Kang R, Coyne CB, Zeh HJ, Lotze MT. PAMPs and DAMPs: signal 0s that spur autophagy and immunity. *Immunol Rev*. Sep 2012;249(1):158-75. doi:10.1111/j.1600-065X.2012.01146.x
4. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *Int Rev Immunol*. Feb 2011;30(1):16-34. doi:10.3109/08830185.2010.529976
5. Murphy K, Weaver C, Janeway C. *Janeway's immunobiology*. 9th edition ed. Immunobiology. Garland Science; 2017.
6. Koenderman L, Buurman W, Daha MR. The innate immune response. *Immunol Lett*. Dec 2014;162(2 Pt B):95-102. doi:10.1016/j.imlet.2014.10.010
7. Hewitt EW. The MHC class I antigen presentation pathway: strategies for viral immune evasion. *Immunology*. Oct 2003;110(2):163-9. doi:10.1046/j.1365-2567.2003.01738.x
8. Roche PA, Furuta K. The ins and outs of MHC class II-mediated antigen processing and presentation. *Nat Rev Immunol*. Apr 2015;15(4):203-16. doi:10.1038/nri3818
9. Actor JK. 2 - Cells and Organs of the Immune System. In: Actor JK, ed. *Elsevier's Integrated Review Immunology and Microbiology (Second Edition)*. W.B. Saunders; 2012:7-16.
10. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. Mar 2013;13(3):159-75. doi:10.1038/nri3399
11. Hickey MJ, Kubes P. Intravascular immunity: the host-pathogen encounter in blood vessels. *Nat Rev Immunol*. May 2009;9(5):364-75. doi:10.1038/nri2532
12. Urban CF, Ermert D, Schmid M, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog*. Oct 2009;5(10):e1000639. doi:10.1371/journal.ppat.1000639
13. Mehmeti-Ajradini M, Bergenfelz C, Larsson AM, et al. Human G-MDSCs are neutrophils at distinct maturation stages promoting tumor growth in breast cancer. *Life Sci Alliance*. Nov 2020;3(11)doi:10.26508/lsa.202000893
14. Hashimoto D, Miller J, Merad M. Dendritic cell and macrophage heterogeneity in vivo. *Immunity*. Sep 23 2011;35(3):323-35. doi:10.1016/j.immuni.2011.09.007

15. Forkner CE. THE ORIGIN OF MONOCYTES IN CERTAIN LYMPH NODES AND THEIR GENETIC RELATION TO OTHER CONNECTIVE TISSUE CELLS. *J Exp Med*. Aug 31 1930;52(3):385-404. doi:10.1084/jem.52.3.385
16. Boyette LB, Macedo C, Hadi K, et al. Phenotype, function, and differentiation potential of human monocyte subsets. *PLoS One*. 2017;12(4):e0176460. doi:10.1371/journal.pone.0176460
17. Cros J, Cagnard N, Woollard K, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. *Immunity*. Sep 24 2010;33(3):375-86. doi:10.1016/j.immuni.2010.08.012
18. Varol C, Yona S, Jung S. Origins and tissue-context-dependent fates of blood monocytes. *Immunol Cell Biol*. Jan 2009;87(1):30-8. doi:10.1038/icb.2008.90
19. Mildner A, Marinkovic G, Jung S. Murine Monocytes: Origins, Subsets, Fates, and Functions. *Microbiol Spectr*. Oct 2016;4(5)doi:10.1128/microbiolspec.MCHD-0033-2016
20. Ginhoux F, Guilliams M. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity*. Mar 15 2016;44(3):439-449. doi:10.1016/j.immuni.2016.02.024
21. Mantovani A, Marchesi F, Jaillon S, Garlanda C, Allavena P. Tumor-associated myeloid cells: diversity and therapeutic targeting. *Cell Mol Immunol*. Mar 2021;18(3):566-578. doi:10.1038/s41423-020-00613-4
22. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. Dec 2008;8(12):958-69. doi:10.1038/nri2448
23. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol*. Oct 14 2011;11(11):723-37. doi:10.1038/nri3073
24. Ovchinnikov DA. Macrophages in the embryo and beyond: much more than just giant phagocytes. *Genesis*. Sep 2008;46(9):447-62. doi:10.1002/dvg.20417
25. Epelman S, Lavine KJ, Randolph GJ. Origin and functions of tissue macrophages. *Immunity*. Jul 17 2014;41(1):21-35. doi:10.1016/j.immuni.2014.06.013
26. Stein M, Keshav S, Harris N, Gordon S. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *J Exp Med*. Jul 1 1992;176(1):287-92. doi:10.1084/jem.176.1.287
27. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. *J Immunol*. Jun 15 2000;164(12):6166-73. doi:10.4049/jimmunol.164.12.6166
28. Hesketh M, Sahin KB, West ZE, Murray RZ. Macrophage Phenotypes Regulate Scar Formation and Chronic Wound Healing. *Int J Mol Sci*. Jul 17 2017;18(7)doi:10.3390/ijms18071545
29. Mantovani A, Sica A, Allavena P, Garlanda C, Locati M. Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum Immunol*. May 2009;70(5):325-30. doi:10.1016/j.humimm.2009.02.008

30. Murray PJ, Allen JE, Biswas SK, et al. Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity*. Jul 17 2014;41(1):14-20. doi:10.1016/j.immuni.2014.06.008
31. Rőszer T. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm*. 2015;2015:816460. doi:10.1155/2015/816460
32. Standring S. *Gray's anatomy : the anatomical basis of clinical practice*. 2016.
33. Aoyama S, Nakagawa R, Mulé JJ, Mailloux AW. Inducible Tertiary Lymphoid Structures: Promise and Challenges for Translating a New Class of Immunotherapy. Review. *Frontiers in Immunology*. 2021-May-14 2021;12(1766)doi:10.3389/fimmu.2021.675538
34. Sautès-Fridman C, Petitprez F, Calderaro J, Fridman WH. Tertiary lymphoid structures in the era of cancer immunotherapy. *Nat Rev Cancer*. Jun 2019;19(6):307-325. doi:10.1038/s41568-019-0144-6
35. Munoz-Erazo L, Rhodes JL, Marion VC, Kemp RA. Tertiary lymphoid structures in cancer - considerations for patient prognosis. *Cell Mol Immunol*. Jun 2020;17(6):570-575. doi:10.1038/s41423-020-0457-0
36. Gray EE, Cyster JG. Lymph node macrophages. *J Innate Immun*. 2012;4(5-6):424-36. doi:10.1159/000337007
37. Szakal AK, Holmes KL, Tew JG. Transport of immune complexes from the subcapsular sinus to lymph node follicles on the surface of nonphagocytic cells, including cells with dendritic morphology. *J Immunol*. Oct 1983;131(4):1714-27.
38. Phan TG, Grigorova I, Okada T, Cyster JG. Subcapsular encounter and complement-dependent transport of immune complexes by lymph node B cells. *Nat Immunol*. Sep 2007;8(9):992-1000. doi:10.1038/ni1494
39. Junt T, Moseman EA, Iannacone M, et al. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. *Nature*. Nov 1 2007;450(7166):110-4. doi:10.1038/nature06287
40. Carrasco YR, Batista FD. B cells acquire particulate antigen in a macrophage-rich area at the boundary between the follicle and the subcapsular sinus of the lymph node. *Immunity*. Jul 2007;27(1):160-71. doi:10.1016/j.immuni.2007.06.007
41. Asano K, Nabeyama A, Miyake Y, et al. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity*. Jan 28 2011;34(1):85-95. doi:10.1016/j.immuni.2010.12.011
42. MacDonald KP, Palmer JS, Cronau S, et al. An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. *Blood*. Nov 11 2010;116(19):3955-63. doi:10.1182/blood-2010-02-266296
43. Witmer-Pack MD, Hughes DA, Schuler G, et al. Identification of macrophages and dendritic cells in the osteopetrotic (op/op) mouse. *J Cell Sci*. Apr 1993;104 (Pt 4):1021-9.
44. Komohara Y, Ohnishi K, Takeya M. Possible functions of CD169-positive sinus macrophages in lymph nodes in anti-tumor immune responses. *Cancer Sci*. Mar 2017;108(3):290-295. doi:10.1111/cas.13137

45. Hartnell A, Steel J, Turley H, Jones M, Jackson DG, Crocker PR. Characterization of human sialoadhesin, a sialic acid binding receptor expressed by resident and inflammatory macrophage populations. *Blood*. Jan 1 2001;97(1):288-96. doi:10.1182/blood.v97.1.288
46. Grabowska J, Lopez-Venegas MA, Affandi AJ, den Haan JMM. CD169(+) Macrophages Capture and Dendritic Cells Instruct: The Interplay of the Gatekeeper and the General of the Immune System. *Front Immunol*. 2018;9:2472. doi:10.3389/fimmu.2018.02472
47. Mildner A, Jung S. Development and function of dendritic cell subsets. *Immunity*. May 15 2014;40(5):642-56. doi:10.1016/j.immuni.2014.04.016
48. Tang-Huau TL, Gueguen P, Goudot C, et al. Human in vivo-generated monocyte-derived dendritic cells and macrophages cross-present antigens through a vacuolar pathway. *Nat Commun*. Jul 2 2018;9(1):2570. doi:10.1038/s41467-018-04985-0
49. Huang AY, Golumbek P, Ahmadzadeh M, Jaffee E, Pardoll D, Levitsky H. Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens. *Science*. May 13 1994;264(5161):961-5. doi:10.1126/science.7513904
50. Sigal LJ, Crotty S, Andino R, Rock KL. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature*. Mar 4 1999;398(6722):77-80. doi:10.1038/18038
51. Heath WR, Carbone FR. Cross-presentation in viral immunity and self-tolerance. *Nat Rev Immunol*. Nov 2001;1(2):126-34. doi:10.1038/35100512
52. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol*. Jul 13 2012;12(8):557-69. doi:10.1038/nri3254
53. Vivier E, Artis D, Colonna M, et al. Innate Lymphoid Cells: 10 Years On. *Cell*. Aug 23 2018;174(5):1054-1066. doi:10.1016/j.cell.2018.07.017
54. O'Sullivan TE. Dazed and Confused: NK Cells. *Front Immunol*. 2019;10:2235. doi:10.3389/fimmu.2019.02235
55. Colonna M. Innate Lymphoid Cells: Diversity, Plasticity, and Unique Functions in Immunity. *Immunity*. Jun 19 2018;48(6):1104-1117. doi:10.1016/j.immuni.2018.05.013
56. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol*. Feb 1975;5(2):112-7. doi:10.1002/eji.1830050208
57. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *Int J Cancer*. Aug 15 1975;16(2):230-9. doi:10.1002/ijc.2910160205
58. Wu SY, Fu T, Jiang YZ, Shao ZM. Natural killer cells in cancer biology and therapy. *Mol Cancer*. Aug 6 2020;19(1):120. doi:10.1186/s12943-020-01238-x
59. Screpanti V, Wallin RP, Grandien A, Ljunggren HG. Impact of FASL-induced apoptosis in the elimination of tumor cells by NK cells. *Mol Immunol*. Feb 2005;42(4):495-9. doi:10.1016/j.molimm.2004.07.033

60. Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol*. Jun 2015;15(6):388-400. doi:10.1038/nri3839
61. Fauriat C, Long EO, Ljunggren HG, Bryceson YT. Regulation of human NK-cell cytokine and chemokine production by target cell recognition. *Blood*. Mar 18 2010;115(11):2167-76. doi:10.1182/blood-2009-08-238469
62. Iannello A, Debbeche O, Samarani S, Ahmad A. Antiviral NK cell responses in HIV infection: I. NK cell receptor genes as determinants of HIV resistance and progression to AIDS. *J Leukoc Biol*. Jul 2008;84(1):1-26. doi:10.1189/jlb.0907650
63. Lanier LL. NK cell recognition. *Annu Rev Immunol*. 2005;23:225-74. doi:10.1146/annurev.immunol.23.021704.115526
64. Bryceson YT, March ME, Ljunggren HG, Long EO. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood*. Jan 1 2006;107(1):159-66. doi:10.1182/blood-2005-04-1351
65. Zwirner NW, Domaica CI. Cytokine regulation of natural killer cell effector functions. *Biofactors*. Jul-Aug 2010;36(4):274-88. doi:10.1002/biof.107
66. Janeway CA, Jr., Medzhitov R. Innate immune recognition. *Annu Rev Immunol*. 2002;20:197-216. doi:10.1146/annurev.immunol.20.083001.084359
67. Germain RN. T-cell development and the CD4-CD8 lineage decision. *Nat Rev Immunol*. May 2002;2(5):309-22. doi:10.1038/nri798
68. Roth DB. V(D)J Recombination: Mechanism, Errors, and Fidelity. *Microbiol Spectr*. Dec 2014;2(6)doi:10.1128/microbiolspec.MDNA3-0041-2014
69. Hoehn KB, Fowler A, Lunter G, Pybus OG. The Diversity and Molecular Evolution of B-Cell Receptors during Infection. *Mol Biol Evol*. May 2016;33(5):1147-57. doi:10.1093/molbev/msw015
70. Mårtensson IL, Almqvist N, Grimsholm O, Bernardi AI. The pre-B cell receptor checkpoint. *FEBS Lett*. Jun 18 2010;584(12):2572-9. doi:10.1016/j.febslet.2010.04.057
71. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. *Blood*. Sep 1 2008;112(5):1570-80. doi:10.1182/blood-2008-02-078071
72. Palmer E. Negative selection--clearing out the bad apples from the T-cell repertoire. *Nat Rev Immunol*. May 2003;3(5):383-91. doi:10.1038/nri1085
73. Mueller DL. Mechanisms maintaining peripheral tolerance. *Nat Immunol*. Jan 2010;11(1):21-7. doi:10.1038/ni.1817
74. Wang Y, Liu J, Burrows PD, Wang JY. B Cell Development and Maturation. *Adv Exp Med Biol*. 2020;1254:1-22. doi:10.1007/978-981-15-3532-1_1
75. Parker DC. T cell-dependent B cell activation. *Annu Rev Immunol*. 1993;11:331-60. doi:10.1146/annurev.iy.11.040193.001555
76. Okada T, Miller MJ, Parker I, et al. Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells. *PLoS Biol*. Jun 2005;3(6):e150. doi:10.1371/journal.pbio.0030150

77. Nutt SL, Hodgkin PD, Tarlinton DM, Corcoran LM. The generation of antibody-secreting plasma cells. *Nat Rev Immunol*. Mar 2015;15(3):160-71. doi:10.1038/nri3795

78. Dal Porto JM, Gauld SB, Merrell KT, Mills D, Pugh-Bernard AE, Cambier J. B cell antigen receptor signaling 101. *Mol Immunol*. Jul 2004;41(6-7):599-613. doi:10.1016/j.molimm.2004.04.008

79. Chen X, Jensen PE. The role of B lymphocytes as antigen-presenting cells. *Arch Immunol Ther Exp (Warsz)*. Mar-Apr 2008;56(2):77-83. doi:10.1007/s00005-008-0014-5

80. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, Ahmed R. Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol*. Nov 15 2003;171(10):4969-73. doi:10.4049/jimmunol.171.10.4969

81. McHeyzer-Williams M, Okitsu S, Wang N, McHeyzer-Williams L. Molecular programming of B cell memory. *Nat Rev Immunol*. Dec 9 2011;12(1):24-34. doi:10.1038/nri3128

82. Vazquez MI, Catalan-Dibene J, Zlotnik A. B cells responses and cytokine production are regulated by their immune microenvironment. *Cytokine*. Aug 2015;74(2):318-26. doi:10.1016/j.cyto.2015.02.007

83. Pistoia V, Tumino N, Vacca P, et al. Human γδ T-Cells: From Surface Receptors to the Therapy of High-Risk Leukemias. Mini Review. *Frontiers in Immunology*. 2018-May-07 2018;9(984)doi:10.3389/fimmu.2018.00984

84. Bonilla FA, Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol*. Feb 2010;125(2 Suppl 2):S33-40. doi:10.1016/j.jaci.2009.09.017

85. Janssen EM, Lemmens EE, Wolfe T, Christen U, von Herrath MG, Schoenberger SP. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature*. Feb 20 2003;421(6925):852-6. doi:10.1038/nature01441

86. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science*. Apr 11 2003;300(5617):337-9. doi:10.1126/science.1082305

87. Sun JC, Williams MA, Bevan MJ. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat Immunol*. Sep 2004;5(9):927-33. doi:10.1038/ni1105

88. Geginat J, Paroni M, Maglie S, et al. Plasticity of human CD4 T cell subsets. *Front Immunol*. 2014;5:630. doi:10.3389/fimmu.2014.00630

89. Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest*. May 2007;117(5):1119-27. doi:10.1172/jci31720

90. Luckheeram RV, Zhou R, Verma AD, Xia B. $CD4^+$T cells: differentiation and functions. *Clin Dev Immunol*. 2012;2012:925135. doi:10.1155/2012/925135

91. Kondělková K, Vokurková D, Krejsek J, Borská L, Fiala Z, Ctirad A. Regulatory T cells (TREG) and their roles in immune system with respect to immunopathological disorders. *Acta Medica (Hradec Kralove)*. 2010;53(2):73-7. doi:10.14712/18059694.2016.63

92. Bakshi RK, Cox MA, Zajac AJ. Cytotoxic T Lymphocytes. In: Mackay IR, Rose NR, Diamond B, Davidson A, eds. *Encyclopedia of Medical Immunology: Autoimmune Diseases*. Springer New York; 2014:332-342.
93. Akbar AN, Terry L, Timms A, Beverley PC, Janossy G. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. *J Immunol*. Apr 1 1988;140(7):2171-8.
94. Kumar BV, Connors TJ, Farber DL. Human T Cell Development, Localization, and Function throughout Life. *Immunity*. Feb 20 2018;48(2):202-213. doi:10.1016/j.immuni.2018.01.007
95. Golubovskaya V, Wu L. Different Subsets of T Cells, Memory, Effector Functions, and CAR-T Immunotherapy. *Cancers (Basel)*. Mar 15 2016;8(3)doi:10.3390/cancers8030036
96. Godfrey DI, Uldrich AP, McCluskey J, Rossjohn J, Moody DB. The burgeoning family of unconventional T cells. *Nat Immunol*. Nov 2015;16(11):1114-23. doi:10.1038/ni.3298
97. Jameson J, Havran WL. Skin gammadelta T-cell functions in homeostasis and wound healing. *Immunol Rev*. Feb 2007;215:114-22. doi:10.1111/j.1600-065X.2006.00483.x
98. Holtmeier W, Kabelitz D. gammadelta T cells link innate and adaptive immune responses. *Chem Immunol Allergy*. 2005;86:151-183. doi:10.1159/000086659
99. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell*. Jan 7 2000;100(1):57-70. doi:10.1016/s0092-8674(00)81683-9
100. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. Mar 4 2011;144(5):646-74. doi:10.1016/j.cell.2011.02.013
101. Shankaran V, Ikeda H, Bruce AT, et al. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*. Apr 26 2001;410(6832):1107-11. doi:10.1038/35074122
102. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. *Annu Rev Immunol*. 2004;22:329-60. doi:10.1146/annurev.immunol.22.012703.104803
103. Swann JB, Smyth MJ. Immune surveillance of tumors. *J Clin Invest*. May 2007;117(5):1137-46. doi:10.1172/jci31405
104. Penn I, Halgrimson CG, Starzl TE. De novo malignant tumors in organ transplant recipients. *Transplant Proc*. Mar 1971;3(1):773-8.
105. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol*. Nov 2002;3(11):991-8. doi:10.1038/ni1102-991
106. Rilke F, Colnaghi MI, Cascinelli N, et al. Prognostic significance of HER-2/neu expression in breast cancer and its relationship to other prognostic factors. *Int J Cancer*. Aug 19 1991;49(1):44-9. doi:10.1002/ijc.2910490109
107. Lipponen PK, Eskelinen MJ, Jauhiainen K, Harju E, Terho R. Tumour infiltrating lymphocytes as an independent prognostic factor in transitional cell bladder cancer. *Eur J Cancer*. 1992;29a(1):69-75. doi:10.1016/0959-8049(93)90579-5

108. Naito Y, Saito K, Shiiba K, et al. CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res*. Aug 15 1998;58(16):3491-4.
109. Jass JR. Lymphocytic infiltration and survival in rectal cancer. *J Clin Pathol*. Jun 1986;39(6):585-9. doi:10.1136/jcp.39.6.585
110. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell*. Apr 2 2010;141(1):39-51. doi:10.1016/j.cell.2010.03.014
111. Gasser S, Raulet DH. The DNA damage response arouses the immune system. *Cancer Res*. Apr 15 2006;66(8):3959-62. doi:10.1158/0008-5472.Can-05-4603
112. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. *Immunology*. May 2007;121(1):1-14. doi:10.1111/j.1365-2567.2007.02587.x
113. Borrego F, Ulbrecht M, Weiss EH, Coligan JE, Brooks AG. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J Exp Med*. Mar 2 1998;187(5):813-8. doi:10.1084/jem.187.5.813
114. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases--elimination, equilibrium and escape. *Curr Opin Immunol*. Apr 2014;27:16-25. doi:10.1016/j.coi.2014.01.004
115. Paul P, Rouas-Freiss N, Khalil-Daher I, et al. HLA-G expression in melanoma: a way for tumor cells to escape from immunosurveillance. *Proc Natl Acad Sci U S A*. Apr 14 1998;95(8):4510-5. doi:10.1073/pnas.95.8.4510
116. Martin LJ, Boyd NF. Mammographic density. Potential mechanisms of breast cancer risk associated with mammographic density: hypotheses based on epidemiological evidence. *Breast Cancer Res*. 2008;10(1):201. doi:10.1186/bcr1831
117. Blagosklonny MV. Antiangiogenic therapy and tumor progression. *Cancer Cell*. Jan 2004;5(1):13-7. doi:10.1016/s1535-6108(03)00336-2
118. Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW. Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts. *Cancer Res*. Jul 15 1991;51(14):3753-61.
119. Van Linthout S, Miteva K, Tschöpe C. Crosstalk between fibroblasts and inflammatory cells. *Cardiovascular Research*. 2014;102(2):258-269. doi:10.1093/cvr/cvu062
120. Weber CE, Kuo PC. The tumor microenvironment. *Surg Oncol*. Sep 2012;21(3):172-7. doi:10.1016/j.suronc.2011.09.001
121. Syed V. TGF-β Signaling in Cancer. *J Cell Biochem*. Jun 2016;117(6):1279-87. doi:10.1002/jcb.25496
122. Nishida N, Yano H, Nishida T, Kamura T, Kojiro M. Angiogenesis in cancer. *Vasc Health Risk Manag*. 2006;2(3):213-9. doi:10.2147/vhrm.2006.2.3.213
123. Kubouchi Y, Yurugi Y, Wakahara M, et al. Podoplanin expression in cancer-associated fibroblasts predicts unfavourable prognosis in patients with pathological

stage IA lung adenocarcinoma. *Histopathology*. Feb 2018;72(3):490-499. doi:10.1111/his.13390

124. Zhang SY, Song XY, Li Y, Ye LL, Zhou Q, Yang WB. Tumor-associated macrophages: A promising target for a cancer immunotherapeutic strategy. *Pharmacol Res*. Nov 2020;161:105111. doi:10.1016/j.phrs.2020.105111
125. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Front Physiol*. 2014;5:75. doi:10.3389/fphys.2014.00075
126. Kuang DM, Zhao Q, Peng C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. *J Exp Med*. Jun 8 2009;206(6):1327-37. doi:10.1084/jem.20082173
127. Kryczek I, Zou L, Rodriguez P, et al. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *J Exp Med*. Apr 17 2006;203(4):871-81. doi:10.1084/jem.20050930
128. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity*. Jul 17 2014;41(1):49-61. doi:10.1016/j.immuni.2014.06.010
129. Ostuni R, Kratochvill F, Murray PJ, Natoli G. Macrophages and cancer: from mechanisms to therapeutic implications. *Trends Immunol*. Apr 2015;36(4):229-39. doi:10.1016/j.it.2015.02.004
130. Argyle D, Kitamura T. Targeting Macrophage-Recruiting Chemokines as a Novel Therapeutic Strategy to Prevent the Progression of Solid Tumors. *Front Immunol*. 2018;9:2629. doi:10.3389/fimmu.2018.02629
131. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol*. Mar 2002;196(3):254-65. doi:10.1002/path.1027
132. Leek RD, Hunt NC, Landers RJ, Lewis CE, Royds JA, Harris AL. Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J Pathol*. Mar 2000;190(4):430-6. doi:10.1002/(sici)1096-9896(200003)190:4<430::Aid-path538>3.0.Co;2-6
133. Saccani A, Schioppa T, Porta C, et al. p50 nuclear factor-kappaB overexpression in tumor-associated macrophages inhibits M1 inflammatory responses and antitumor resistance. *Cancer Res*. Dec 1 2006;66(23):11432-40. doi:10.1158/0008-5472.Can-06-1867
134. Qiu SQ, Waaijer SJH, Zwager MC, de Vries EGE, van der Vegt B, Schroder CP. Tumor-associated macrophages in breast cancer: Innocent bystander or important player? *Cancer Treat Rev*. Nov 2018;70:178-189. doi:10.1016/j.ctrv.2018.08.010
135. Medrek C, Ponten F, Jirstrom K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer*. Jul 23 2012;12:306. doi:10.1186/1471-2407-12-306
136. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol*. Apr 2010;22(2):231-7. doi:10.1016/j.coi.2010.01.009

137. Serafini P. Myeloid derived suppressor cells in physiological and pathological conditions: the good, the bad, and the ugly. *Immunol Res*. Dec 2013;57(1-3):172-84. doi:10.1007/s12026-013-8455-2

138. Pinto ML, Rios E, Durães C, et al. The Two Faces of Tumor-Associated Macrophages and Their Clinical Significance in Colorectal Cancer. *Front Immunol*. 2019;10:1875. doi:10.3389/fimmu.2019.01875

139. Najafi M, Hashemi Goradel N, Farhood B, et al. Macrophage polarity in cancer: A review. *J Cell Biochem*. Mar 2019;120(3):2756-2765. doi:10.1002/jcb.27646

140. Jayasingam SD, Citartan M, Thang TH, Mat Zin AA, Ang KC, Ch'ng ES. Evaluating the Polarization of Tumor-Associated Macrophages Into M1 and M2 Phenotypes in Human Cancer Tissue: Technicalities and Challenges in Routine Clinical Practice. *Front Oncol*. 2019;9:1512. doi:10.3389/fonc.2019.01512

141. Yuan X, Zhang J, Li D, et al. Prognostic significance of tumor-associated macrophages in ovarian cancer: A meta-analysis. *Gynecol Oncol*. Oct 2017;147(1):181-187. doi:10.1016/j.ygyno.2017.07.007

142. Williams CB, Yeh ES, Soloff AC. Tumor-associated macrophages: unwitting accomplices in breast cancer malignancy. *NPJ Breast Cancer*. 2016;2:15025-. doi:10.1038/npjbcancer.2015.25

143. Gabrilovich DI, Bronte V, Chen SH, et al. The terminology issue for myeloid-derived suppressor cells. *Cancer Res*. Jan 1 2007;67(1):425; author reply 426. doi:10.1158/0008-5472.Can-06-3037

144. Kong YY, Fuchsberger M, Xiang SD, Apostolopoulos V, Plebanski M. Myeloid derived suppressor cells and their role in diseases. *Curr Med Chem*. 2013;20(11):1437-44. doi:10.2174/0929867311320110006

145. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. Mar 2009;9(3):162-74. doi:10.1038/nri2506

146. Poschke I, Kiessling R. On the armament and appearances of human myeloid-derived suppressor cells. *Clin Immunol*. Sep 2012;144(3):250-68. doi:10.1016/j.clim.2012.06.003

147. Millrud CR, Bergenfelz C, Leandersson K. On the origin of myeloid-derived suppressor cells. *Oncotarget*. Jan 10 2017;8(2):3649-3665. doi:10.18632/oncotarget.12278

148. Almand B, Clark JI, Nikitina E, et al. Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. *J Immunol*. Jan 1 2001;166(1):678-89. doi:10.4049/jimmunol.166.1.678

149. Mundy-Bosse BL, Young GS, Bauer T, et al. Distinct myeloid suppressor cell subsets correlate with plasma IL-6 and IL-10 and reduced interferon-alpha signaling in $CD4^+$ T cells from patients with GI malignancy. *Cancer Immunol Immunother*. Sep 2011;60(9):1269-79. doi:10.1007/s00262-011-1029-z

150. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. *J Immunol*. Jan 15 2004;172(2):989-99. doi:10.4049/jimmunol.172.2.989

151. Mantovani A. The growing diversity and spectrum of action of myeloid-derived suppressor cells. *Eur J Immunol*. Dec 2010;40(12):3317-20. doi:10.1002/eji.201041170
152. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Clin Orthop Relat Res*. Jan 1991;(262):3-11.
153. Tang J, Shalabi A, Hubbard-Lucey VM. Comprehensive analysis of the clinical immuno-oncology landscape. *Ann Oncol*. Jan 1 2018;29(1):84-91. doi:10.1093/annonc/mdx755
154. Dunn GP, Koebel CM, Schreiber RD. Interferons, immunity and cancer immunoediting. *Nat Rev Immunol*. Nov 2006;6(11):836-48. doi:10.1038/nri1961
155. Razaghi A, Owens L, Heimann K. Review of the recombinant human interferon gamma as an immunotherapeutic: Impacts of production platforms and glycosylation. *J Biotechnol*. Dec 20 2016;240:48-60. doi:10.1016/j.jbiotec.2016.10.022
156. Atkins MB, Lotze MT, Dutcher JP, et al. High-dose recombinant interleukin 2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993. *J Clin Oncol*. Jul 1999;17(7):2105-16. doi:10.1200/jco.1999.17.7.2105
157. Coventry BJ, Ashdown ML. The 20th anniversary of interleukin-2 therapy: bimodal role explaining longstanding random induction of complete clinical responses. *Cancer Manag Res*. 2012;4:215-21. doi:10.2147/cmar.S33979
158. Wrangle JM, Patterson A, Johnson CB, et al. IL-2 and Beyond in Cancer Immunotherapy. *J Interferon Cytokine Res*. Feb 2018;38(2):45-68. doi:10.1089/jir.2017.0101
159. Geukes Foppen MH, Donia M, Svane IM, Haanen JB. Tumor-infiltrating lymphocytes for the treatment of metastatic cancer. *Mol Oncol*. Dec 2015;9(10):1918-35. doi:10.1016/j.molonc.2015.10.018
160. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L. 'Off-the-shelf' allogeneic CAR T cells: development and challenges. *Nat Rev Drug Discov*. Mar 2020;19(3):185-199. doi:10.1038/s41573-019-0051-2
161. June CH, Sadelain M. Chimeric Antigen Receptor Therapy. *N Engl J Med*. Jul 5 2018;379(1):64-73. doi:10.1056/NEJMra1706169
162. Klichinsky M, Ruella M, Shestova O, et al. Human chimeric antigen receptor macrophages for cancer immunotherapy. *Nat Biotechnol*. Aug 2020;38(8):947-953. doi:10.1038/s41587-020-0462-y
163. Xie G, Dong H, Liang Y, Ham JD, Rizwan R, Chen J. CAR-NK cells: A promising cellular immunotherapy for cancer. *EBioMedicine*. Sep 2020;59:102975. doi:10.1016/j.ebiom.2020.102975
164. Sadeghzadeh M, Bornehdeli S, Mohahammadrezakhani H, et al. Dendritic cell therapy in cancer treatment; the state-of-the-art. *Life Sci*. Aug 1 2020;254:117580. doi:10.1016/j.lfs.2020.117580
165. Plosker GL. Sipuleucel-T: in metastatic castration-resistant prostate cancer. *Drugs*. Jan 1 2011;71(1):101-8. doi:10.2165/11206840-000000000-00000

166. Anassi E, Ndefo UA. Sipuleucel-T (provenge) injection: the first immunotherapy agent (vaccine) for hormone-refractory prostate cancer. *P t*. Apr 2011;36(4):197-202.
167. Weiner LM, Surana R, Wang S. Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat Rev Immunol*. May 2010;10(5):317-27. doi:10.1038/nri2744
168. Seidel UJ, Schlegel P, Lang P. Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. *Front Immunol*. 2013;4:76. doi:10.3389/fimmu.2013.00076
169. Gelderman KA, Tomlinson S, Ross GD, Gorter A. Complement function in mAb-mediated cancer immunotherapy. *Trends Immunol*. Mar 2004;25(3):158-64. doi:10.1016/j.it.2004.01.008
170. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. Mar 22 2012;12(4):252-64. doi:10.1038/nrc3239
171. Buchbinder EI, Desai A. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol*. Feb 2016;39(1):98-106. doi:10.1097/coc.0000000000000239
172. Pico de Coana Y, Choudhury A, Kiessling R. Checkpoint blockade for cancer therapy: revitalizing a suppressed immune system. *Trends Mol Med*. Aug 2015;21(8):482-91. doi:10.1016/j.molmed.2015.05.005
173. Ribas A, Camacho LH, Lopez-Berestein G, et al. Antitumor activity in melanoma and anti-self responses in a phase I trial with the anti-cytotoxic T lymphocyte-associated antigen 4 monoclonal antibody CP-675,206. *J Clin Oncol*. Dec 10 2005;23(35):8968-77. doi:10.1200/jco.2005.01.109
174. Larkin J, Chiarion-Sileni V, Gonzalez R, et al. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med*. Jul 2 2015;373(1):23-34. doi:10.1056/NEJMoa1504030
175. Robert C, Schachter J, Long GV, et al. Pembrolizumab versus Ipilimumab in Advanced Melanoma. *N Engl J Med*. Jun 25 2015;372(26):2521-32. doi:10.1056/NEJMoa1503093
176. Schmid P, Adams S, Rugo HS, et al. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. *N Engl J Med*. Nov 29 2018;379(22):2108-2121. doi:10.1056/NEJMoa1809615
177. Gordon SR, Maute RL, Dulken BW, et al. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature*. May 25 2017;545(7655):495-499. doi:10.1038/nature22396
178. Peranzoni E, Lemoine J, Vimeux L, et al. Macrophages impede CD8 T cells from reaching tumor cells and limit the efficacy of anti-PD-1 treatment. *Proc Natl Acad Sci U S A*. Apr 24 2018;115(17):E4041-e4050. doi:10.1073/pnas.1720948115
179. Wolchok JD, Chiarion-Sileni V, Gonzalez R, et al. Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med*. Oct 5 2017;377(14):1345-1356. doi:10.1056/NEJMoa1709684
180. Overman MJ, Lonardi S, Wong KYM, et al. Durable Clinical Benefit With Nivolumab Plus Ipilimumab in DNA Mismatch Repair-Deficient/Microsatellite

Instability-High Metastatic Colorectal Cancer. *J Clin Oncol*. Mar 10 2018;36(8):773-779. doi:10.1200/jco.2017.76.9901

181. Socialstyreslsen. Statistik um nyupptäckta cancerfall 2019. 2020;
182. Cancerfonden S-. Cancer i siffror 2018. 2019;2018-6-10
183. Miki Y, Swensen J, Shattuck-Eidens D, et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science*. Oct 7 1994;266(5182):66-71. doi:10.1126/science.7545954
184. Wooster R, Bignell G, Lancaster J, et al. Identification of the breast cancer susceptibility gene BRCA2. *Nature*. Dec 21-28 1995;378(6559):789-92. doi:10.1038/378789a0
185. Reeder JG, Vogel VG. Breast cancer prevention. *Cancer Treat Res*. 2008;141:149-64. doi:10.1007/978-0-387-73161-2_10
186. Shuen AY, Foulkes WD. Inherited mutations in breast cancer genes--risk and response. *J Mammary Gland Biol Neoplasia*. Apr 2011;16(1):3-15. doi:10.1007/s10911-011-9213-5
187. Cancer CGoHFiB. Breast cancer and hormone replacement therapy: collaborative reanalysis of data from 51 epidemiological studies of 52,705 women with breast cancer and 108,411 women without breast cancer. Collaborative Group on Hormonal Factors in Breast Cancer. *Lancet*. Oct 11 1997;350(9084):1047-59.
188. Hulka BS, Moorman PG. Breast cancer: hormones and other risk factors. *Maturitas*. Sep-Oct 2008;61(1-2):203-13; discussion 213. doi:10.1016/j.maturitas.2008.11.016
189. McDonald JA, Goyal A, Terry MB. Alcohol Intake and Breast Cancer Risk: Weighing the Overall Evidence. *Curr Breast Cancer Rep*. Sep 2013;5(3)doi:10.1007/s12609-013-0114-z
190. Nazari SS, Mukherjee P. An overview of mammographic density and its association with breast cancer. *Breast Cancer*. May 2018;25(3):259-267. doi:10.1007/s12282-018-0857-5
191. Cleary MP, Grossmann ME. Minireview: Obesity and breast cancer: the estrogen connection. *Endocrinology*. Jun 2009;150(6):2537-42. doi:10.1210/en.2009-0070
192. Javed A, Lteif A. Development of the human breast. *Semin Plast Surg*. Feb 2013;27(1):5-12. doi:10.1055/s-0033-1343989
193. Goff SL, Danforth DN. The Role of Immune Cells in Breast Tissue and Immunotherapy for the Treatment of Breast Cancer. *Clin Breast Cancer*. Feb 2021;21(1):e63-e73. doi:10.1016/j.clbc.2020.06.011
194. McGee SF, Lanigan F, Gilligan E, Groner B. Mammary gland biology and breast cancer. Conference on Common Molecular Mechanisms of Mammary Gland Development and Breast Cancer Progression. *EMBO Rep*. Nov 2006;7(11):1084-8. doi:10.1038/sj.embor.7400839
195. Robbins SLKVCRS. Robbins and Cotran pathologic basis of disease. 2010;
196. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*. Nov 1991;19(5):403-10. doi:10.1111/j.1365-2559.1991.tb00229.x

197. Alkabban FM, Ferguson T. Breast Cancer. *StatPearls*. StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.; 2021.
198. Tonellotto F, Bergmann A, de Souza Abrahão K, de Aguiar SS, Bello MA, Thuler LCS. Impact of Number of Positive Lymph Nodes and Lymph Node Ratio on Survival of Women with Node-Positive Breast Cancer. *Eur J Breast Health*. Apr 2019;15(2):76-84. doi:10.5152/ejbh.2019.4414
199. Sørlie T, Perou CM, Tibshirani R, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A*. Sep 11 2001;98(19):10869-74. doi:10.1073/pnas.191367098
200. Cheang MC, Chia SK, Voduc D, et al. Ki67 index, HER2 status, and prognosis of patients with luminal B breast cancer. *J Natl Cancer Inst*. May 20 2009;101(10):736-50. doi:10.1093/jnci/djp082
201. Mitri Z, Constantine T, O'Regan R. The HER2 Receptor in Breast Cancer: Pathophysiology, Clinical Use, and New Advances in Therapy. *Chemother Res Pract*. 2012;2012:743193. doi:10.1155/2012/743193
202. Rakha EA, Reis-Filho JS, Ellis IO. Basal-like breast cancer: a critical review. *J Clin Oncol*. May 20 2008;26(15):2568-81. doi:10.1200/jco.2007.13.1748
203. Fougner C, Bergholtz H, Norum JH, Sørlie T. Re-definition of claudin-low as a breast cancer phenotype. *Nat Commun*. Apr 14 2020;11(1):1787. doi:10.1038/s41467-020-15574-5
204. Maughan KL, Lutterbie MA, Ham PS. Treatment of breast cancer. *Am Fam Physician*. Jun 1 2010;81(11):1339-46.
205. Saini KS, Taylor C, Ramirez AJ, et al. Role of the multidisciplinary team in breast cancer management: results from a large international survey involving 39 countries. *Ann Oncol*. Apr 2012;23(4):853-9. doi:10.1093/annonc/mdr352
206. Moo TA, Sanford R, Dang C, Morrow M. Overview of Breast Cancer Therapy. *PET Clin*. Jul 2018;13(3):339-354. doi:10.1016/j.cpet.2018.02.006
207. Beniey M, Haque T, Hassan S. Translating the role of PARP inhibitors in triple-negative breast cancer. *Oncoscience*. Jan 2019;6(1-2):287-288. doi:10.18632/oncoscience.474
208. Kwapisz D. Pembrolizumab and atezolizumab in triple-negative breast cancer. *Cancer Immunol Immunother*. Mar 2021;70(3):607-617. doi:10.1007/s00262-020-02736-z
209. Mittendorf EA, Philips AV, Meric-Bernstam F, et al. PD-L1 expression in triple-negative breast cancer. *Cancer Immunol Res*. Apr 2014;2(4):361-70. doi:10.1158/2326-6066.Cir-13-0127
210. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ. Strategies for subtypes--dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. *Ann Oncol*. Aug 2011;22(8):1736-47. doi:10.1093/annonc/mdr304
211. Laoui D, Movahedi K, Van Overmeire E, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions. *Int J Dev Biol*. 2011;55(7-9):861-7. doi:10.1387/ijdb.113371dl

212. Stossi F, Madak-Erdoğan Z, Katzenellenbogen BS. Macrophage-elicited loss of estrogen receptor-α in breast cancer cells via involvement of MAPK and c-Jun at the ESR1 genomic locus. *Oncogene*. Apr 5 2012;31(14):1825-34. doi:10.1038/onc.2011.370

213. Lindsten T, Hedbrant A, Ramberg A, et al. Effect of macrophages on breast cancer cell proliferation, and on expression of hormone receptors, uPAR and HER-2. *Int J Oncol*. Jul 2017;51(1):104-114. doi:10.3892/ijo.2017.3996

214. Asano T, Ohnishi K, Shiota T, et al. CD169-positive sinus macrophages in the lymph nodes determine bladder cancer prognosis. *Cancer Sci*. May 2018;109(5):1723-1730. doi:10.1111/cas.13565

215. Ohnishi K, Komohara Y, Saito Y, et al. CD169-positive macrophages in regional lymph nodes are associated with a favorable prognosis in patients with colorectal carcinoma. *Cancer Sci*. Sep 2013;104(9):1237-44. doi:10.1111/cas.12212

216. Ohnishi K, Yamaguchi M, Erdenebaatar C, et al. Prognostic significance of CD169-positive lymph node sinus macrophages in patients with endometrial carcinoma. *Cancer Sci*. Jun 2016;107(6):846-52. doi:10.1111/cas.12929

217. Strömvall K, Sundkvist K, Ljungberg B, Halin Bergström S, Bergh A. Reduced number of CD169(+) macrophages in pre-metastatic regional lymph nodes is associated with subsequent metastatic disease in an animal model and with poor outcome in prostate cancer patients. *Prostate*. Nov 2017;77(15):1468-1477. doi:10.1002/pros.23407

218. Shiota T, Miyasato Y, Ohnishi K, et al. The Clinical Significance of CD169-Positive Lymph Node Macrophage in Patients with Breast Cancer. *PLoS One*. 2016;11(11):e0166680. doi:10.1371/journal.pone.0166680

219. Cassetta L, Fragkogianni S, Sims AH, et al. Human Tumor-Associated Macrophage and Monocyte Transcriptional Landscapes Reveal Cancer-Specific Reprogramming, Biomarkers, and Therapeutic Targets. *Cancer Cell*. Apr 15 2019;35(4):588-602.e10. doi:10.1016/j.ccell.2019.02.009

220. Shaabani N, Duhan V, Khairnar V, et al. CD169(+) macrophages regulate PD-L1 expression via type I interferon and thereby prevent severe immunopathology after LCMV infection. *Cell Death Dis*. Nov 3 2016;7(11):e2446. doi:10.1038/cddis.2016.350

221. Ryden L, Jonsson PE, Chebil G, et al. Two years of adjuvant tamoxifen in premenopausal patients with breast cancer: a randomised, controlled trial with long-term follow-up. *Eur J Cancer*. Jan 2005;41(2):256-64. doi:10.1016/j.ejca.2004.06.030

222. Group SBCC. Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer. Swedish Breast Cancer Cooperative Group. *J Natl Cancer Inst*. Nov 6 1996;88(21):1543-9. doi:10.1093/jnci/88.21.1543

223. Chebil G, Bendahl PO, Idvall I, Ferno M. Comparison of immunohistochemical and biochemical assay of steroid receptors in primary breast cancer--clinical associations and reasons for discrepancies. *Acta Oncol*. 2003;42(7):719-25. doi:10.1080/02841860310004724

Paper I

Experimental Cell Research 390 (2020) 111932

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Inflammatory macrophage derived TNFα downregulates estrogen receptor α via FOXO3a inactivation in human breast cancer cells

Frida Björk Gunnarsdóttir[a], Catharina Hagerling[a,b], Caroline Bergenfelz[a], Meliha Mehmeti[a], Eva Källberg[a], Roni Allaoui[a], Sofie Mohlin[c], Sven Påhlman[d], Christer Larsson[d], Karin Jirström[e], Daniel Bexell[d], Karin Leandersson[a,*]

[a] Cancer Immunology, Department of Translational Medicine, 214 28, Malmö, Lund University, Sweden
[b] Division of Clinical Genetics, Department of Laboratory Medicine Lund, Lund University, 221 00, Lund, Sweden
[c] Division of Pediatrics, Department of Clinical Sciences Lund, Lund University, 221 00, Lund, Sweden
[d] Translational Cancer Research, TCR, Medicon Village, Lund University, 221 00, Lund, Sweden
[e] Division of Oncology and Pathology, Department of Clinical Sciences, Lund University, 221 00, Lund, Sweden

ARTICLE INFO

Keywords:
Breast cancer
Macrophage
TNFalpha
Estrogen receptor
FOXO3a
Endocrine resistance

ABSTRACT

Patients with estrogen receptor α positive (ERα$^+$) breast cancer can respond to endocrine therapy, but treatment resistance is common and associated with downregulation of ERα expression in the dormant residual cells. Here we show, using long-term NSG xenograft models of human breast cancer and primary human monocytes, *in vitro* primary cell cultures and tumors from breast cancer patients, that macrophage derived tumor necrosis factor alpha (TNFα) downregulates ERα in breast cancer cells via inactivation of the transcription factor Forkhead box O transcription factor 3a (FOXO3a). Moreover, presence of tumor associated macrophages in the primary tumor of breast cancer patients, was associated with ERα negativity, and with worse prognosis in patients with ERα$^+$ tumors. We propose that pro-inflammatory macrophages, despite being tumoricidal, may have direct effects on tumor progression and endocrine resistance in breast cancer patients. Our findings suggest that TNFα antagonists should be evaluated for treatment of ERα$^+$ breast cancer.

1. Introduction

Breast cancers are divided into different subtypes based on receptor status (Estrogen Receptor α [ER], Progesterone Receptor [PR] and Human Epidermal growth factor Receptor 2 [HER2]). The luminal A subtype of breast cancer (ER$^+$PR$^+$HER2$^-$Ki67^{low}; according to the St Gallen molecular classification [1]) is most often associated with a good prognosis, partly because endocrine therapy can be used to target the ERα signaling pathway, while the triple negative breast cancers (TNBC; ER$^-$PR$^-$HER2$^-$) have a poor prognosis [2,3]. Different breast cancer subtypes tend to metastasize with varying aggressiveness, a process that often is associated with epithelial mesenchymal transition (EMT) [4]. For patients with ERα$^+$ breast cancer, resistance to endocrine therapy is commonly caused by downregulation of ERα in the disseminated cancer cells [2,3].

Myeloid immune cells are known to affect tumor development and progression, and infiltration of tumor associated macrophages is therefore associated with a worse prognosis [5]. Conceptually however, macrophages can be both beneficial for the patient (pro-inflammatory macrophages [CD68$^+$ CD163$^-$]); by eliminating cancer cells and activating anti-tumor immune responses, or detrimental for the patient (anti-inflammatory macrophages [CD68$^+$ CD163$^+$] or myeloid suppressor cells [CD68$^-$CD163$^+$]); by promoting tumor progression, inhibiting pro-inflammatory immune responses, and inducing wound healing reactions such as angiogenesis and matrix degradation, all of which promote metastatic spread [6–8].

Forkhead box O transcription factors (FOXO) are a subgroup of Forkhead box (FOX) transcription factors that are involved in normal physiological as well as pathological processes [9,10]. FOXO proteins (FOXO1, 3a, 4 and 6) are tumor-suppressors that are regulated by the

Abbreviations: EMT, Epithelial mesenchymal transition; ERα, estrogen receptor α; FOXO3a, Forkhead box O3; M0, unpolarized primary human macrophages; M1, pro-inflammatory macrophages; M2, anti-inflammatory macrophages; TMA, tissue microarray; TNBC, Triple negative breast cancer; TNFα, tumor necrosis factor alpha

* Corresponding author. Cancer Immunology, Department for Translational Medicine, Clinical Research Center, Jan Waldenströmsg 35, Lund University, SE-214 28, Malmö, Sweden.
E-mail address: Karin.Leandersson@med.lu.se (K. Leandersson).

https://doi.org/10.1016/j.yexcr.2020.111932
Received 4 December 2019; Received in revised form 24 February 2020; Accepted 27 February 2020
Available online 04 March 2020

PI3K/Akt pathway and are involved in crucial processes such as apoptosis, cell-cycle arrest, metabolism and differentiation [9,10]. In breast cancer, inactivated FOXO3a that is sequestered in the cytoplasm, has been linked to both EMT and aggressive breast cancer subtypes [11–14]. In line with this, FOXO3a has been shown to inhibit breast tumor growth, angiogenesis, invasion and metastasis [9,10]. Lately, FOXO3a has also been suggested to be involved in endocrine resistance [10]. A functional interaction between FOXO proteins and ERα signaling was proposed, mainly as mediators in cross-talks of ER and growth factor receptor signaling [12,15], although FOXO3a induced *ESR1* transcription has also been proposed [14].

We hypothesized that primary human macrophages can modify ERα expression in human breast cancer cells *in vivo*. We show that co-transplantation of primary human monocytes together with ERα$^+$ breast cancer cells in NSG mice induced a long-term significant downregulation of ERα expression. This was supported by similar findings *in vitro*, where primary human monocyte derived macrophage cultures also downregulated ERα in human breast cancer cells. We further show that the mechanism behind the downregulation was pro-inflammatory macrophage derived TNFα. The molecular mechanism was TNFα-induced Akt, that inhibited and sequestered FOXO3a in the cytoplasm of the breast cancer cells *in vivo*. The inhibition of FOXO3a lead to lack of *ESR1* (ERα) transcription. Our findings were supported by clinical specimens, where the presence of CD68$^+$ macrophages in the primary tumor of breast cancer patients was associated with ERα negativity, and to worse prognosis primarily in ERα$^+$ breast cancer patients. We propose that pro-inflammatory macrophages, despite being tumoricidal, may have direct effects on tumor progression and metastasis as well as on endocrine resistance in breast cancer patients.

2. Materials and methods

2.1. Ethical approval

Ethical permit was obtained from the regional ethical committee at Lund University (Dnr 613/02, Dnr 2012/689, Dnr 2014/669, Dnr 2017/949) whereby written consent was given, or when not required patients were offered the option to opt out, as approved by the regional ethical committee at Lund University, according to the Declaration of Helsinki. The NSG models (approvals M11-15) were approved by the regional ethics committee for animal research at Lund University, Sweden.

2.21. Isolation of primary human monocytes and macrophage differentiation

Concentrated leukocytes were obtained from healthy donors. PBS containing 5 mM EDTA and 2.5% w/v sucrose was used to dilute the concentrated leukocytes and Ficoll-Paque Plus (GE Healthcare Bio-sciences, Uppsala, Sweden) gradient used to isolate peripheral blood mononuclear cells (PBMCs). Monocytes were isolated from PBMCs by magnetic cell sorting (MACS) using Monocyte Isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. M0/M1/M2 monocyte-derived macrophages were generated as previously described [16] and flow cytometry used to verify polarization using CD14, CD86, CD206, HLADR antibodies (BD Biosciences). All cytokines used in differentiation cultures or stimulation cultures were from R&D Systems. Conditioned media (CM) was harvested and stored at −80 °C. Human Inflammatory Cytokine bead array (CBA) was used to measure cytokines secreted by macrophages (BD Biosciences). Samples were analyzed using a FACSVerse (BD Biosciences). TGFβ was measured using Human TGF-beta 1 quantikine ELISA kit (R&D systems).

2.3. In vitro cultures

MCF-7 and T47D (ERα$^+$) and MDA-MB-231 (TNBC) breast cancer cells were purchased from and characterized by ATCC and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Biosera, Boussens, France), 1% sodium pyruvate (Hyclone), 1% HEPES (Hyclone) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). *Mycoplasma* testing was performed as a routine. Thawing of cells were done from low passages of the original expanded ATCC vials. For macrophage effect study, cancer cells were cultured for 48 h in M0, M1 or M2 CM. Control samples were cultured in Opti-MEM medium supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The Akt inhibitor MK-2206 was obtained from Selleckchem (Houston, TX, US) and reconstituted in DMSO. MK-2206 was added (2,5 μM/well) for 48 h with DMSO added to control wells. TNFα inhibitor Etanercept (Merck) was added (10 μg/ml) with Opti-MEM medium 30 min before CM addition. For cytokine stimulation, cancer cells were serum starved for 12 h after which the following cytokines were added: IL8 (100 ng/ml), IL1β (25 ng/ml), IL6 (50 ng/ml), IL10 (10 ng/ml) and TNFα (25 ng/ml). The control samples were treated identically but left unstimulated.

2.4. Western blots

All antibodies used for Western blot are shown in Supplementary table 1 (clone; dilution; company). Antibodies used for detection of proteins of interest were: ERα, anti-FOXO3a that detects total FOXO3a expression, anti-FOXO3a (phospho S253) that only detects FOXO3a phosphorylated at Serine 253, anti-ERK1/2, anti-phospho ERK/P-p44/42 MAPK, anti-Akt pan, anti-phospho Akt S473, anti-CAR, and anti-E-Cadherin. For housekeeping control, β-Actin and GAPDH antibodies were used.

2.5. siRNA transfections

Transient siRNA transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). In all, 30 nM of Silencer Select Negative Control #2 and siFOXO3 (s5262) (ThermoFisher) were used. All analyses were performed 72 h post transfection.

2.6. RNA extraction, cDNA synthesis and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from monocytes, M0, M1 and M2 macrophages harvested in TRIzol™ Reagent according to manufacturer's protocol for cells grown in monolayer. Total RNA was extracted from breast cancer cells using RNeasy Plus Mini Kit (Qiagen) according to manufacturer's protocol. qRT–PCR was performed in triplicates using Maxima SYBR Green/Rox (Thermo Scientific) according to manufacturer's instructions. qRT–PCR analysis was performed on the Mx3005 P QPCR system (Agilent Technologies, Santa Clara, CA, USA) and the relative mRNA expression was normalized to *ACTB*, *GAPDH* and *SDHA* and calculated using the comparative Ct method. List of primer sequences can be found in Supplementary Table 2.

2.7. Gene expression analyses

The publicly available database R2: microarray analysis and visualization platform [17]; TCGA 1097 was used for gene expression profile analyses. For breast cancer subtype RNA expression profiles Metabric was used [18,19].

2.8. Animal procedures and xenografts

Female 8-week old NSG-mice (NOD.Cg-*Prkdc(scid)Il2rg(tm1Wji)*/

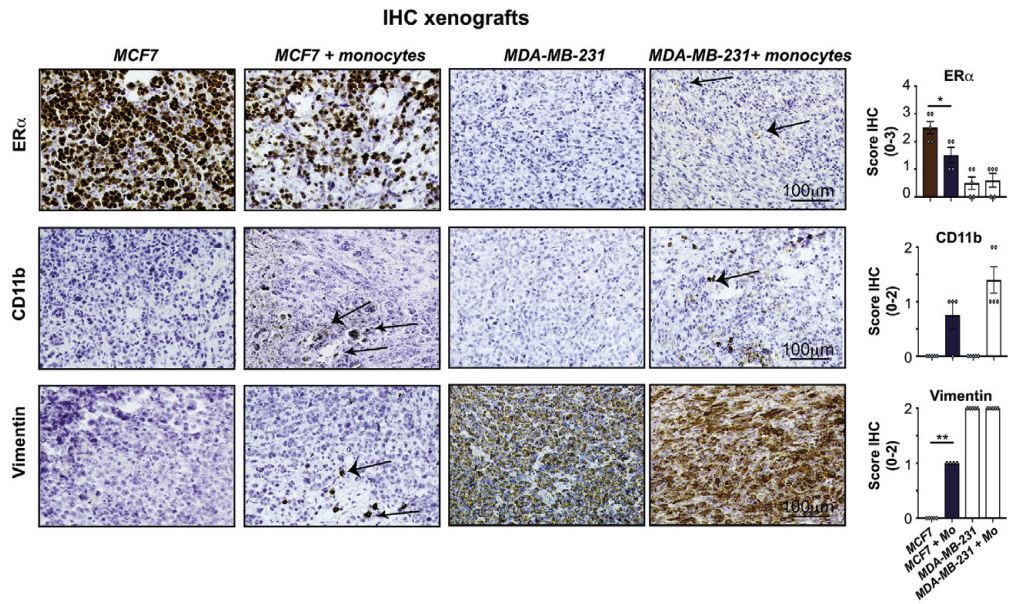


Fig. 1. Downregulation of ERα in breast cancer cells by primary human macrophages *in vivo*. Primary human monocytes were long term co-transplanted together with $ERα^+$ (MCF-7 for 90 days) or ER^- (MDA-MB-231 for 21 days) breast cancer cell lines in NSG mice [20]. Controls were only MCF-7 or MDA-MB-231 transplanted cells. The xenografts were resected and immunohistochemistry was performed. A significant downregulation of ERα was seen in the MCF-7 + monocytes xenografts. Myeloid cells were detected using CD11b (MCF-7 + monocytes and MDA-MB-231 + monocytes) and Vimentin (MCF-7 + monocytes). Histograms represent the mean score of the xenografts for each staining. Representative pictures are shown. Unpaired *t*-test (Vimentin Mann Whitney *t*-test; due to identical numbers). Error bars indicate SEM. N = 5 for each group. *p < 0.05, **p < 0.01, ***p < 0.001.

SzJ strain, The Jackson Laboratory, Maine, USA) were housed in a controlled environment and all procedures were approved by the regional ethics committee for animal research at Lund University, Sweden (M11-15). Mice were anesthetized by isoflurane and injected with human breast cancer cells (MCF-7, T47D or MDA-MB-231 cells (1×10^6 cells)) on the right flank, alone or in combination with primary human monocytes (1×10^6 cells/mouse) as previously described [20]. For sacrifice, mice were anesthetized by isoflurane and euthanized by cervical dislocation. Tumors were excised on day 21 (for T47D, MCF-7 and TNBC MDA-MB-231 cells) after injection, or for long-term evaluation on day 90 (for MCF-7 and MCF-7 + primary human monocytes) and subsequently fixed in 4% paraformaldehyde and embedded in paraffin. Five (N = 5) mice were used in each group except for 21 d MCF-7 and 21 d MCF-7 + primary human monocytes (N = 3). The animal work was performed in accordance with the ARRIVE reporting guidelines.

2.9. Breast cancer patients and samples

The breast cancer study cohort has been previously described [21–23] and included 498 patients who were diagnosed with invasive breast cancer between January 1, 1988 and December 31, 1992 at the Department of Pathology, Skåne University Hospital, Malmö. Ethical approval for the primary breast cancer cohort study was obtained from the Ethics Committee at Lund University (Dnr 613/02). Informed consent was not required and patients were offered the option to opt out.

2.10. TMA

Tissue microarrays (TMA) were constructed as previously described [21–23]. Analysis of ER, PR and HER2 status of the tumors in the TMA, was performed according to current Swedish guidelines. CD68 had been scored previously [24]. Pearson Chi square-test was used for crosstabs. Kaplan-Meier analysis was used to evaluate the impact of a low macrophage infiltration (CD68 *low*) or high macrophage infiltration (CD68 *high*) on breast cancer recurrence free survival (RFS) in patients with ERα negative tumors and ERα positive tumors respectively. Log rank test was applied to analyze any significant differences in Kaplan-Meier survival plots. All P values were two-tailed. $p \leq 0.05$ was considered significant. All calculations and statistical analyses were performed with IBM SPSS Statistics version 23.0 (SPSS Inc).

2.11. Immunohistochemistry

Antibodies used were: anti-CD68, anti-ERα, anti-E-cadherin, anti-CD11b (specific for human), anti-vimentin, anti-P-FOXO3a (phospho S253). All primary antibodies used for IHC are shown in Supplementary table 3 (clone; dilution; company). The E-cadherin and ERα staining was scored by intensity (0–3). In the primary human breast cancers, expression of ERα in tumor cells and of CD68 in immune cells had been annotated previously [22–24].

2.12. Statistics

Analysis of variance (ANOVA) or t-tests according to figure legends, was performed for the *in vitro* experiments using the Graph Pad Prism

software. For the primary human breast cancer cohort, and Pearson Chi Square tests for E-cadherin/CD11b cross tabular IHC NSG expression, calculations were performed with IBM SPSS Statistics version 23.0 (SPSS Inc). All statistical tests were two sided and $p \leq 0.05$ was considered significant. Correlations between TNF and ESR1 expressed in the human breast cancer 1097 TCGA database was performed via R2: microarray analysis and visualization platform http://r2.amc.nl. For breast cancer subtype RNA expression profiles Metabric was used [18,19].

3. Results

3.1. Primary human monocytes downregulate ERα in cancer cells xenografts

To investigate what specific long term effects primary human macrophages might have on ERα$^+$ human breast cancer cells *in vivo*, we co-transplanted primary human monocytes together with ERα$^+$-breast cancer cells (MCF-7) for 90 days in a previously described NSG xenograft model from our group [20]. One of the questions we asked was whether primary human macrophages would downregulate ERα in human breast cancer cells *in vivo*. The intensity levels of ERα were significantly reduced in the tumors with co-transplanted primary human monocytes (a mixed population of MCF-7 cells and primary human monocytes were injected, and tumors resected at 90 days), as compared to the xenografts without (MCF-7; 90 days) (Fig. 1). As control xenografts, we used a TNBC cell line lacking ERα (MDA-MB-231; resected at 21 days for ethical reasons), and as shown in Fig. 1 the lack of ERα in the control TNBC xenografts remained unaffected by co-transplanting primary human monocytes, although the transplanted monocytes themselves expressed ERα as previously published [25] and verified by the myeloid human specific CD11b marker (arrows Fig. 1). Vimentin was also used to detect the human myeloid cells (arrows Fig. 1), as we have previously shown that transplanted monocytes specifically express vimentin in MCF-7 xenografts [20]. Vimentin is constitutively expressed in MDA-MB-231 cells. Despite the slow growth, MCF-7 xenografts at 21 d, as well as another ERα$^+$ cell line T47D, were performed as controls for 21 d, with similar but non-significant effect on ERα expression (Supplementary Figure 1A).

3.2. Macrophages induce ERα-downregulation in breast cancer cells in vitro through TNFα

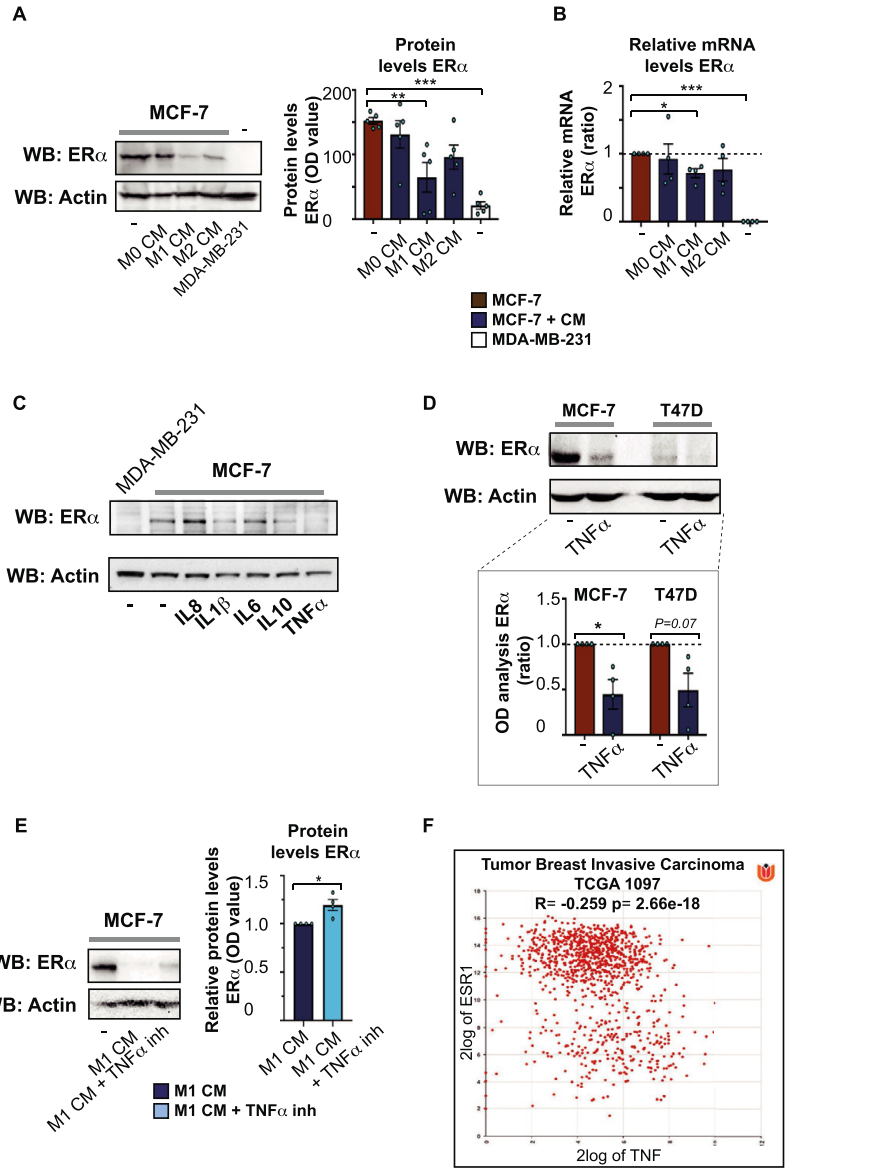
To elaborate on the finding that primary human macrophages may affect the ERα expression of breast cancer cells *in vivo*, we next set out to investigate a possible molecular mechanism. We therefore cultured primary human monocyte derived macrophages differentiated into either an M0 (unpolarized), M1 (pro-inflammatory) or an M2 (anti-inflammatory) phenotype, harvested the supernatant (conditioned media; CM), and cultured MCF-7 breast cancer cells in the media for 48 h. As shown in Fig. 2a, ERα was clearly downregulated both at the protein (Fig. 2a) and mRNA levels (Fig. 2b), by soluble mediators produced by the M1 pro-inflammatory macrophages, but only slightly by M0 or M2 macrophages. Different macrophage subtypes secrete overlapping but also unique mediators, and to test which mediators were selectively secreted by the M1 pro-inflammatory macrophages in our experiments, we investigated protein levels of some of the most common macrophage-derived cytokines IL8, IL1β, IL6, IL10 and TNFα. Both M1 and M2 macrophages secreted IL8, IL1β, IL6 and IL10, but only M1 macrophages secreted TNFα (Supplementary Fig. 1b). To investigate whether any of the cytokines analyzed could have a direct effect on ERα expression levels, we next stimulated MCF-7 cells with the corresponding recombinant cytokines for 48 h (Fig. 2c). A clear downregulation of ERα in MCF-7 cells was observed by TNFα (Fig. 2c and d) and a slight downregulation by IL1β (Fig. 2c). This was confirmed using another ERα$^+$ breast cancer cell line (T47D; Fig. 2d) although with larger variations between experiments, and by adding the TNFα antagonist Etanercept that hindered the M1 CM induced ER downregulation (Fig. 2e). We also investigated whether *TNF* expression in human primary breast cancers correlated to *ESR1* (ERα) expression using the publicly available database R2 (TCGA 1097; http://r2.amc.nl) (Fig. 2f). Indeed, a significant inverse correlation between *TNF* and *ESR1* was present in primary human breast cancers (R = −0.259; P = 2.66e-18). In summary, pro-inflammatory primary human macrophages have the potential to downregulate ERα in human breast cancer cells via secreted TNFα.

3.3. Macrophage derived TNFα phosphorylates FOXO3a leading to ERα-downregulation

We wanted to understand the molecular mechanism of how TNFα can promote ERα-downregulation in breast cancer cells. Analyses of signals downstream of TNFα in the breast cancer cells indicated that the Akt pathway was specifically activated by pro-inflammatory M1 CM (Fig. 3a) rather than the MAPK/ERK pathway (Supplementary Fig. 1c). We therefore searched for transcription factors involved in *ESR1*-regulation and found FOXO3a, that in its un-phosphorylated form is active in the nucleus transcribing *ESR1* [11,14], but upon P-Akt-induced phosphorylation of Ser253 [26,27], or IκB induced phosphorylation of Ser 644 [12], is inactivated and sequestered in the cytoplasm. We confirmed that the M1 CM induced downregulation of ERα was relieved by addition of an Akt inhibitor (Fig. 3b), and that indeed TNFα was able to induce phosphorylation of FOXO3a also in MCF-7 cells (Fig. 3c), while IL1β was not (Supplementary Fig. 1d). The total levels of FOXO3a was slightly higher at both mRNA (Fig. 3d) and protein (Supplementary Fig. 1e) level in the MCF-7 breast cancer cell line, and slightly higher in the M1 CM treated MCF-7 cells (Fig. 3d and Supplementary Fig. 1e), also in combination with the Akt inhibitor (Fig. 3b). However, studying a large cohort of breast cancer signatures using Metabric [18,19] (Fig. 3e) we could not confirm a higher level of total *FOXO3* in any breast cancer subtype, which is in sharp contrast to the *FOXA1* gene that represents one of the molecular hallmarks for ERα$^+$ breast cancers (Fig. 3d–e). Silencing of total *FOXO3* in MCF-7 cells confirmed that FOXO3a expression correlates with *ESR1* transcription (Fig. 3f). We finally stained representative xenografts with an antibody specific for P-Ser253-FOXO3a, and found a clear pattern of P-Ser253-FOXO3a in the cytoplasm primarily in xenografts with co-transplanted primary human monocytes (Fig. 3g). Together this indicates that TNFα produced by pro-inflammatory human macrophages induce activation of the Akt pathway, that subsequently phosphorylates Ser253-FOXO3a thus sequestering it to the cytoplasm, leading to a reduced transcription of *ESR1* (ERα).

3.4. FOXO3a induced ERα downregulation is associated with EMT traits

ERα negativity has previously been associated with downregulation of E-cadherin and silencing of ERα has even been shown to induce EMT [28–30]. Furthermore, indications that anti-inflammatory macrophages can induce EMT in cancer cells has been raised [31,32], but also that pro-inflammatory mediators like TNFα can induce EMT [33,34]. Recently, downregulation of FOXO3a was shown to promote EMT in pancreatic ductal adenocarcinoma via SPRY2 [35]. We also found EMT associated changes in relation to the TNFα-FOXO3a induced ERα downregulation, both *in vitro* and *in vivo*. As shown in Supplementary Fig. 2a, the EMT regulated proteins E-cadherin (Supplementary Fig. 2a) and Coxsackie and Adenovirus Receptor (CAR) (Supplementary Fig. 2b) were downregulated in MCF-7 cells under M1 CM conditions. Silencing of total *FOXO3* in MCF-7 cells, which decreased *ESR1* transcription (Fig. 3f), also lead to an increase in *SNAI2* (Slug), but to decreased *TWIST1* and *SNAI1* (Supplementary Fig. 2c). TGFβ was not induced in our primary macrophage cultures (Supplementary Fig. 2d). In xenografts co-transplanted with primary human monocytes, E-cadherin was



(caption on next page)

also downregulated, although not significantly as compared to the xenografts without primary human monocytes, (Supplementary Fig. 2e). Low E-cadherin expression levels did however correlate significantly to high presence of primary human monocytes ($P = 0.046$; Supplementary Fig. 2F), also visualized using double IHC staining of E-cadherin and CD11b (Supplementary Fig. 2G). Thus, the macrophage derived TNFα that causes downregulation of ERα, might eventually have severe consequences on breast cancer progression by Slug induced EMT traits.

Fig. 2. Downregulation of ERα in breast cancer cells by primary human macrophages *in vitro*. (A) ERα$^+$ human breast cancer cells (MCF-7) were cultured in supernatants (conditioned media [CM]) generated from *in vitro* cultures of human macrophages of M0, M1 and M2 type. The Western blot shows a representative experiment. The histogram represents the OD values of the ERα bands in order to visualize the ERα protein downregulation, compared to the untreated MCF-7 cells or untreated MDA-MB-231 as a negative control. One-way ANOVA multiple comparison Holm-Sidaks' test. N = 5. (B) The histogram represents the relative mRNA levels of *ESR1* (ERα), compared to the untreated MCF-7 cells or untreated MDA-MB-231 as a negative control. N = 4. Ratio Paired *t*-test. (C) The corresponding recombinant cytokines were tested to analyze their individual ability to downregulate ERα. The cytokines were added for 48 h at the concentrations: IL8 (100 ng/ml), IL1β (25 ng/ml), IL6 (50 ng/ml), IL10 (10 ng/ml) and TNFα (25 ng/ml). Control samples were treated identically but left unstimulated. The blot is a representative experiment. (D) TNFα downregulates ERα in two different ERα$^+$ breast cancer cell lines; MCF-7 and T47D. The blot is a representative experiment. The lower histograms represent the relative OD values of the ERα bands in order to visualize the ERα downregulation. N = 4. Paired *t*-test. (E) A TNFα inhibitor (Etanercept) reverses the M1 CM caused ERα downregulation in MCF-7 cells. The blot is a representative experiment. The histogram represents the relative OD values of the ERα bands in order to visualize the ERα upregulation. N = 4. Ratio Paired *t*-test. (F) Correlation between TNF and ESR1 expression in primary human breast cancers using the publicly available database R2: microarray analysis and visualization platform [17] (TCGA 1097; R = −259, p = 2.66e-18). Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

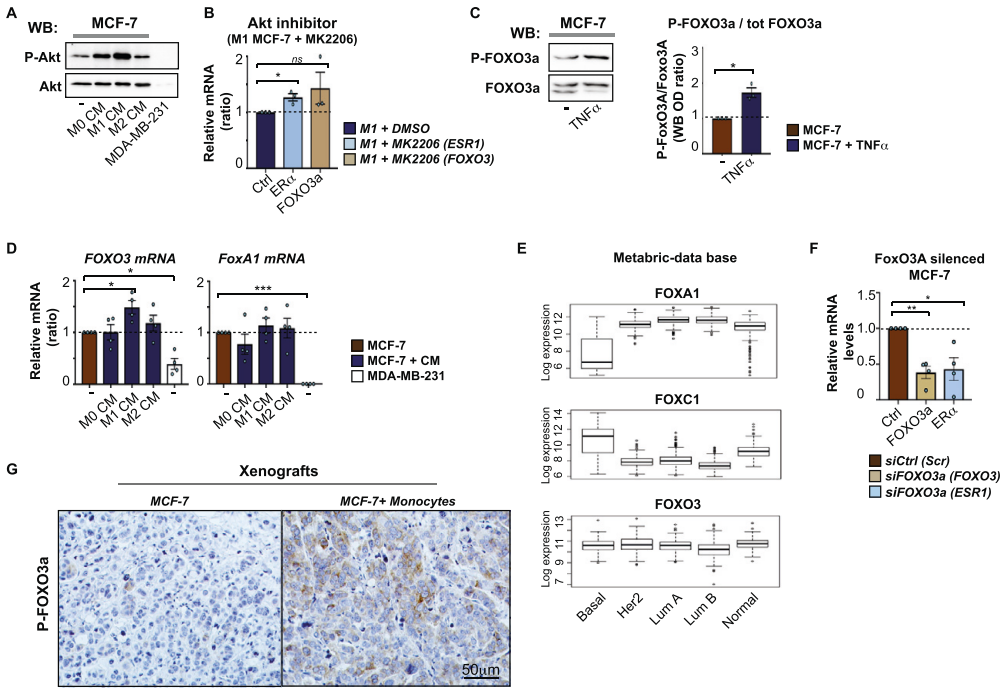


Fig. 3. FOXO3a in breast cancer cells is inhibited by TNFα induced Akt phosphorylation. (A) CM generated from primary human M1 macrophages induce phosphorylation of Akt in MCF-7 cells, but not as much from cultures of M0 and M2 type human macrophages. The Western blot shows a representative experiment. (B) *ESR1* expression (ERα) is significantly increased in MCF-7 cells treated with M1 CM when the Akt pathway is inhibited. The histogram represents the relative mRNA levels of ERα in MCF-7 cells cultured in M1 CM, with (MK2206) or without (DMSO) Akt-inhibition. N = 3. Ratio Paired *t*-test. (C) Recombinant TNFα induced phosphorylation of FOXO3a in human breast cancer MCF-7 cells. The Western blot shows a representative experiment. The histogram represents the relative OD values of the Ser253 P-FOXO3a bands in relation to total FOXO3a, in order to visualize the phosphorylation, and compared to the untreated MCF-7 cells as a negative control. Paired Ratio *t*-test. N = 3. (D) The relative mRNA levels of *FOXO3* and *FOXA1* in MCF-7 cells cultured in M0, M1 or M2 CM, compared to the untreated MCF-7 cells or untreated MDA-MB-231 as a negative control. N = 4. Paired Ratio *t*-test. (E) Expression levels of the FOX genes *FOXA1, FOXC1* and *FOXO3* in different human breast cancer subtypes according to the Metabric database [18,19]. (F) Relative mRNA levels of *FOXO3* (FOXO3a) and *ESR1* (ERα) in MCF-7 cells transfected with siFOXO3 to inhibit FOXO3a, and compared to the MCF-7 cells treated with scrambled siRNA (Scr). N = 4. Paired *t*-test. (G) Phosphorylation and sequestration of FOXO3a (P-FOXO3a) in the cytoplasm of breast cancer xenografts of primary human macrophages cells co-transplanted with MCF-7 cells in NSG mice, as controlled to only MCF-7 breast cancer cells. Immunohistochemistry was performed using Ser253 P-FOXO3a specific antibodies. Error bars indicate SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

3.5. Primary tumor macrophage infiltration correlates with ERα-negative tumors

Using a cohort consisting of primary tumors from 498 breast cancer patients, we show that high overall presence of infiltrating macrophages (CD68$^+$ *high*) in the primary tumor is significantly associated with lack of ERα expression (Table 1; Pearson Chi-Square, Linear by Linear association $p = 0.018$). Representative images for immunohistochemical (IHC) staining are shown in Fig. 4A. A dense infiltration of macrophages (CD68$^+$ *high*) in the primary tumor also

Table 1
Cross-correlations of ERα expression (0, 1) and presence of CD68$^+$ macrophages (*low, high*) in primary human breast tumors.

		ERα		Total
		0	1	
CD68	***Low***	10	112	122
	high	63	309	372
Total		73	421	494[a]

[a] Pearson Chi-Square, Linear by Linear association $p = 0.018$.

correlated to a shorter recurrence free survival (RFS), but only significantly so for patients with ERα$^+$ breast tumors ($p = 0.379$ for patients with ERα$^-$ tumors; $p = 0.006$ for patients with ERα$^+$ tumors using Log Rank test) (Fig. 4B). In summary, infiltration of macrophages is associated with a shorter RFS in breast cancer patients with ERα$^+$ tumors, but ERα$^-$ breast cancers are most likely to be associated with infiltration of tumor associated macrophages, thus making it difficult to assess a direct correlation to ERα-downregulation *in vivo*. Therefore, future studies should evaluate the putative effect of macrophages on ERα-downregulation in remaining metastasizing cancer cells in cohorts with paired specimens from the primary tumor and multiple metastatic sites.

4. Discussion

Endocrine therapy is a valuable treatment option for patients with ERα$^+$ tumors [36]. The endocrine treatments offered are usually tamoxifen or aromatase inhibitor therapy, which both target estrogen actions, hence hindering tumor progression. One of the main resistance mechanisms for ERα-positive tumors is downregulation of ERα in the remaining cancer cells [37] and the identification of additional therapies that may reverse this ERα downregulation are warranted. In this study we show that primary human macrophages are capable of downregulating ERα expression in breast cancer cells, both *in vivo* and *in vitro*, via secreted TNFα. The downregulation is persistent and associated with a TNFα-P-Akt induced inactivation of FOXO3a, thus sequestering it from the nucleus and disrupting *ESR1* transcription.

Our findings that presence of tumor infiltrating macrophages in the primary tumor *per se* correlate with lack of ERα, is consistent with previous findings [5,38]. These findings make the evaluation of a potential local downregulation of ERα in the primary tumor of patients difficult to address, especially since ERα negative tumors have a more profound effect on skewing infiltrating macrophages to an anti-inflammatory profile [20,38]. The potential effect of macrophages on ERα-downregulation in metastasizing cells (lymph nodes and distant metastases) should ideally be evaluated in cohorts with paired primary tumor and metastases. The observation that presence of macrophages in the primary tumor of breast cancer patients, is associated with an increased recurrence rate only in patients with ERα$^+$ tumors, strengthens our proposed mechanisms, but could also be explained by the fact that ERα$^-$ breast cancer patients in general have a poorer prognosis. As we also noted, presence of macrophages and downregulation of ERα may correlate to initiation of EMT as assessed by reduced levels of E-cadherin and CAR *in vitro* and *in vivo*. In our hands, we claim to having observed only a partial initiation of EMT for several reasons, one being that vimentin was not upregulated in the malignant cells *in vivo* in xenografts co-transplanted with human monocytes. Two mediators that have been linked to initiation of EMT are TGFβ and TNFα [4], but a direct link to human macrophage derived TNFα has to our knowledge not been reported previously. Likewise, ERα$^-$ tumors often have an EMT phenotype [39]. Our observation that silencing of total *FOXO3,* and thus *ESR1* in breast cancer cells, led to increased *SNAI2* (Slug), but not *TWIST1* and *SNAI1*, is interesting and fully in line with a previous report on ERα-Slug linked EMT [40].

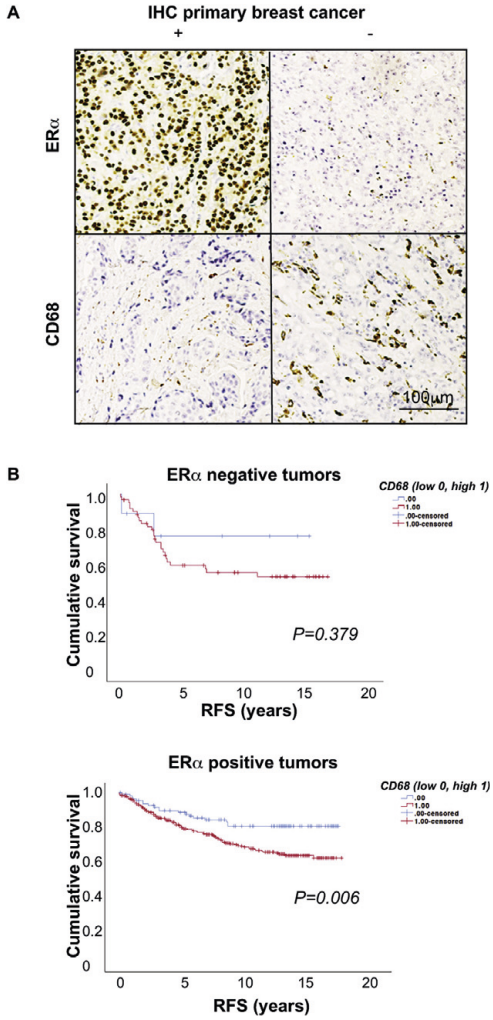


Fig. 4. High infiltration of macrophages correlates with ERα$^-$ breast cancers, but also to a worse prognosis primarily in ERα$^+$ breast cancer patients. (A) Immunohistochemical staining of ERα and CD68 in primary human breast cancer. The images show representative primary tumor samples with high or low expression levels of ERα or densities of CD68$^+$ macrophages. (B) RFS according to the infiltration of the pan-macrophage cell marker CD68 (low macrophage infiltration (CD68 *low*) or high macrophage infiltration (CD68 *high*)) in ERα$^-$ and ERα$^+$ breast cancer patients respectively. Log-rank P value < 0.05 was considered significant.

ERα-negative breast cancers, especially TNBC, explicitly affect immune infiltration [20,32,38] and the connection to ERα expression and presence of immune cells is therefore ambiguous. The results we obtained in this study indicate that pro-inflammatory CD68$^+$ macrophages present in the primary tumors may indeed downregulate ERα via secreted cytokines, such as TNFα. Macrophages have previously

been proposed to downregulate ERα in breast cancer cells, albeit through an unknown molecular mechanism [41,42]. Pro-inflammatory cytokines have in common that they induce activation of NFκB and STAT3 [43]. It is well documented that activation of NFκB can induce downregulation of ERα [44]. However, in our study we found that TNFα was the only cytokine tested that significantly downregulated ERα, thus reducing the possibility for NFκB activation as the only explanation. Instead we found that TNFα inhibits the *ESR1* transcriptional regulator FOXO3a. TNFα has been shown to downregulate ERα in an Akt-dependent manner previously, but an involvement of FOXO3a phosphorylation was not indicated [45]. TNFα has also been shown to phosphorylate FOXO3a previously via IκB in breast cancer cells [11,46], but a connection with Akt and *ESR1* regulation was not suggested. It is also still possible that macrophages regulate ERα expression at the post-transcriptional level [47], as ERα protein levels seem to be more drastically affected as compared to *ESR1* mRNA levels. Contrasting the effect on FOXO3a, TNFα has been shown to be a positive regulator of FOXO1 [48].

In the present study, we could not see a correlation between anti-inflammatory M2 macrophages and ERα downregulation *in vitro*, indicating that it is pro-inflammatory macrophages that have this potential. Our findings suggest that different macrophage subtypes have various and unique impacts on breast cancer progression, and that pro-inflammatory macrophages, despite being tumoricidal, may have unwanted, direct effects on endocrine resistance mechanisms in breast cancer patients. A nationwide cohort study on breast cancer patients with rheumatoid arthritis, showed no increase in breast cancer recurrences in patients treated with TNFα antagonists, as compared to the untreated group [49]. A relevant follow up study should investigate TNFα antagonist treatment and survival in patients with $ERα^+$ tumors specifically. We propose that human macrophages, in a breast tumor context, have the capacity to induce endocrine resistance through downregulation of ERα via TNFα. Since TNFα antagonists have been shown to be tolerable in a large cohort of breast cancer patients [49,50], the combination of TNFα antagonists and endocrine therapy should be re-evaluated. We hypothesize that TNFα antagonists could provide a dual-hit effect in $ERα^+$ breast cancer, targeting both inflammation and endocrine therapy treatment resistance.

Ethics approval and consent to participate

Ethical permit was obtained from the regional ethical committee at Lund University (Dnr 613/02, Dnr 2012/689, Dnr 2014/669, Dnr 2017/949) whereby written consent was given, or when not required patients were offered the option to opt out, as approved by the regional ethical committee at Lund University. The NSG models (approvals M11-15) were approved by the regional ethics committee for animal research at Lund University, Sweden. The study was performed in accordance with the Declaration of Helsinki.

Consent for publication

No individual person's data is included in this study. All authors have read and agreed on publishing this study.

Data availability

All datasets generated in the course of the current study are presented in the main text and the Supplementary Information available online. Gene expression data was analyzed using publicly available database R2: microarray analysis and visualization platform [17]; TCGA 1097 was used for gene expression profile analyses. For breast cancer subtype RNA expression profiles Metabric was used [18,19].

Funding

This work was supported by grants from the Swedish Cancer Society; the Swedish Research Council; the Governmental Funding of Clinical Research within the National Health Service (ALF), the UMAS Cancer foundation, the Gunnar Nilsson's Cancer Foundation, the Åke Wibergs foundation, the Percy Falks Foundation, and the Gyllenstiernska Krapperups foundation.

Author contribution

FBG performed the majority of the experiments, but also analyzed and interpreted data and wrote the initial manuscript together with KL. CH and CB were responsible for initiating the study together with KL. CH, CB, MM, EK and RA performed additional experiments. SM, SP and DB performed animal experiments and DB was responsible for experimental design. CL was responsible for gene expression database analyses. KJ was responsible for the patient breast cancer cohort. KL was responsible for the study, design, writing, analysis and interpreting data. All authors contributed to writing and revising the manuscript.

Declaration of competing 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 authors otherwise declare no competing interest.

Acknowledgements

The authors thank Elise Nilsson and Kristina Ekström-Holka for professional technical skills in preparation of the IHC and TMA and Professor Lisa M. Coussens for the initial CD68 patient cohort data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexcr.2020.111932.

References

[1] A. Goldhirsch, W.C. Wood, A.S. Coates, R.D. Gelber, B. Thurlimann, H.J. Senn, m. Panel, Strategies for subtypes–dealing with the diversity of breast cancer: highlights of the St. Gallen international expert consensus on the primary therapy of early breast cancer 2011, Ann. Oncol. 22 (2011) 1736–1747.
[2] D.C. Allred, P. Brown, D. Medina, The origins of estrogen receptor alpha-positive and estrogen receptor alpha-negative human breast cancer, Breast Cancer Res. 6 (2004) 240–245.
[3] T. Sorlie, C.M. Perou, R. Tibshirani, T. Aas, S. Geisler, H. Johnsen, T. Hastie, M.B. Eisen, M. van de Rijn, S.S. Jeffrey, T. Thorsen, H. Quist, J.C. Matese, P.O. Brown, D. Botstein, P.E. Lonning, A.L. Borresen-Dale, Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 10869–10874.
[4] J. Felipe Lima, S. Nofech-Mozes, J. Bayani, J.M. Bartlett, EMT in breast carcinoma-A review, J. Clin. Med. 5 (2016).
[5] C. Medrek, F. Ponten, K. Jirstrom, K. Leandersson, The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients, BMC Canc. 12 (2012) 306.
[6] D. Laoui, K. Movahedi, E. Van Overmeire, J. Van den Bossche, E. Schouppe, C. Mommer, A. Nikolaou, Y. Morias, P. De Baetselier, J.A. Van Ginderachter, Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions, Int. J. Dev. Biol. 55 (2011) 861–867.
[7] A. Mantovani, A. Sica, Macrophages, innate immunity and cancer: balance, tolerance, and diversity, Curr. Opin. Immunol. 22 (2010) 231–237.
[8] P. Serafini, Myeloid derived suppressor cells in physiological and pathological conditions: the good, the bad, and the ugly, Immunol. Res. 57 (2013) 172–184.
[9] M.E. Carter, A. Brunet, FOXO transcription factors, Curr. Biol. 17 (2007) R113–R114.
[10] M. Bullock, FOXO factors and breast cancer: outfoxing endocrine resistance, Endocr. Relat. Canc. 23 (2016) R113–R130.
[11] M.C. Hu, D.F. Lee, W. Xia, L.S. Golfman, F. Ou-Yang, J.Y. Yang, Y. Zou, S. Bao, N. Hanada, H. Saso, R. Kobayashi, M.C. Hung, IkappaB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a, Cell 117 (2004) 225–237.

[12] Y. Zou, W.B. Tsai, C.J. Cheng, C. Hsu, Y.M. Chung, P.C. Li, S.H. Lin, M.C. Hu, Forkhead box transcription factor FOXO3a suppresses estrogen-dependent breast cancer cell proliferation and tumorigenesis, Breast Cancer Res. 10 (2008) R21.

[13] J.Y. Yang, C.S. Zong, W. Xia, H. Yamaguchi, Q. Ding, X. Xie, J.Y. Lang, C.C. Lai, C.J. Chang, W.C. Huang, H. Huang, H.P. Kuo, D.F. Lee, L.Y. Li, H.C. Lien, X. Cheng, K.J. Chang, C.D. Hsiao, F.J. Tsai, C.H. Tsai, A.A. Sahin, W.J. Muller, G.B. Mills, D. Yu, G.N. Hortobagyi, M.C. Hung, ERK promotes tumorigenesis by inhibiting FOXO3a via MDM2-mediated degradation, Nat. Cell Biol. 10 (2008) 138–148.

[14] K. Belguise, S. Guo, G.E. Sonenshein, Activation of FOXO3a by the green tea polyphenol epigallocatechin-3-gallate induces estrogen receptor alpha expression reversing invasive phenotype of breast cancer cells, Canc. Res. 67 (2007) 5763–5770.

[15] E.R. Schuur, A.V. Loktev, M. Sharma, Z. Sun, R.A. Roth, R.J. Weigel, Ligand-dependent interaction of estrogen receptor-alpha with members of the forkhead transcription factor family, J. Biol. Chem. 276 (2001) 33554–33560.

[16] C. Bergenfelz, C. Medrek, E. Ekstrom, K. Jirstrom, H. Janols, M. Wullt, A. Bredberg, K. Leandersson, Wnt5a induces a tolerogenic phenotype of macrophages in sepsis and breast cancer patients, J. Immunol. 188 (2012) 5448–5458.

[17] **R.m.a.a.v. platform,** http://r2.amc.nl.

[18] C. Curtis, S.P. Shah, S.F. Chin, G. Turashvili, O.M. Rueda, M.J. Dunning, D. Speed, A.G. Lynch, S. Samarajiwa, Y. Yuan, S. Graf, G. Ha, G. Haffari, A. Bashashati, R. Russell, S. McKinney, M. Group, A. Langerod, A. Green, E. Provenzano, G. Wishart, S. Pinder, P. Watson, F. Markowetz, L. Murphy, I. Ellis, A. Purushotham, A.L. Borresen-Dale, J.D. Brenton, S. Tavare, C. Caldas, S. Aparicio, The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups, Nature 486 (2012) 346–352.

[19] B. Pereira, S.F. Chin, O.M. Rueda, H.K. Vollan, E. Provenzano, H.A. Bardwell, M. Pugh, L. Jones, R. Russell, S.J. Sammut, D.W. Tsui, B. Liu, S.J. Dawson, J. Abraham, H. Northen, J.F. Peden, A. Mukherjee, G. Turashvili, A.R. Green, S. McKinney, A. Oloumi, S. Shah, N. Rosenfeld, L. Murphy, D.R. Bentley, I.O. Ellis, A. Purushotham, S.E. Pinder, A.L. Borresen-Dale, H.M. Earl, P.D. Pharoah, M.T. Ross, S. Aparicio, C. Caldas, The somatic mutation profiles of 2,433 breast cancers refines their genomic and transcriptomic landscapes, Nat. Commun. 7 (2016) 11479.

[20] R. Allaoui, C. Bergenfelz, S. Mohlin, C. Hagerling, K. Salari, Z. Werb, R.L. Anderson, S.P. Ethier, K. Jirstrom, S. Pahlman, D. Bexell, B. Tahin, M.E. Johansson, C. Larsson, K. Leandersson, Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers, Nat. Commun. 7 (2016) 13050.

[21] R. Allaoui, C. Hagerling, E. Desmond, C.F. Warfvinge, K. Jirstrom, K. Leandersson, Infiltration of gammadelta T cells, IL-17+ T cells and FoxP3+ T cells in human breast cancer, Canc. Biomarkers 20 (2017) 395–409.

[22] S. Borgquist, C. Holm, M. Stendahl, L. Anagnostaki, G. Landberg, K. Jirstrom, Oestrogen receptors alpha and beta show different associations to clinicopathological parameters and their co-expression might predict a better response to endocrine treatment in breast cancer, J. Clin. Pathol. 61 (2008) 197–203.

[23] F. Lanigan, G. Gremel, R. Hughes, D.J. Brennan, F. Martin, K. Jirstrom, W.M. Gallagher, Homeobox transcription factor muscle segment homeobox 2 (Msx2) correlates with good prognosis in breast cancer patients and induces apoptosis in vitro, Breast Cancer Res. 12 (2010) R59.

[24] D.G. DeNardo, D.J. Brennan, E. Rexhepaj, B. Ruffell, S.L. Shiao, S.F. Madden, W.M. Gallagher, N. Wadhwani, S.D. Keil, S.A. Junaid, H.S. Rugo, E.S. Hwang, K. Jirstrom, B.L. West, L.M. Coussens, Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy, Canc. Discov. 1 (2011) 54–67.

[25] A.J. Murphy, P.M. Guyre, C.R. Wira, P.A. Pioli, Estradiol regulates expression of estrogen receptor ERalpha46 in human macrophages, PLoS One 4 (2009) e5539.

[26] A. Brunet, A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, M.E. Greenberg, Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor, Cell 96 (1999) 857–868.

[27] X. Wang, S. Hu, L. Liu, Phosphorylation and acetylation modifications of FOXO3a: independently or synergistically? Oncol Lett 13 (2017) 2867–2872.

[28] S. Singh, R. Chakrabarti, Consequences of EMT-driven changes in the immune microenvironment of breast cancer and therapeutic response of cancer cells, J. Clin. Med. 8 (2019).

[29] P. Bouris, S.S. Skandalis, Z. Piperigkou, N. Afratis, K. Karamanou, A.J. Aletras, A. Moustakas, A.D. Theocharis, N.K. Karamanos, Estrogen receptor alpha mediates epithelial to mesenchymal transition, expression of specific matrix effectors and functional properties of breast cancer cells, Matrix Biol. 43 (2015) 42–60.

[30] S. Al Saleh, F. Al Mulla, Y.A. Luqmani, Estrogen receptor silencing induces epithelial to mesenchymal transition in human breast cancer cells, PLoS One 6 (2011) e20610.

[31] A.K. Bonde, V. Tischler, S. Kumar, A. Soltermann, R.A. Schwendener, Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors, BMC Canc. 12 (2012) 35.

[32] S. Su, Q. Liu, J. Chen, J. Chen, F. Chen, C. He, D. Huang, W. Wu, L. Lin, W. Huang, J. Zhang, X. Cui, F. Zheng, H. Li, H. Yao, F. Su, E. Song, A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis, Canc. Cell 25 (2014) 605–620.

[33] J. Zhang, H. Yao, G. Song, X. Liao, Y. Xian, W. Li, Regulation of epithelial-mesenchymal transition by tumor-associated macrophages in cancer, Am J Transl Res 7 (2015) 1699–1711.

[34] C.W. Li, W. Xia, L. Huo, S.O. Lim, Y. Wu, J.L. Hsu, C.H. Chao, H. Yamaguchi, N.K. Yang, Q. Ding, Y. Wang, Y.J. Lai, A.M. LaBaff, T.J. Wu, B.R. Lin, M.H. Yang, G.N. Hortobagyi, M.C. Hung, Epithelial-mesenchymal transition induced by TNF-alpha requires NF-kappaB-mediated transcriptional upregulation of Twist1, Canc. Res. 72 (2012) 1290–1300.

[35] J. Li, R. Yang, Y. Dong, M. Chen, Y. Wang, G. Wang, Knockdown of FOXO3a induces epithelial-mesenchymal transition and promotes metastasis of pancreatic ductal adenocarcinoma by activation of the beta-catenin/TCF4 pathway through SPRY2, J. Exp. Clin. Canc. Res. 38 (2019) 38.

[36] C.K. Osborne, R. Schiff, Mechanisms of endocrine resistance in breast cancer, Annu. Rev. Med. 62 (2011) 233.

[37] E.A. Musgrove, R.L. Sutherland, Biological determinants of endocrine resistance in breast cancer, Nat. Rev. Canc. 9 (2009) 631–643.

[38] S. Sousa, R. Brion, M. Lintunen, P. Kronqvist, J. Sandholm, J. Monkkonen, P.L. Kellokumpu-Lehtinen, S. Lauttia, O. Tynninen, H. Joensuu, D. Heymann, J.A. Maatta, Human breast cancer cells educate macrophages toward the M2 activation status, Breast Cancer Res. 17 (2015) 101.

[39] D. Sarrio, S.M. Rodriguez-Pinilla, D. Hardisson, A. Cano, G. Moreno-Bueno, J. Palacios, Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype, Canc. Res. 68 (2008) 989–997.

[40] Y. Ye, Y. Xiao, W. Wang, K. Yearsley, J.X. Gao, B. Shetuni, S.H. Barsky, ERalpha signaling through slug regulates E-cadherin and EMT, Oncogene 29 (2010) 1451–1462.

[41] F. Stossi, Z. Madak-Erdogan, B.S. Katzenellenbogen, Macrophage-elicited loss of estrogen receptor-alpha in breast cancer cells via involvement of MAPK and c-Jun at the ESR1 genomic locus, Oncogene 31 (2012) 1825–1834.

[42] T. Lindsten, A. Hedbrant, A. Ramberg, J. Wijkander, A. Solterbeck, M. Eriksson, D. Delbro, A. Erlandsson, Effect of macrophages on breast cancer cell proliferation, and on expression of hormone receptors, uPAR and HER-2, Int. J. Oncol. 51 (2017) 104–114.

[43] M.M. Chaturvedi, B. Sung, V.R. Yadav, R. Kannappan, B.B. Aggarwal, NF-kappaB addiction and its role in cancer: 'one size does not fit all, Oncogene 30 (2011) 1615–1630.

[44] L. Sas, F. Lardon, P.B. Vermeulen, J. Hauspy, P. Van Dam, P. Pauwels, L.Y. Dirix, S.J. Van Laere, The interaction between ER and NFkappaB in resistance to endocrine therapy, Breast Cancer Res. 14 (2012) 212.

[45] S.H. Lee, H.S. Nam, TNF alpha-induced down-regulation of estrogen receptor alpha in MCF-7 breast cancer cells, Mol. Cell 26 (2008) 285–290.

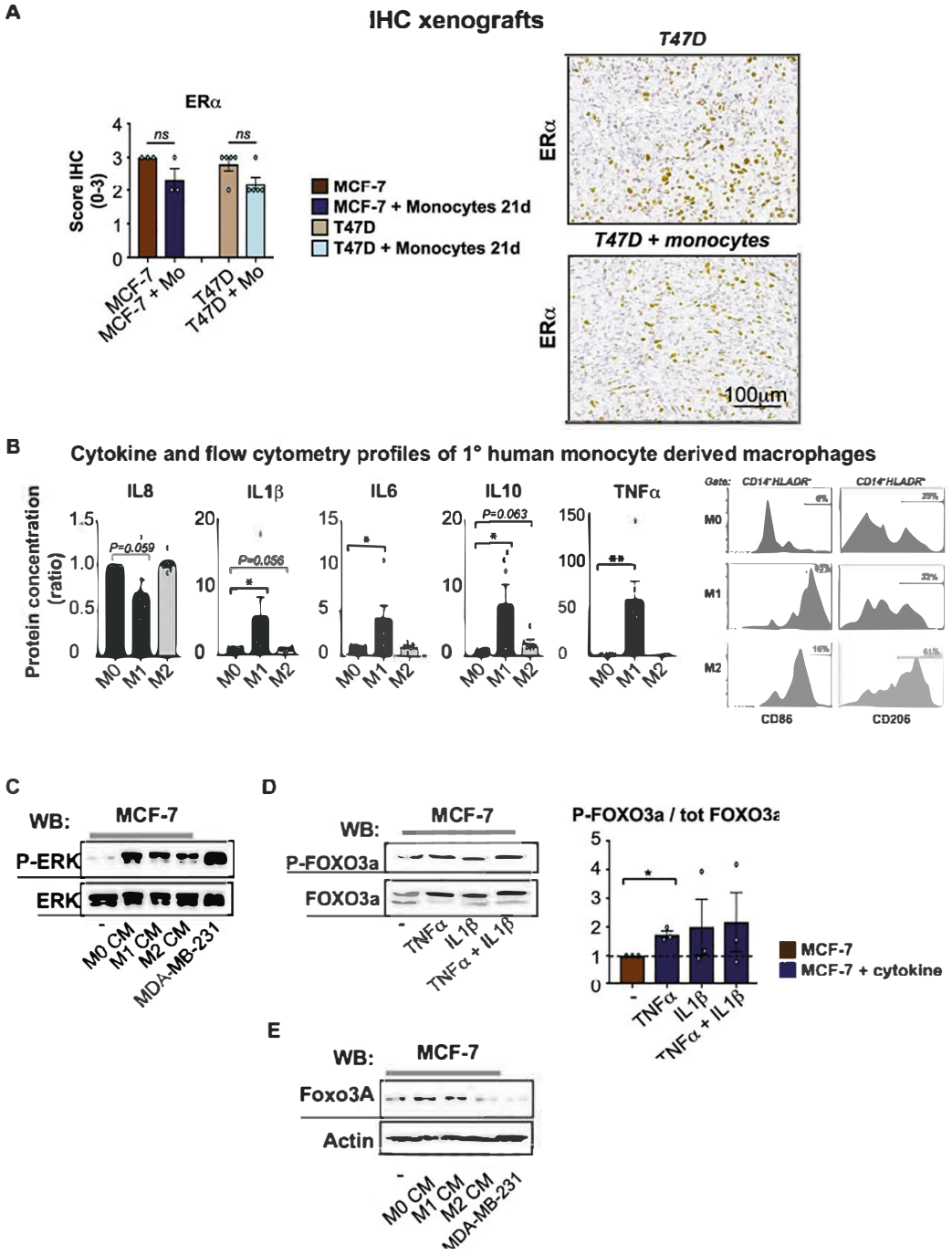
[46] S.M. Kornblau, N. Singh, Y. Qiu, W. Chen, N. Zhang, K.R. Coombes, Highly phosphorylated FOXO3A is an adverse prognostic factor in acute myeloid leukemia, Clin. Canc. Res. 16 (2010) 1865–1874.

[47] J.C. Keen, Q. Zhou, B.H. Park, C. Pettit, K.M. Mack, B. Blair, K. Brenner, N.E. Davidson, Protein phosphatase 2A regulates estrogen receptor alpha (ER) expression through modulation of ER mRNA stability, J. Biol. Chem. 280 (2005) 29519–29524.

[48] Y. Wang, Y. Zhou, D.T. Graves, FOXO transcription factors: their clinical significance and regulation, BioMed Res. Int. 2014 (2014) 925350.

[49] P. Raaschou, T. Frisell, J. Askling, A.S. Group, TNF inhibitor therapy and risk of breast cancer recurrence in patients with rheumatoid arthritis: a nationwide cohort study, Ann. Rheum. Dis. 74 (2015) 2137–2143.

[50] L.K. Mercer, M. Lunt, A.L. Low, W.G. Dixon, K.D. Watson, D.P. Symmons, K.L. Hyrich, B.C.C. Consortium, Risk of solid cancer in patients exposed to anti-tumour necrosis factor therapy: results from the British Society for Rheumatology Biologics Register for Rheumatoid Arthritis, Ann. Rheum. Dis. 74 (2015) 1087–1093.


A
IHC xenografts
ERα
Score IHC (0-3)
ns
ns
MCF-7
MCF-7 + Mo
T47D
T47D + Mo
MCF-7
MCF-7 + Monocytes 21d
T47D
T47D + Monocytes 21d
T47D
T47D + monocytes
ERα
100µm
B
Cytokine and flow cytometry profiles of 1° human monocyte derived macrophages
Protein concentration (ratio)
IL8
IL1β
IL6
IL10
TNFα
P=0.059
P=0.056
P=0.063
M0
M1
M2
Gate: CD14+HLADR-
CD14+HLADR+
CD86
CD206
C
WB:
MCF-7
P-ERK
ERK
-
M0 CM
M1 CM
M2 CM
MDA-MB-231
D
WB:
MCF-7
P-FOXO3a
FOXO3a
-
TNFα
IL1β
TNFα + IL1β
P-FOXO3a / tot FOXO3a
MCF-7
MCF-7 + cytokine
E
WB:
MCF-7
Foxo3A
Actin
-
M0 CM
M1 CM
M2 CM
MDA-MB-231

Supplementary Figure 1.

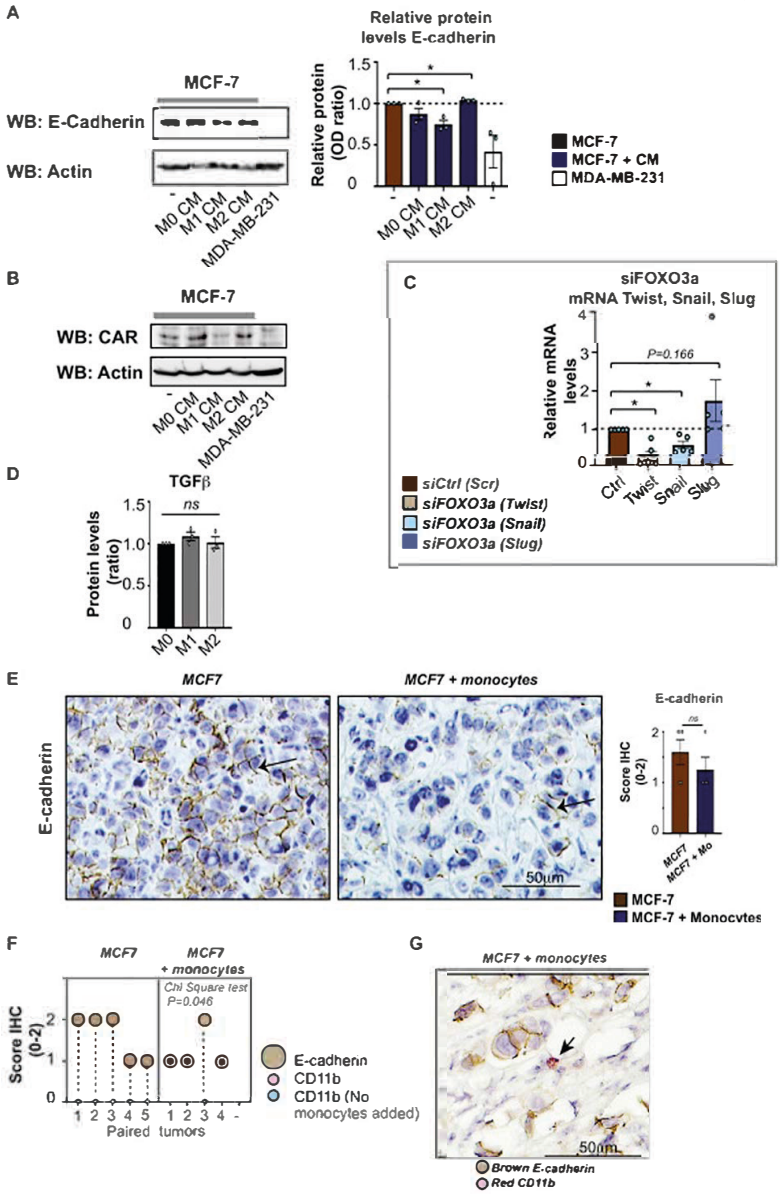
(A) Primary human monocytes were co-transplanted together with MCF-7 or T47D cells for 21 days [1]. Controls were only MCF-7 or T47D transplanted cells. The xenografts were resected and immunohistochemistry was performed. The histograms represent the mean IHC ER intensity score of the xenografts. Representative IHC ER staining of the T47D xenografts are shown. Unpaired t-test. Error bars indicate SEM. N=3 for MCF-7 21d and N=5 for T47D 21d. *p<0.05, **p<0.01, ***p<0.001.

(B) Human inflammatory cytokine bead array (CBA) of supernatants (CM) generated from *in vitro* cultures of human macrophages of M0, M1 and M2 type (left). The histogram panels indicate the relative protein level for each cytokine as compared to M0. N=5. Ratio Paired t-test. To the right in the panel, histogram mean fluorescence intensity (MFI) from representative flow cytometry profiles on primary human macrophages of M0, M1 and M2 type is shown. Gated on $CD14^{+}HLADR^{+}$; histogram MFI showing $CD86^{high}$ (M1 marker) and $CD206^{high}$ (M2 marker) expression.

(C) CM generated from primary human M0, M1 and M2 macrophages induce similar phosphorylation levels of P-ERK1/2 in human breast cancer MCF-7cells, compared to the untreated MCF-7 cells or untreated MDA-MB-231 as a negative control. The western blot shows a representative experiment. N=4.

(D) Recombinant TNFα, but not IL-1β, induce phosphorylation of FOXO3a in human breast cancer MCF-7cells. The western blot shows a representative experiment. The histogram represents the relative OD values of the Ser253 P-FOXO3a bands in relation to total FOXO3a, in order to visualize the phosphorylation, and compared to the untreated MCF-7 cells as a negative control. Paired Ratio t-test. N=3.

(E) The protein levels of FOXO3a in MCF-7 cells treated with CM generated from primary human M0, M1 and M2 macrophages, compared to the untreated MCF-7 cells or untreated MDA-MB-231 as a negative control. The western blot shows a representative experiment.


A
MCF-7
WB: E-Cadherin
WB: Actin
- M0 CM M1 CM M2 CM MDA-MB-231
Relative protein levels E-cadherin
Relative protein (OD ratio)
MCF-7
MCF-7 + CM
MDA-MB-231
B
MCF-7
WB: CAR
WB: Actin
C
siFOXO3a
mRNA Twist, Snail, Slug
Relative mRNA levels
P=0.166
Ctrl Twist Snail Slug
siCtrl (Scr)
siFOXO3a (Twist)
siFOXO3a (Snail)
siFOXO3a (Slug)
D
TGFβ
ns
Protein levels (ratio)
M0 M1 M2
E
MCF7
MCF7 + monocytes
E-cadherin
50µm
E-cadherin
Score IHC (0-2)
MCF7 MCF7 + Mo
MCF-7
MCF-7 + Monocytes
F
MCF7
MCF7 + monocytes
Chi Square test P=0.046
Score IHC (0-2)
Paired tumors
E-cadherin
CD11b
CD11b (No monocytes added)
G
MCF7 + monocytes
50µm
Brown E-cadherin
Red CD11b

Supplementary Figure 2.

The primary human M1-macrophage induced ERα downregulation is accompanied by EMT associated traits *in vitro* and *in vivo*. (A) M1 CM generated from primary human M1 macrophages induce downregulation of the EMT markers E-cadherin (A) and CAR (B) in human breast cancer MCF-7 cells, in contrast to CM from cultures of M0 and M2 type primary human macrophages. The western blots show representative experiments. The histogram represents the relative OD values of the E-cadherin bands in order to visualize the E-cadherin protein downregulation, compared to the untreated MCF-7 cells or untreated MDA-MB-231 as a negative control. Ratio Paired t-test. N=3. (C) Relative mRNA levels of Twist, Snail and Slug in MCF-7 cells transfected with siFOXO3 to inhibit FOXO3a, and compared to the untreated MCF-7 cells. N=5. Ratio Paired t-test. (D) TGFβ ELISA of supernatants (CM) generated from *in vitro* cultures of human macrophages of M0, M1 and M2 type. The histogram panels indicate the relative protein level for TGFβ as compared to M0. N=5. Ratio Paired t-test. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001.

(E) Downregulation of E-cadherin on breast cancer xenografts of primary human macrophages cells co-transplanted with MCF-7 cells in NSG mice, as controlled to only MCF-7 breast cancer cells. The histogram represents the mean score of the xenografts for the E-cadherin staining. N=5. Unpaired t-test. Error bars indicate SEM. *p<0.05, **p<0.01, ***p<0.001.

(F) Low E-cadherin expression levels correlate significantly to high presence of primary human monocytes as measured by IHC (**P=0.046*; N=4. Pearson Chi Square test) (G) Double IHC staining of E-cadherin (brown) and CD11b (red).

Supplementary Tables

Supplementary Table 1. WB antibodies

Antibody	Clone	Company	Dilution
β-Actin	AC-15	Sigma	1:5000
Akt (pan)	40D4	Cell Signaling	1:2000
Phospho-Akt (Ser47)	D9E	Cell Signaling	1:2000
CAR	E1-1	Santa Cruz	1:200
E-Cadherin	36/E-Cadherin	BD Biosciences	1:200
ERK 1/2	C-9	Santa Cruz	1:1000
ERα	D-12	Santa Cruz	1:200
FOXO3A	EP1949Y	Abcam	1:5000
FOXO3A phospho S253	EPR1951(2)	Abcam	1:5000
GAPDH	0411	Santa Cruz	1:5000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	197G2	Cell Signaling	1:1000

Supplementary Table 2. Primer sequences for RT-qPCR

Gene	Sequence
ERα	F: 5'-GCAGGGAGAGGAGTTTGTGT-3' R: 5'-ATGTGGGAGAGGATGAGGAG-3'
FoxA1	F: 5'-GGGGGTTTGTCTGGCATAGC-3' R: 5'-GCACTGGGGGAAAGGTTGTG-3'
FoxO3	F: 5'-CAAACCCAGGGCGCTCTT-3' R: 5'-CTCACTCAAGCCCATGTTGCT-3'
Twist1	F: 5'-GCC AGG TAC ATC GAC TTC CTC T-3' R: 5'-TCC ATC CTC CAG ACC GAG AAG G-3'
ACTB	F: 5'-CTGGAACGGTGAAGGTGACA-3' R: 5'-AAGGGACTTCCTGTAACAATGCA-3'
GAPDH	F: 5'-TGCACCACCAACTGCTTAGC-3' R: 5'-GGCATGGACTGTGGTCATGAG-3'
SDHA	F: 5′-TGGGAACAAGAGGGCATCTG-3′ R: 5′-CCACCACTGCATCAAATTCATG-3′

Supplementary Table 3. IHC antibodies

Antibody	Clone	Company	Dilution
CD11b	EP1345Y	Abcam	1:100
CD68[a]	KP1	DAKO	1:1500
E-Cadherin M3612	NCH-38	DAKO	1:100
ERα M7047	1D5	DAKO	1:50
P-FoxO3a phospho S253	EPR1951(2)	Abcam	1:100
Vimentin	V9	DAKO	1:1000

[a] For CD68 staining in the primary human breast cancer cohort see [2]

References Supplementary files

[1] R. Allaoui, C. Bergenfelz, S. Mohlin, C. Hagerling, K. Salari, Z. Werb, R.L. Anderson, S.P. Ethier, K. Jirstrom, S. Pahlman, D. Bexell, B. Tahin, M.E. Johansson, C. Larsson, K. Leandersson, Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers, Nat Commun 7 (2016) 13050.

[2] D.G. DeNardo, D.J. Brennan, E. Rexhepaj, B. Ruffell, S.L. Shiao, S.F. Madden, W.M. Gallagher, N. Wadhwani, S.D. Keil, S.A. Junaid, H.S. Rugo, E.S. Hwang, K. Jirstrom, B.L. West, L.M. Coussens, Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy, Cancer Discov 1 (2011) 54-67.

Paper II

ONCOIMMUNOLOGY
2020, VOL. 9, NO. 1, e1848067 (13 pages)
https://doi.org/10.1080/2162402X.2020.1848067

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

OPEN ACCESS Check for updates

Co-localization of CD169$^+$ macrophages and cancer cells in lymph node metastases of breast cancer patients is linked to improved prognosis and PDL1 expression

Frida Björk Gunnarsdottir[a], Nathalie Auoja[a], Pär-Ola Bendahl[b], Lisa Rydén[b,c], Mårten Fernö[b], and Karin Leandersson[a]

[a]Cancer Immunology, Department of Translational Medicine, Lund University, Malmö, Sweden; [b]Division of Oncology, Department of Clinical Sciences Lund, Lund University, Lund, Sweden; [c]Department of Surgery, Skåne University Hospital, Lund, Sweden

ABSTRACT

Breast cancer is the most common form of cancer in women worldwide. Although the survival among breast cancer patients has improved, there is still a large group of patients with dismal prognosis. One of the most important prognostic factors for poor prognosis is lymph node metastasis. Increasing knowledge concerning the lymph nodes of breast cancer patients indicates that they are affected by the primary tumor. In this study we show that presence of CD169$^+$ subcapsular sinus macrophages in contact with lymph node metastases in breast cancer patients, is related to better prognosis after adjuvant tamoxifen treatment, but only in patients with PDL1$^+$ primary tumors. This is in contrast to the prognostic effect of CD169$^+$ primary tumor-associated macrophages (TAMs). We further show that CD169$^+$ macrophages were spatially associated with expression of PDL1 on nearby cells, both in primary tumors and metastatic lymph node, although PDL1 expression in metastatic lymph node as such did not have further prognostic impact. Our data suggest that CD169$^+$ resident lymph node macrophages have a unique function in targeting immune responses against breast cancer and should be further investigated in detail.

ARTICLE HISTORY
Received 14 June 2020
Revised 4 November 2020
Accepted 5 November 2020

KEYWORDS
Breast cancer; macrophage; CD169; lymph node; PDL1

Introduction

Breast cancer is the most common type of cancer among women and is divided into different subtypes depending on the status of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), Ki67, and histological grade.[1] Whereas breast cancers with a hormone receptor-positive status (ER$^+$PR$^+$) have a beneficial short-term prognosis, those that lack all three receptors (ER$^-$PR$^-$HER2$^-$; triple-negative breast cancers; TNBC) have a worse prognosis.[1] Still, for all breast cancer subtypes, the dissemination of tumor cells to the lymph nodes is one of the most significant prognostic factors associated with worse prognosis.[2]

Lymph nodes are secondary lymphoid organs where immune responses are mounted.[2] It is here that the tumor-associated antigens are transported to be recognized by the adaptive immune response, so that a tumor-specific immune attack can be started. In the lymph nodes, cells of the innate immune response are present, with various functions, but one important function is to act as antigen-pesenting cells (APCs); to phagocytose and present foreign substances (antigens) to the adaptive lymphocytes (T cells and B cells). The most important APC for the activation of naïve T cells are dendritic cells (DCs), while macrophages can induce activation of effector or memory T cells and naïve B cells.[3] Tumor antigens are mutated proteins that are present in the malignant cells. Evidence suggest that tumor-draining lymph nodes are affected by the tumor, and that the immune balance in the lymph node affects the anti-tumor immune response.[4,5]

Conventional tumor-associated macrophages (TAMs) located in the primary tumor are mostly associated with a worse prognosis in cancer patients.[6–9] In lymph nodes however, there are resident macrophages, that are subdivided into specific populations. One subtype of lymph node resident macrophages is the subcapsular sinus macrophages (CD169$^+$).[10,11] These specialized CD169$^+$ macrophages surround the lymphoid follicles in lymph nodes and act as gate-keepers for antigen delivery.[3] In mice, they have been proposed to be involved in the activation of B, T and NK cells, but also in regulating overt immune responses and T_{regs}.[10,12–15] The CD169$^+$ macrophages have also been shown to be specialized in phagocytosing and bringing distant tumor cell antigens to the lymph nodes in mice.[16] In humans, the presence of CD169$^+$ macrophages in metastasis-free lymph nodes of cancer patients with endometrial, bladder, prostate, and colorectal cancer has previously been correlated to an improved prognosis.[17–20] In contrast, a similar study on breast cancer patients showed that presence of lymph node CD169$^+$ macrophages, in lymph nodes without metastasis, correlated to early tumor stage, but not to prognosis.[21] High expression of *SIGLEC1* (CD169) in primary breast tumors, on the other hand, is associated with shorter disease-specific survival in public datasets derived from tumor samples from breast cancer patients.[22]

During the last years, immune checkpoint inhibitors have revolutionized clinical care in oncology.[23] Antibodies targeting CTLA4, PD1, and PDL1 have been evaluated with therapeutic success in many types of cancer. In breast cancer however, the success is hitherto more limited.[24] The only example in breast cancer is the positive effect of anti-PDL1 (atezolizumab) – nab-

CONTACT Karin Leandersson karin.leandersson@med.lu.se Cancer Immunology, Department of Translational Medicine, Lund University, Malmö SE 214 28, Sweden.
Supplemental data for this article can be accessed on the publisher's website.

paclitaxel combination therapy in advanced TNBC.[25] The reason to this is unknown and more information is needed to understand breast cancer-induced immune responses.[24,26] PDL1 is expressed on both APCs and tumor cells.[26] In cervical cancer patients, PDL1-expressing macrophages with immunosuppressive character have been found surrounding metastatic tumor cells in lymph nodes with metastasis,[27] and this correlated with non-responsive, tolerogenic lymphocytes.[28] Interestingly, $CD169^+$ macrophages are responsible for induction of PDL1 expression via local type I IFN production in viral infections, which lead to a local T cell exhaustion.[12] Whether PDL1 is co-expressed with CD169, in vicinity of, or on the subset of $CD169^+$ subcapsular sinus macrophages in lymph nodes with metastases and primary tumors of cancer patients, and what the consequences this would have on immune escape, is not known.

This study included patients with primary breast cancer who received 2 years of adjuvant tamoxifen. We retrieved tissue samples from primary tumors and synchronous lymph nodes with metastases. We investigated whether $CD169^+$ subcapsular sinus lymph node macrophages, present in direct contact with cancer cells in lymph node metastases, as compared to $CD169^+$ macrophages located in primary tumor (TAMs), would be a prognostic factor for breast cancer patients or not. We further investigated whether $CD169^+$ lymph node and $CD169^+$ primary tumor-associated macrophages were associated with PDL1 expression in breast cancer patients, as they are in viral infections,[12] and how this correlated to prognosis.

Materials and methods

Patients

This study was based on a representative cohort of primary stage 2 breast cancer patients (N = 445) from two prospective-randomized clinical trials that included patients from the South-Swedish Health Care Region during 1985–1994.[29–31] At that time neither adjuvant chemotherapy nor anti-HER2 therapy were included in general treatment guidelines for primary breast cancer in the South Swedish Health Care Region. Only patients treated with 2 years of tamoxifen were included. Two of the premenopausal patients received adjuvant chemotherapy in addition to tamoxifen. 159 patients were excluded due to loss of primary tumor and metastatic lymph node tissue, leaving 286 for the present study. 272 samples were annotated for CD169 and PDL1 expression in primary tumor and 180 for metastatic lymph node. Matched primary tumor and lymph node samples were obtained from 166 patients (Figure 1(a)). For CD68 staining, 261 samples were annotated for primary tumor and 184 for metastatic lymph node. Matched samples were obtained from 169 patients. For PD1 staining, 263 samples were annotated for primary tumor and 177 for metastatic lymph node. Matched samples were obtained from 159 patients. Patient and tumor characteristics for the patients included, as well as those excluded, are summarized in Table 1. Ethical approval for the use of retrospective breast cancer and lymph node specimens (Dnr 240–01), IHC control lymph node (Dnr 2010/477), and IHC control tonsil (Dnr 2017/941) was obtained from the Regional Ethics Committee in Lund, Sweden, and have been handled all in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Tissue microarray and immunohistochemistry

The expression levels of ER, PR, Ki67, and HER2 had been reevaluated on whole sections or tissue microarrays (TMAs) from paraffin-embedded tumor material as previously described.[31,34,35] The experimental biomarkers in the present study were analyzed on TMAs. All cores were 1.0 mm in diameter.

Following antibodies and dilutions were used: anti-CD169 (dilution 1:500, Spring M5160), anti-PDL1 (dilution 1:500, Cell Signaling 29122), anti-CD68 (dilution 1:1500, DAKO M0876) chosen at a dilution and time to highlight the variations in intensity between macrophages located in different areas of a human lymph node as previously discussed by us in a recent review,[36] anti-PD1 (dilution 1:100, Abcam 137132). For control staining of metastasis-free lymph node and human tonsil see Figure 1(b,c). 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. 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).

Biomarker evaluation

CD169, CD68, and PDL1 staining was scored independently by three of the authors (FGB, NA and KL) and discordant scorings were discussed until consensus was reached. The density of $CD169^+$ or $CD68^+$ macrophages or $PDL1^+$ cells, either in the primary tumor or in direct contact with the cancer cells in lymph node metastases of breast cancer patients, was scored as 0 (absent), 1 (<10%) or 2 (≥10%). If at least one of two cores was positive for biomarker expression, this tumor was classified as positive. For statistical analysis, these categories were dichotomized into absent (0) or present (1–2) biomarker expression. Figures 1(b,c) and 2 show examples of immunohistochemical (IHC) staining of CD169, CD68, and PDL1. In addition to individual biomarker scoring, samples were also scored positive for CD169 and PDL1 co-expression ($CD169^+PDL1^+$), but only if the cells expressing the markers were in close proximity or both markers were expressed on the same cell. We also evaluated PD1 to visualize PD1 expressing lymphocytes in relation to PDL1 expression and macrophage distribution, and found PD1 to be expressed in the T cell zone, lymphoid follicles and germinal centers mainly (Figure 2(a) right). In the primary tumor and metastatic lymph node specimens, PD1 was scored as 0 (absent), 1 (<10%), 2 (≥10-25%) or 3 (>25%), whereby categories were dichotomized into low (0–2) or high (3) biomarker expression.

Statistical analysis

The association between primary tumor (PT) and metastatic lymph node (MLN) expression of CD169, CD68, PDL1, and

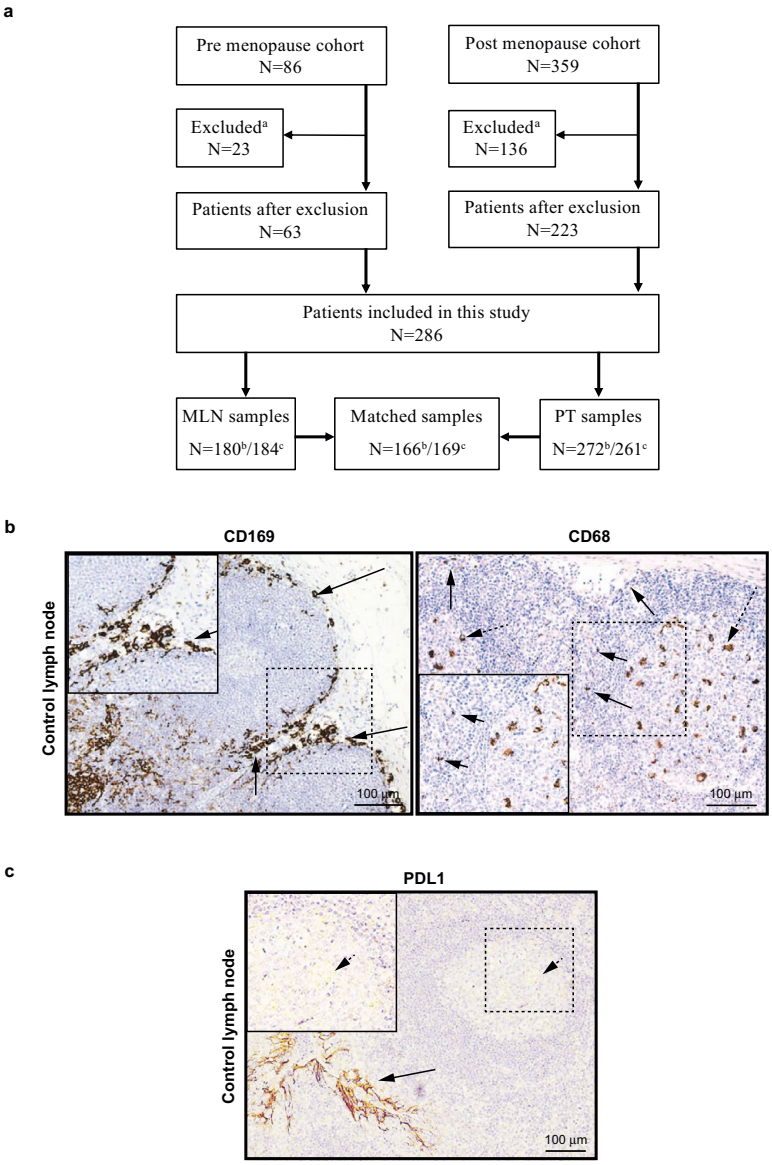


Figure 1. Cohort flow diagram and immunohistochemical staining of biomarkers in lymph node. (a) Cohort flow diagram for biomarker evaluation. [a]Excluded = both primary tumor (PT) and metastatic lymph node (MLN) material missing. [b] CD169 and PDL1 evaluation. [c] CD68 evaluation. (b) Positive staining control for CD169 (left) and CD68 (right) macrophages in a metastasis free control lymph node from a breast cancer patient. Arrows point to Subcapsular sinus macrophages ($CD169^+$) surrounding the lymphoid follicles. The CD68 staining was titrated to show differences in intensity of CD68 in the various macrophage compartments in human lymph node, where black arrows point to subcapsular sinus macrophages with weak CD68 expression and dashed arrows point to germinal center tingible body macrophages with a strong CD68 expression.[32] (c) Positive staining control for PDL1 in a human tonsil. Arrows point to epithelial crypt cells (black arrows) and to a small extent and of weak expression in the germinal center macrophages in lymphoid follicles (dashed arrows) as previously described.[33]

Table 1. Patient characteristics and clinicopathological features.

Variable	Included (*n = 286*)	(%)	Excluded (*n = 159*)	(%)
Age (years)				
Median (range)	62	(26–81)	64	(33–80)
<50	57	(20)	21	(13)
≥50	229	(80)	138	(87)
Menopausal status				
Pre	63	(22)	23	(14)
Post	223	(78)	136	(86)
Tumor size				
<20 mm	88	(31)	42	(26)
≥20 mm	198	(69)	117	(74)
Histological grade				
G1	15	(6)	2	(10)
G2	181	(66)	10	(45)
G3	78	(28)	10	(45)
missing	12		137	
Ki67				
Low (≤20%)	172	(63)	12	(67)
High (>20%)	99	(37)	6	(33)
missing	15		141	
ER				
Neg (<10%)	77	(29)	38	(25)
Pos (≥10%)	193	(71)	114	(75)
missing	16		7	
PR				
Neg (<10%)	123	(47)	84	(56)
Pos (≥10%)	140	(53)	67	(44)
missing	23		8	
HER2				
Neg	205	(87)	125	(84)
Pos	30	(13)	24	(16)
missing	51		10	
TNBC				
No	173	(79)	119	(84)
Yes	45	(21)	22	(16)
missing	68		18	

ER = estrogen receptor, PR = progesterone receptor, HER2 = Human epidermal growth factor receptor 2, TNBC = triple negative breast cancer.

PD1, and different patient and tumor characteristics was analyzed using Fisher's exact test, logistic regression, or Mann–Whitney U test, where appropriate. When planning the study, 5-year distant recurrence-free interval (DRFi) was chosen as endpoint for the prognostic analyses of the experimental markers. Longer follow-up could have been used, but prognostic effects of biomarkers, measured at the time of diagnosis, tend to weaken with follow-up time leading to non-proportional hazards. DRFi was defined as the time from surgery of the primary tumor until radiological and/or biopsy-verified recurrence or breast cancer-related death. Kaplan–Meier graphs were used to illustrate differences in 5-year DRFi according to CD169, CD68, PDL1, and PD1 expression and log-rank tests used to quantify the evidence against the null hypotheses of equality. Cox regression models were used for estimation of hazard ratios (HR) with 95% confidence interval (CI) according to CD169 expression in metastatic lymph node in both uni- and multivariable analysis. Proportional hazards assumptions were checked graphically. The established prognostic factors tumor size, histological grade, ER, PR, Ki67, HER2, and age, were included in multivariable Cox analyses. Statistical calculations were performed using IBM SPSS Statistics (version 26.0). All *P* values presented are two-sided and should in general be regarded as continuous measures of evidence, but following Benjamin et al., two thresholds will be used throughout this paper: suggestive evidence for *P* values between 0.05 and 0.005 and significant evidence for $P < 0.005$.[37]

Results

Distribution and characterization of CD169$^+$ macrophages

To investigate CD169$^+$ lymph node macrophages and CD169$^+$ TAMs, antibodies were chosen that recognize resident subcapsular sinus CD169$^+$ macrophages surrounding the lymphoid follicles in lymph nodes (Figure 1(b)), and the pan-macrophage marker CD68 used at a concentration and time to visualize the various staining intensities that macrophages have in different locations of human lymph node (Figure 1(b)).[36] A PDL1 antibody that recognized cells primarily in the epithelial crypt cells of human tonsil and to a small extent and of weak expression in the germinal center macrophages as previously shown,[33] was chosen and verified (Figures 1(c) and 2(a) left).

In lymph node with metastasis, the investigated lymph node CD169$^+$ macrophages were located in direct contact with lymph node metastases, mostly surrounding and not preferentially infiltrating (Figure 2(a) left). Lymph node macrophages in general (CD68$^+$), and PD1$^+$ lymphocytes, were present also in the metastases and in lymphoid follicles (Figure 2(a) right). When PDL1 expression was present in the lymph node metastases, it was found primarily in the malignant cells *per se* or co-expressed on CD169$^+$ macrophages (Figure 2(a) left and Figure 2(b)). In the primary tumor, CD169$^+$ tumor-associated macrophages (CD169$^+$ TAMs) were often associated near or in direct contact with PDL1$^+$ malignant cells, and co-expression of CD169$^+$PDL1$^+$ on macrophages was also observed (Figure 2(c) right).

Association between the experimental biomarkers and clinicopathological parameters

Presence of CD169$^+$ macrophages in primary tumor (CD169$^+$ PT) showed evidence of correlation with high Ki67 in the primary tumor, as well as with premenopausal status (Table 2). Presence of CD169$^+$ cells in metastatic lymph node (CD169$^+$ MLN) on the other hand, correlated with smaller primary tumor size, and to a lesser degree with PR-positivity (PR$^+$) of the lymph node metastasis (Table 2).

Just like CD169$^+$ macrophages, presence of any CD68$^+$ TAMs in the primary tumor in general (CD68$^+$ PT), correlated with high Ki67 in the primary tumor and to premenopausal status. It further correlated with ER-negativity (ER$^-$) of the primary tumor and higher histological grade. Interestingly, presence of CD68 in metastatic lymph node (CD68$^+$ MLN) only showed evidence of correlation with high Ki67 in primary tumor and to some extent with high Ki67 in lymph node metastasis (Table 2).

PDL1 expression in the primary tumor (PDL1$^+$ PT) showed evidence for correlation with ER$^-$ in both primary tumor and lymph node metastases, as well as to a TNBC primary tumor subtype (Table 3). It further correlated with high Ki67 in both primary tumor and lymph node metastases and PR-negativity (PR$^-$) in the primary tumor (Table 3). PDL1 expression in metastatic lymph node (PDL1$^+$ MLN) correlated both with younger age and a premenopausal status (Table 3).

Since most patients had PD1$^+$ cells present in the primary tumor, and all had PD1$^+$ cells present in the metastatic lymph node, high infiltration of PD1$^+$ immune cells (PD1^{high}) was used for statistical evaluation. As shown in Supplementary Table 1, PD1^{high} in the primary tumor correlated with ER$^-$, high Ki67,

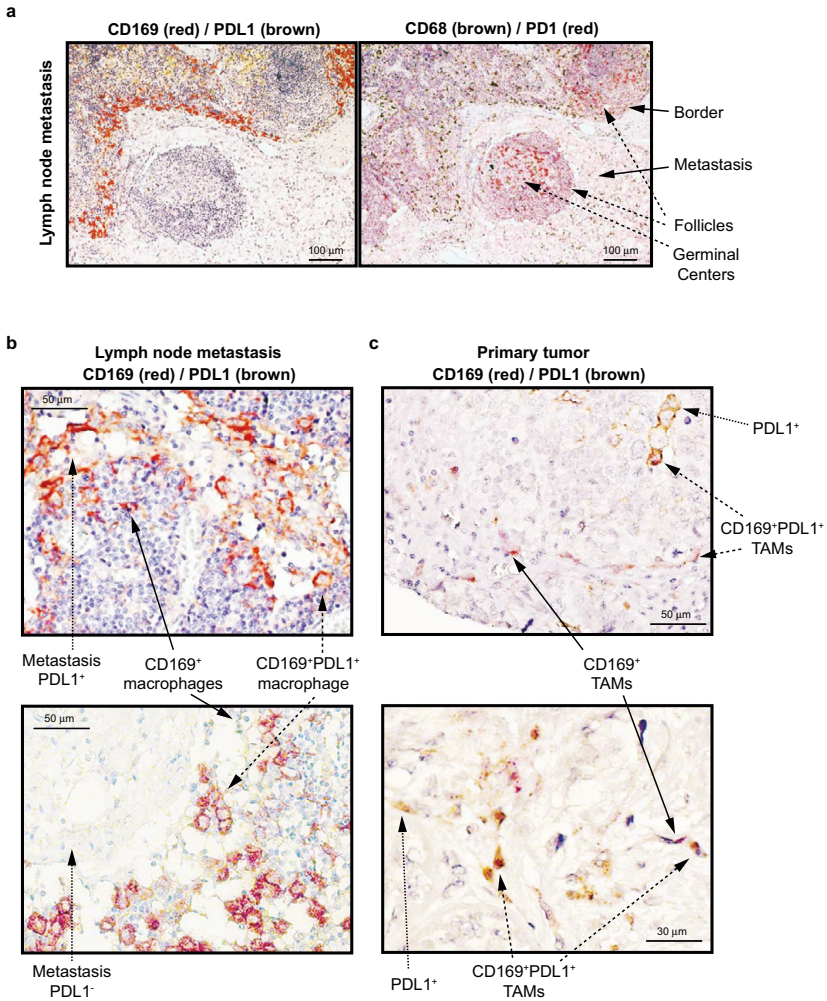


Figure 2. Immunohistochemical staining of CD169, PDL1 and CD68 in primary human breast tumors with paired lymph node metastases. (a) CD169 expression (red) and PDL1 expression (brown) (left) and CD68 expression (brown) and PD1 expression (red) (right) in lymph node metastases of breast cancer patients. Arrows point to the indicated histological structures. (b) CD169 expression (red) and PDL1 expression (brown) in lymph node metastases of breast cancer patients. The images show two metastases representing a PDL1$^+$ (upper) and a PDL1$^-$ (lower) metastasis. Arrows point to single PDL1$^+$ malignant cells, or co-expressing CD169$^+$ PDL1$^+$ macrophages. (c) CD169 expression (red) and PDL1 expression (brown) in primary tumor. Arrows point to single PDL1$^+$ malignant cells, single CD169$^+$ tumor associated macrophages (TAMs) or co-expressing CD169$^+$ PDL1$^+$ TAMs. The images show two representative primary tumors.

and a TNBC subtype in the primary tumor. PD1^{high} in metastatic lymph node did not correlate with any of the clinicopathological features.

Correlation of CD169$^+$ macrophages with PDL1, and PD1 expression

We next investigated whether CD169 expression would correlate with PDL1 expression as previously shown in viral infections.[12] Indeed, CD169 expression correlated positively with PDL1 expression both in primary tumor (OR = 8.4, 95% CI: (3.8–18.6), $P < 0.001$) and metastatic lymph node (OR = 3.6, 95% CI: (2.1–-6.4), $P < 0.001$), although PDL1 expression was mostly present on adjacent cells, and only occasionally on the same cell (CD169$^+$PDL1$^+$) (Figure 2(c)). Co-expression of CD169 and PDL1 in the primary tumor (CD169$^+$PDL1$^+$ PT) correlated with ER$^-$ in primary tumor and lymph node, PR$^-$ in primary tumor, high Ki67 in primary tumor, and positively with a TNBC primary

Table 2. Odds ratios of presence of CD169$^+$ and CD68$^+$ macrophages in metastatic lymph node (MLN) and primary tumor (PT) by patient and tumor clinicopathological features.

		CD169$^+$ PT				CD169$^+$ MLN				CD68$^+$ PT				CD68$^+$ MLN			
Clinicopathological features		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		63[†]	26–81[‡]	0.15[b]	272	63[†]	26–81[‡]	0.21[b]	180	63[†]	26–81[‡]	0.11[b]	261	63[†]	26–81[‡]	0.087[b]	184
Menopausal status	Pre	1			58	1			41	1			56	1			42
	Post	0.38	0.17–0.84	0.012	214	0.64	0.26–1.57	0.40	139	0.40	0.19–0.84	0.015	205	0.83	0.37–1.84	0.70	142
Tumor size	≤20 mm	1			78	1			81	1			76	1			82
	>20 mm	1.13	0.63–2.04	0.76	194	0.31	0.14–0.68	0.003	99	1.20	0.68–2.12	0.56	185	0.64	0.33–1.26	0.24	102
Histological grade	G1	1			15	1			10	1			15	1			11
	G2	0.87	0.27–2.86	0.82[c]	173	0.91	0.18–4.56	0.91[c]	116	2.09	0.72–6.05	0.18[c]	164	2.17	0.62–7.60	0.23[c]	119
	G3	1.24	0.35–4.40	0.74[c]	75	0.61	0.11–3.23	0.56[c]	48	5.81	1.77–19.06	0.004[c]	73	2.80	0.72–10.97	0.14[c]	48
Ki67 PT	Low (≤20%)	1			169	1			109	1			162	1			113
	High (>20%)	2.31	1.24–4.33	0.009	97	1.45	0.68–3.11	0.36	60	7.19	3.38–15.28	<0.001	94	3.29	1.42–7.63	0.004	60
Ki67 MLN	Low (≤20%)	1			48	1			46	1			45	1			47
	High (>20%)	2.93	0.75–11.48	0.15	19	1.82	0.51–6.42	0.55	19	3.84	0.78–18.92	0.12	19	6.88	0.83–56.92	0.051	19
ER PT	Neg (<10%)	1			74	1			43	1			72	1			43
	Pos (≥10%)	0.93	0.51–1.72	0.88	185	1.37	0.62–3.01	0.53	127	0.40	0.20–0.78	0.006	176	0.91	0.41–1.99	1.00	131
ER MLN	Neg (<10%)	1			43	1			45	1			42	1			46
	Pos (≥10%)	1.39	0.64–3.01	0.42	122	1.27	0.58–2.78	0.54	126	0.59	0.27–1.33	0.25	116	1.10	0.52–2.34	0.85	129
PR PT	Neg (<10%)	1			119	1			76	1			113	1			77
	Pos (≥10%)	1.10	0.63–1.91	0.78	135	1.63	0.80–3.33	0.21	90	0.58	0.33–1.02	0.070	130	0.53	0.26–1.06	0.085	93
PR MLN	Neg (<10%)	1			82	1			84	1			77	1			86
	Pos (≥10%)	1.65	0.81–3.37	0.21	81	2.27	1.09–4.75	0.031	85	0.98	0.50–1.92	1.00	79	0.85	0.44–1.67	0.73	87
HER2 PT	Neg	1			202	1			124	1			159	1			127
	Pos	1.12	0.45–2.77	1.00	29	0.48	0.17–1.33	0.16	19	2.38	0.87–6.53	0.090	28	1.00	0.36–2.79	1.00	20
HER2 MLN	Neg	1			72	1			76	1			67	1			76
	Pos	1.33	0.34–5.26	1.00	15	0.56	0.19–1.68	0.38	16	1.47	0.37–5.82	0.75	15	0.96	0.30–3.06	1.00	16
TNBC	No	1			170	1			113	1			164	1			117
	Yes	1.82	0.79–4.19	0.18	45	1.17	0.40–3.47	1.00	22	1.91	0.86–4.25	0.14	45	0.95	0.36–2.54	1.00	22

Abbreviations: ER = estrogen receptor. PR = progesterone receptor. HER2 = human epidermal growth factor receptor 2. TNBC = triple negative breast cancer. PT = primary tumor. MLN = metastatic lymph node.
OR = Odds ratio. 95%CI = 95% confidence interval.
[†]Median age in years. [‡]Range in years.
[a]Fisher's exact test unless otherwise stated. [b]Mann-Whitney U test. [c]Logistic regression.

Table 3. Odds ratios of presence of PDL1$^+$ cells and co-expressing CD169$^+$PDL1$^+$ cells in metastatic lymph node (MLN) and primary tumor (PT) by patient and tumor clinicopathological features.

		PDL1$^+$ PT				PDL1$^+$ MLN				CD169$^+$PDL1$^+$ PT				CD169$^+$PDL1$^+$ MLN			
Clinicopathological features		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		63[†]	26–81[‡]	0.21[b]	272	63[†]	26–81[‡]	0.013[b]	180	63[†]	26–81[‡]	0.16[b]	272	63[†]	26–81[‡]	0.011[b]	180
Menopausal status	Pre	1			58	1			41	1			58	1			41
	Post	0.67	0.35–1.29	0.27	214	0.43	0.19–0.97	0.042	139	0.65	0.36–1.19	0.20	214	0.73	0.36–1.47	0.47	139
Tumor size	≤20 mm	1			78	1			81	1			78	1			81
	>20 mm	1.25	0.72–2.18	0.47	194	1.02	0.55–1.88	1.00	99	1.40	0.78–2.50	0.32	194	0.74	0.41–1.33	0.36	99
Histological grade	G1	1			15	1			10	1			15	1			10
	G2	0.56	0.17–1.84	0.34[c]	173	1.09	0.29–4.08	0.90[c]	116	2.29	0.50–10.52	0.29[c]	173	0.55	0.15–2.00	0.36[c]	116
	G3	1.58	0.44–5.72	0.48[c]	75	1.62	0.40–6.63	0.50[c]	48	6.00	1.27–28.44	0.024[c]	75	1.29	0.33–5.03	0.72[c]	48
Ki67 PT	Low (≤20%)	1			169	1			109	1			169	1			109
	High (>20%)	3.58	1.93–6.64	<0.001	97	1.53	0.77–3.02	0.24	60	3.93	2.29–6.76	<0.001	97	1.48	0.78–2.79	0.26	60
Ki67 MLN	Low (≤20%)	1			48	1			46	1			48	1			46
	High (>20%)	8.18	1.00–67.09	0.028	19	1.49	0.46–4.90	0.57	19	1.96	0.65–5.95	0.26	19	1.58	0.54–4.63	0.43	19
ER PT	Neg (<10%)	1			74	1			43	1			74	1			43
	Pos (≥10%)	0.38	0.20–0.72	0.003	185	0.55	0.25–1.19	0.14	127	0.40	0.23–0.71	0.002	185	0.73	0.36–1.46	0.38	127
ER MLN	Neg (<10%)	1			43	1			45	1			43	1			45
	Pos (≥10%)	0.42	0.19–0.96	0.040	122	0.59	0.28–1.25	0.20	126	0.45	0.22–0.94	0.050	122	0.94	0.47–1.86	0.86	126
PR PT	Neg (<10%)	1			119	1			76	1			119	1			76
	Pos (≥10%)	0.53	0.31–0.90	0.023	135	0.80	0.42–1.52	0.52	90	0.51	0.30–0.88	0.020	135	0.67	0.36–1.24	0.21	90
PR MLN	Neg (<10%)	1			82	1			84	1			82	1			84
	Pos (≥10%)	0.98	0.51–1.87	1.00	81	0.79	0.42–1.48	0.52	85	0.85	0.43–1.68	0.73	81	0.77	0.42–1.42	0.44	85
HER2 PT	Neg	1			202	1			124	1			202	1			124
	Pos	1.86	0.72–4.79	0.28	29	0.59	0.22–1.56	0.31	19	2.91	1.32–6.43	0.010	29	0.66	0.24–1.85	0.46	19
HER2 MLN	Neg	1			72	1			76	1			72	1			76
	Pos	1.54	0.39–6.03	0.75	15	0.79	0.27–2.36	0.44	16	2.28	0.73–7.10	0.22	15	0.93	0.27–3.23	1.00	16
TNBC	No	1			170	1			113	1			170	1			113
	Yes	2.59	1.13–5.91	0.020	45	2.87	0.91–9.04	0.088	22	2.66	1.35–5.23	0.006	45	2.54	1.00–6.44	0.058	22

Abbreviations: ER = estrogen receptor. PR = progesterone receptor. HER2 = human epidermal growth factor receptor 2. TNBC = triple negative breast cancer. PT = primary tumor. MLN = metastatic lymph node.
OR = Odds ratio. 95%CI = 95% confidence interval.
[†]Median age in years. [‡]Range in years.
[a]Fisher's exact test unless otherwise stated. [b]Mann-Whitney U test. [c]Logistic regression.

tumor subtype, the same clinicopathological features that correlated with PDL1 expression alone in primary tumor, with the exception of high Ki67 in lymph node. Co-expression of CD169 and PDL1 in primary tumor showed evidence for further correlation with higher histological grade and HER2$^+$ in primary tumor (Table 3). Co-expression of CD169 and PDL1 in metastatic lymph node (CD169$^+$PDL1$^+$ MLN) only showed evidence for a correlation with age, just as younger age correlated with PDL1 expression alone in metastatic lymph node (Table 3).

CD169 expression did not correlate with PD1 expression in the primary tumor (OR = 1.77, 95% CI: (0.70–4.48), *P = 0.22*) or in the metastatic lymph node (OR = 2.08, 95% CI: (0.68–6.38), *P = 0.19*), while PDL1 expression correlated with PD1 expression both in the primary tumor (OR = 9.44, 95% CI: (2.21–-40.40), *P < 0.001*) and the metastatic lymph node (OR = 4.14, 95% CI: (1.37–12.52), *P = 0.007*).

The prognostic importance of the experimental biomarkers when analyzed individually

In univariable analyses, suggestive evidence for an association to better prognosis was seen for patients with CD169$^+$ macrophages in metastatic lymph node compared to patients with no CD169$^+$ macrophages in metastatic lymph node (Figure 3(a) left; HR = 0.46, 95% CI: (0.25–0.85), *P = 0.013*). This association was not seen when considering CD169 macrophages in the primary tumor (Figure 3(a) right; HR = 1.32, 95% CI: (0.73–2.41), *P = 0.35*). In contrast, patients with CD68$^+$ macrophages in the primary tumor had worse prognosis compared to patients with no CD68$^+$ macrophages in the primary tumor (Figure 3(b) right; HR = 2.24, 95% CI: (1.17–4.30), *P = 0.016*), an association not seen when considering CD68$^+$ macrophages in the metastatic lymph node (Figure 3(b) left; HR = 0.67, 95% CI: (0.36–1.22), *P = 0.19*). Interestingly, suggestive evidence for the same survival trend as for CD68 was seen for PDL1 expression *per se*, with no association in the metastatic lymph node (Figure 3(c) left; HR = 0.79, 95% CI: (0.43–1.43), *P = 0.44*), but with an association to worse prognosis for patients with PDL1$^+$ primary tumors (Figure 3(c) right; HR = 1.82, 95% CI: (1.00–3.29), *P = 0.049*). Suggestive evidence was also seen for an association between PD1^{high} in the primary tumor and worse prognosis (Supplementary Fig. 1A right; HR = 2.01, 95% CI: (1.09–3.72), *P = 0.025*) in

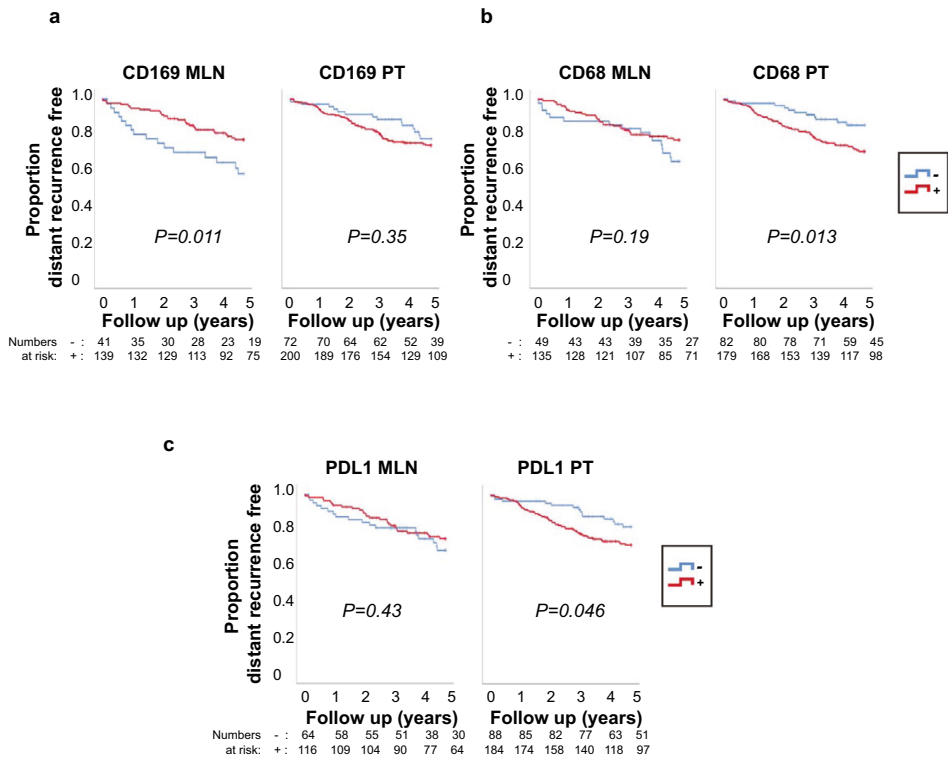


Figure 3. Differences in 5-year distant recurrence-free interval (DRFi) according to CD169, CD68 and PDL1 expression in metastatic lymph node (MLN) and primary tumors (PT) of breast cancer patients. *P* value by log-rank test. (a) CD169 expression – in metastatic lymph node (CD169 MLN) (left) and primary tumor (CD169 PT) (right). (b) CD68 expression (-/+) in metastatic lymph node (CD68 MLN) (left) and primary tumor (CD68 PT) (right). (c) PDL1 expression (-/+) in metastatic lymph node (PDL1 MLN) (left) and primary tumor (PDL1 PT) (right).

agreement with previous studies.[38,39] This association was not seen in the metastatic lymph node (Supplementary Fig. 1A left; HR = 0.81, 95% CI: (0.34–1.93), *P = 0.64*).

We next performed multivariable analyses. The suggestive evidence for a better prognosis for patients with CD169$^+$ macrophages in the metastatic lymph node prompted us to investigate whether the lymph node CD169$^+$ macrophages had an independent prognostic effect on 5-year DRFi. A series of Cox regression analyses adjusting for tumor size, histological grade, ER, PR, Ki67, HER2, and age, both individually and all together, were performed and summarized in a forest plot (Figure 4). Unadjusted, presence of CD169$^+$ macrophages in metastatic lymph node was associated to better prognosis (see above), but the association was considerably weaker after multivariable adjustment (HR = 0.70, 95% CI: (0.32–1.50), *P = 0.36*).

Prognostic importance of experimental biomarker combinations

We continued investigating the prognostic importance of experimental biomarker combinations, starting within the primary tumor and metastatic lymph node, separately. When combining the individual scoring of CD169 and PDL1 expression in the metastatic lymph node (Figure 5(a) left), PDL1 expression did not add prognostic information for either the CD169$^+$ group (red lines Figure 5(a) left; HR = 0.96, 95% CI: (0.41–2.25), *P = 0.93*), or the CD169$^-$ group (blue lines Figure 5(a) left; HR = 0.85, 95% CI: (0.29–2.44), *P = 0.76*). In contrast, in the primary tumor, there was a tendency that patients with PDL1$^-$ tumors had a better prognosis than patients with PDL1$^+$ tumors in both the CD169$^+$ group (red lines Figure 5(a) right; HR = 0.74, 95% CI: (0.37–-1.48), *P = 0.40*) and the CD169$^-$ group (blue lines Figure 5(a) right; HR = 0.31, 95% CI: (0.10–1.00), *P = 0.05*). Based on these results, we decided to compare the two extreme groups. Patients lacking both CD169 and PDL1 expression in primary tumor (solid blue line Figure 5(a) right) had better prognosis compared to patients positive for both CD169 and PDL1 (red dashed line Figure 5(a) right; HR = 0.36, 95% CI: (0.13–1.00), *P = 0.05*).

We next investigated the effect of PDL1$^+$ primary tumors on lymph node macrophages. Since primary tumors have the capacity to modify draining lymph nodes[40] and PDL1 expression is induced by IFNs and proinflammatory cytokines that can be produced at higher levels in breast tumor subtypes like TNBC,[12,36] we investigated whether PDL1$^+$ primary tumors would affect the prognostic importance of metastatic lymph node macrophages to a higher extent than PDL1$^-$ primary tumors would. Interestingly, when stratifying for PDL1 expressing primary tumors (Figure 5(b,c)), we saw that patients with CD169$^+$ macrophages in metastatic lymph node seemed to have a better prognosis only when primary tumors were PDL1$^+$ (Figure 5(b) left; HR = 0.45, 95% CI: (0.22–0.94), *P = 0.033*). This trend was not observed in patients with PDL1$^-$ tumors (Figure 5(b) right; HR = 0.68, 95% CI: (0.20–2.31), *P = 0.53*). When the same division was used to analyze CD68$^+$ macrophages in PDL1$^+$ tumors, no effect was seen (Figure 5(c) left; HR = 0.86, 95% CI: (0.37–1.99), *P = 0.73*). However, patients with CD68$^+$ macrophages in metastatic lymph node and PDL1$^-$ tumor did show a trend toward better prognosis (Figure 5(c) right; HR = 0.30, 95% CI: (0.08–1.16), *P = 0.080*).

Finally, we analyzed CD169 and PDL1 co-expression. Patients with co-expression of CD169 and PDL1 on either the same cell or nearby cells (CD169$^+$PDL1$^+$) in metastatic lymph nodes had slightly better prognosis (Supplementary Fig. 1B left; HR = 0.60, 95% CI: (0.32–1.12), *P = 0.11*) and in primary tumors a slightly worse prognosis compared to all other patients, but the evidence was weak (Supplementary Fig. 1B right; HR = 1.42, 95% CI: (0.85–2.36), *P = 0.18*).

Discussion

In this study we observed that CD169$^+$ macrophages presence near lymph node metastases of breast cancer patients was associated

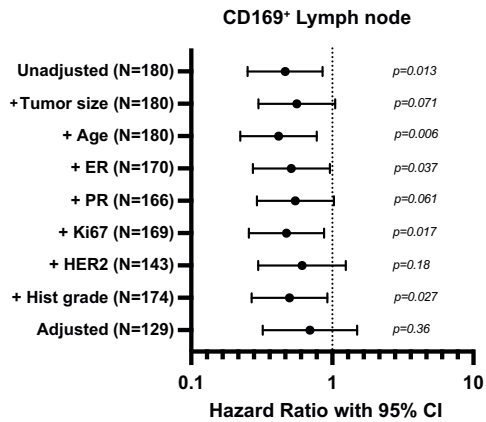


Figure 4. Forest plot showing results from Cox regression analysis on 5-year distant recurrence-free interval (DRFi) in breast cancer patients with CD169 expression in metastatic lymph node. Adjusted for tumor size, age, estrogen receptor (ER), progesterone receptor (PR), Ki67 expression, HER2 status and histological grade, both individually and all together. Dots indicate hazard ratios, horizontal lines indicate 95% confidence interval (95% CI). Note that the scale is logarithmic.

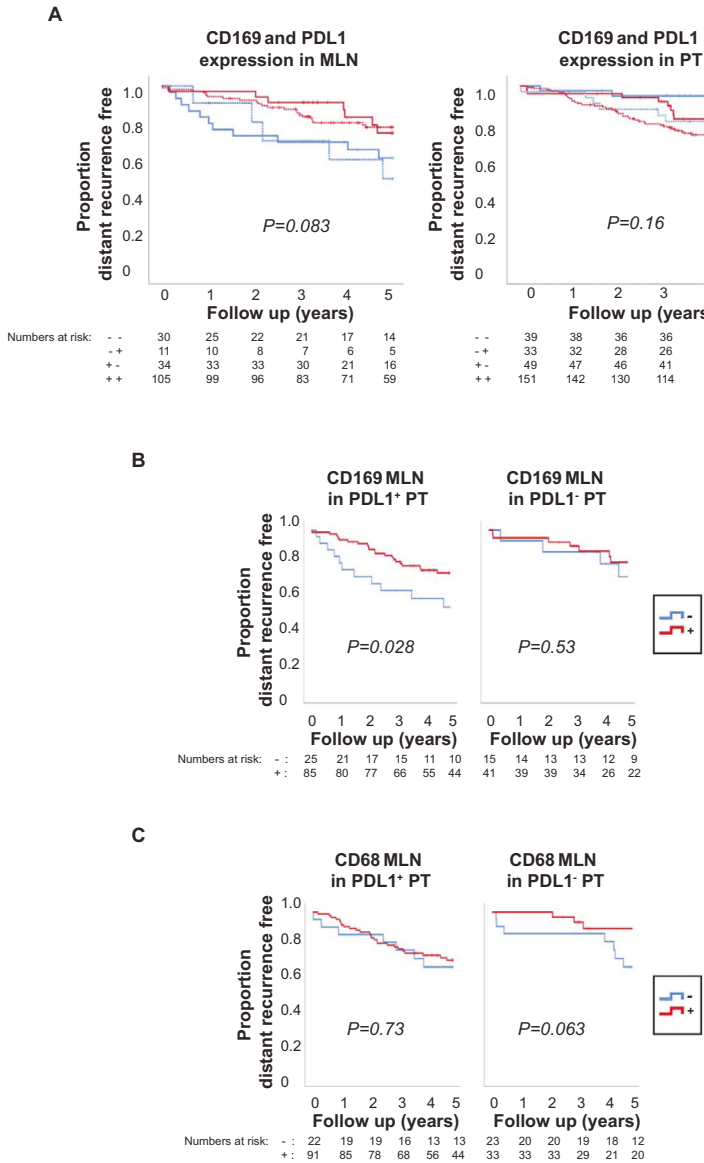


Figure 5. Kaplan-Meier curves illustrating differences in 5-year distant recurrence-free interval (DRFi) according to CD169, CD68 and PDL1 expression in metastatic lymph node (MLN) and primary tumors (PT) of breast cancer patients. *P* value by log-rank test. (a) Combined individual expression of CD169 and PDL1 in metastatic lymph node (left) and primary tumor (right). Solid lines indicate PDL1^{-} tumors, and dashed lines PDL1^{+} tumors, with (red) or without (blue) CD169 expression respectively (3-df test). (b) CD169 expression (-/+) in metastatic lymph node (CD169 MLN) in patients with PDL1 positive primary tumor (PDL1^{+} PT) (left) and PDL1 negative primary tumor (PDL1^{-} PT) (right). (c) CD68 expression (-/+) in metastatic lymph node (CD68 MLN) in patients with PDL1 positive primary tumor (PDL1^{+} PT) (left) and PDL1 negative primary tumor (PDL1^{-} PT) (right).

with smaller tumor size and, in univariable analyses, to improved prognosis after adjuvant tamoxifen. This is in contrast with CD68^{+} macrophages in lymph node metastases, which were not associated with prognosis, although these macrophages were associated with more aggressive tumor characteristics of the primary tumor (higher histological grade, high Ki67, and ER-negativity).

One possible explanation to this difference in prognostic importance may be that patients with advanced tumors have a stronger tumor-derived effect on the draining lymph node follicles, resulting in loss of beneficial CD169$^+$ subcapsular sinus macrophages specifically.[41] Another explanation could be that the CD169$^+$ macrophages present in metastatic lymph nodes reorganize to other sites picking up tumor antigens for cross presentation.[16] Our findings in this study differ from another study published on CD169$^+$ lymph node subcapsular sinus macrophages in breast cancer patients.[21] There, presence of CD169$^+$ lymph node macrophages correlated to small tumor size, no lymph node metastasis, and low Ki67 in the primary tumor, but did not correlate with relapse-free or breast cancer-specific survival. The reason for this may be that in our study we evaluated CD169$^+$ macrophages in direct contact with metastasis, while Shiota *et al.* only used cancer cell-free lymph nodes for analysis and did not analyze CD169 expression in the primary tumor samples, only CD8 expression.[21] To our knowledge, we here show for the first time that CD169$^+$ macrophages located in direct vicinity of lymph node metastasis in breast cancer patients, correlate with improved prognosis. The evidence for a prognostic importance in our study was, however, not retained after adjustment for other clinicopathological features. In multivariable analysis, we found that the presence of CD169$^+$ macrophages in lymph node metastases was not a strong independent risk factor for prognosis. The patients in the cohort used in this study had all received adjuvant tamoxifen, which also could have an impact on outcome of this study, and therefore further studies are needed to verify our results. On the other hand, this fact also excludes any treatment-related effect on the CD169$^+$ macrophages other than tamoxifen.

We also compared the differences between CD169$^+$ macrophages in metastatic lymph node and primary tumor. In many cases, although the correlation with clinicopathological biomarkers was weak, the location of CD169$^+$ macrophages rendered opposite trends in metastatic lymph node and primary tumor. The same was noted for the 5-year DRFi analysis where CD169$^+$ macrophages in metastatic lymph node correlated with better prognosis while CD169$^+$ macrophages in primary tumor did not. At this stage, it is impossible to say whether the CD169$^+$ macrophages in the metastatic lymph nodes are solely resident CD169$^+$ macrophages or a blend of resident and monocyte-derived CD169$^+$ macrophages. Our finding would, however, support that the CD169$^+$ macrophages in these two different locations have different functions with regard to tumor cells, or adaptive immune cells, and that they most likely have different origin, although further evidence is needed to prove this. These findings could also give an explanation to a previous experimental study performed in mice, where depletion of all CD169$^+$ macrophages, and not only lymph node resident, lead to a reduced breast tumor growth and less metastasis.[42] Interestingly, high expression of *SIGLEC1* in primary breast tumors has formerly been associated with shorter recurrence-free survival in public datasets.[22]

Around 30% of the primary tumors were PDL1$^{+,}$ and PDL1 expression in the primary tumor of the breast cancer patients in this cohort correlated with PD1 expression, TNBC primary tumor subtype classification, and hallmarks of TNBC; ER and PR negativity and high Ki67. This is in line with previous research that shows that PDL1 is associated with more aggressive basal subtypes of breast cancer.[43] We further saw that breast cancer patients with PDL1 expression in the primary tumor had worse prognosis than patients with PDL1 negative tumors. The same effect was not seen when PDL1 expression in the lymph node metastasis was examined. PDL1 expression on APCs, as compared to on malignant cells, is of more relevance for successful anti-PDL1 therapy.[44] Interestingly, a recent study showed that it was PDL1 expression on tumor-infiltrating lymphocytes (TILs) in tumors of TNBC patients, but not on the tumor cells themselves, that was associated with poor prognosis.[45] As mentioned before, in viral infections CD169$^+$ macrophages have been shown to induce type I IFNs that promotes PDL1 expression.[12] That supports our findings in this study, where the presence of CD169$^+$ macrophages both in primary tumor and in metastatic lymph node correlated with the presence of PDL1$^+$ cells in the same location. In our hands, the PDL1-expressing nonmalignant cells could probably be of both lymphoid as well as myeloid origin, but the CD169$^+$PDL1$^+$ co-expressing cells are most likely macrophages (APCs) as judged by their morphology and CD169 expression.

When we combined the individual scoring of PDL1 and CD169, we saw that CD169 expression was associated with the prognosis in the metastatic lymph node, while PDL1 expression affected the prognosis in the primary tumor negatively, although this was more pronounced in primary tumors lacking CD169$^+$ TAMs. Interestingly, though, patients with CD169$^+$ macrophages in metastatic lymph node seemed to have a better prognosis only when primary tumors were PDL1$^+$. When assessing co-expression, CD169$^+$PDL1$^+$, on the same or nearby cells, we observed a similar pattern. In the metastatic lymph nodes, the prognostic effect of CD169 alone is stronger than that of CD169$^+$PDL1$^+$ co-expressing cells. This indicates that CD169$^+$ macrophages, independent of PDL1 expression, are important for prognosis when present in metastatic lymph nodes, while in the primary tumors, a subpopulation of CD169$^+$ macrophages co-expressing PDL1 may have a worse effect on tumor progression than CD169$^+$ macrophages alone. Interestingly, the co-expression of CD169 and PDL1 in both primary tumor and metastatic lymph node did not seem to change the correlation to clinicopathological features that PDL1 expression alone had.

In conclusion, we observed that CD169$^+$ macrophages have a positive effect on the prognosis when expressed in the metastatic lymph node, compared to no effect when expressed in the primary tumor, which further supports the theory that CD169$^+$ macrophages differ in the properties between the two locations. This effect was not seen in patients with PDL1$^-$ primary tumors. We also observed that the expression of CD169 was correlated with expression of PDL1, both in metastatic lymph node and in the primary tumor. This merits further research since to our knowledge, the relationship between CD169 and PDL1 expression in breast cancer has not been explored, thus investigating the biological differences between lymph node and primary tumor CD169$^+$ macrophages will be of importance in the near future.

Abbreviations

APC	Antigen presenting cells
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2

PDL1	Programmed death-ligand 1
IHC	Immunohistochemical
MLN	Metastatic lymph node
PR	Progesterone receptor
TAM	Tumor associated macrophages
TMA	Tissue microarray
PT	Primary tumor
TNBC	Triple negative breast cancer

Acknowledgments

The authors thank Kristina Lövgren for professional technical skills in preparation of the TMA. The authors thank Kristina Ekström-Holka for professional technical skills in preparation of the IHC. This work was supported by grants from the Swedish Cancer Society; the Swedish Research Council; the Governmental Funding of Clinical Research within the National Health Service (ALF), the UMAS Cancer foundation, the Gunnar Nilsson's Cancer Foundation, the Åke Wibergs foundation, the Percy Falks Foundation, and the Gyllenstiernska Krapperups foundation.

Author contributions

FBG was responsible for analyzing data and for writing the initial manuscript together with KL. NA and FBG were responsible for annotating the IHC together with KL. LR and MF were responsible for the clinical patient cohort. POB and FBG were responsible for statistical evaluations. KL was responsible for designing the study, for analyzing data and for writing the initial manuscript.

Disclosure of potential conflicts 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 authors otherwise declare no competing interest.

Funding

This work was supported by the Swedish Cancer Society [grant number 18 0693]; Swedish Research Council [2017 02443].

ORCID

Frida Björk Gunnarsdottir http://orcid.org/0000-0002-2112-4382
Pär-Ola Bendahl http://orcid.org/0000-0001-8862-1845
Lisa Rydén http://orcid.org/0000-0001-7515-3130
Karin Leandersson http://orcid.org/0000-0001-8254-3137

Data availability

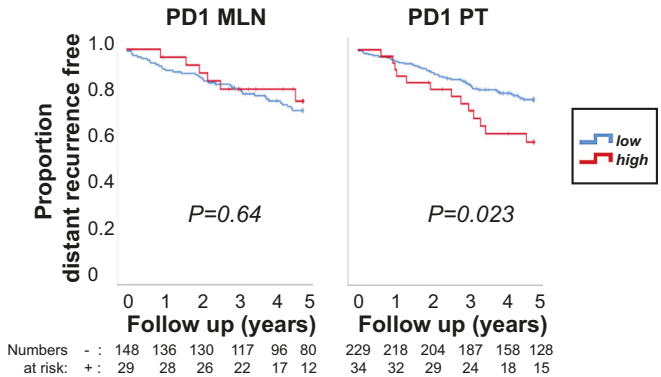
All datasets generated in the course of the current study are presented in the main text and the Supplementary Information available online.

References

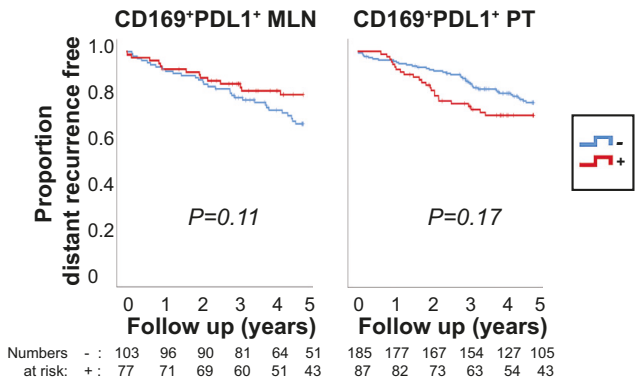
1. Curigliano G, Burstein HJ, Winer EP, Gnant M, Dubsky P, Loibl S, Colleoni M, Regan MM, Piccart-Gebhart M, Senn H-J. De-escalating and escalating treatments for early-stage breast cancer: the St. Gallen international expert consensus conference on the primary therapy of early breast cancer 2017. Ann Oncol. 2017 Aug 1;28(8):1700–1712. doi:10.1093/annonc/mdx308.
2. Sleeman JP. The lymph node pre-metastatic niche. J Mol Med (Berl). 2015 Nov;93(11):1173–1184.
3. Martinez-Pomares L, Gordon S. Antigen presentation the macrophage way. Cell. 2007 Nov 16;131(4):641–643. doi:10.1016/j.cell.2007.10.046.
4. Kohrt HE, Nouri N, Nowels K, Johnson D, Holmes S, Lee PP. Profile of immune cells in axillary lymph nodes predicts disease-free survival in breast cancer. PLoS Med. 2005 Sep;2(9):e284. doi:10.1371/journal.pmed.0020284.
5. Grotz TE, Jakub JW, Mansfield AS, Goldenstein R, Enninga EAL, Nevala WK, Leontovich AA, Markovic SN. Evidence of Th2 polarization of the sentinel lymph node (SLN) in melanoma. Oncoimmunology. 2015 Aug;4(8):e1026504. doi:10.1080/2162402X.2015.1026504.
6. Medrek C, Ponten F, Jirstrom K, Leandersson K. The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients [Research Support, Non-U.S. Gov't]. BMC Cancer. 2012;12(1):306. doi:10.1186/1471-2407-12-306.
7. Laoui D, Movahedi K, Van Overmeire E, Van den Bossche J, Schouppe E, Mommer C, Nikolaou A, Morias Y, De Baetselier P, Van Ginderachter JA, et al. Tumor-associated macrophages in breast cancer: distinct subsets, distinct functions [Review]. Int J Dev Biol. 2011;55(7–9):861–867. doi:10.1387/ijdb.113371dl.
8. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity [Review]. Curr Opin Immunol. 2010 Apr;22(2):231–237. doi:10.1016/j.coi.2010.01.009.
9. Serafini P. Myeloid derived suppressor cells in physiological and pathological conditions: the good, the bad, and the ugly [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S.]. Immunol Res. 2013 Dec;57(1–3):172–184.
10. Asano K, Kikuchi K, Tanaka M. CD169 macrophages regulate immune responses toward particulate materials in the circulating fluid. J Biochem. 2018 Aug 1;164(2):77–85. doi:10.1093/jb/mvy050.
11. Louie DAP, Liao S. Lymph node subcapsular sinus macrophages as the frontline of lymphatic immune defense. Front Immunol. 2019;10:347. doi:10.3389/fimmu.2019.00347.
12. Shaabani N, Duhan V, Khairnar V, Gassa A, Ferrer-Tur R, Häussinger D, Recher M, Zelinskyy G, Liu J, Dittmer U. CD169+ macrophages regulate PD-L1 expression via type I interferon and thereby prevent severe immunopathology after LCMV infection. Cell Death Dis. 2016 Nov 3;7(11):e2446. doi:10.1038/cddis.2016.350.
13. Muerkoster S, Rocha M, Crocker PR, Schirrmacher V, Umansky V. Sialoadhesin-positive host macrophages play an essential role in graft-versus-leukemia reactivity in mice. Blood. 1999 Jun 15;93(12):4375–4386. doi:10.1182/blood.V93.12.4375.
14. Martinez-Pomares L, Gordon S. CD169+ macrophages at the crossroads of antigen presentation. Trends Immunol. 2012 Feb;33(2):66–70. doi:10.1016/j.it.2011.11.001.
15. Garcia Z, Lemaitre F, van Rooijen N, Albert ML, Levy Y, Schwartz O, Bousso P. Subcapsular sinus macrophages promote NK cell accumulation and activation in response to lymph-borne viral particles. Blood. 2012 Dec 6;120(24):4744–4750. doi:10.1182/blood-2012-02-408179.
16. Asano K, Nabeyama A, Miyake Y, Qiu C-H, Kurita A, Tomura M, Kanagawa O, Fujii S-I, Tanaka M. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. Immunity. 2011 Jan 28;34(1):85–95. doi:10.1016/j.immuni.2010.12.011.
17. Ohnishi K, Yamaguchi M, Erdenebaatar C, Saito F, Tashiro H, Katabuchi H, Takeya M, Komohara Y. Prognostic significance of CD 169-positive lymph node sinus macrophages in patients with endometrial carcinoma. Cancer Sci. 2016 Jun;107(6):846–852. doi:10.1111/cas.12929.
18. Ohnishi K, Komohara Y, Saito Y, Miyamoto Y, Watanabe M, Baba H, Takeya M. CD169-positive macrophages in regional lymph nodes are associated with a favorable prognosis in patients with colorectal carcinoma. Cancer Sci. 2013 Sep;104(9):1237–1244. doi:10.1111/cas.12212.
19. Asano T, Ohnishi K, Shiota T, Motoshima T, Sugiyama Y, Yatsuda J, Kamba T, Ishizaka K, Komohara Y. CD169-positive sinus macrophages in the lymph nodes determine bladder cancer prognosis. Cancer Sci. 2018 May;109(5):1723–1730. doi:10.1111/cas.13565.
20. Stromvall K, Sundkvist K, Ljungberg B, Halin Bergström S, Bergh A. Reduced number of CD169 + macrophages in pre-metastatic regional lymph nodes is associated with subsequent metastatic disease in an

animal model and with poor outcome in prostate cancer patients. Prostate. 2017 Nov;77(15):1468–1477. doi:10.1002/pros.23407.
21. Shiota T, Miyasato Y, Ohnishi K, Yamamoto-Ibusuki M, Yamamoto Y, Iwase H, Takeya M, Komohara Y. The clinical significance of CD169-positive lymph node macrophage in patients with breast cancer. PLoS One. 2016;11(11):e0166680. doi:10.1371/journal.pone.0166680.
22. Cassetta L, Fragkogianni S, Sims AH, Swierczak A, Forrester LM, Zhang H, Soong DYH, Cotechini T, Anur P, Lin EY et al. Human tumor-associated macrophage and monocyte transcriptional landscapes reveal cancer-specific reprogramming, biomarkers, and therapeutic targets. Cancer Cell. 2019 Apr 15;35(4):588–602.e10. doi:10.1016/j.ccell.2019.02.009.
23. Pico de Coana Y, Choudhury A, Kiessling R. Checkpoint blockade for cancer therapy: revitalizing a suppressed immune system. Trends Mol Med. 2015 Aug;21(8):482–491. doi:10.1016/j.molmed.2015.05.005.
24. Swoboda A, Nanda R. Immune checkpoint blockade for breast cancer. Cancer Treat Res. 2018;173:155–165.
25. Schmid P, Adams S, Rugo HS, Schneeweiss A, Barrios CH, Iwata H, Diéras V, Hegg R, Im S-A, Shaw Wright G. Atezolizumab and Nab-paclitaxel in advanced triple-negative breast cancer. N Engl J Med. 2018 Nov 29;379(22):2108–2121. doi:10.1056/NEJMoa1809615.
26. Wu Y, Chen W, Xu ZP, Gu W. PD-L1 distribution and perspective for cancer immunotherapy-blockade, knockdown, or inhibition. Front Immunol. 2019;10:2022. doi:10.3389/fimmu.2019.02022.
27. Heeren AM, de Boer E, Bleeker MC, Musters RJP, Buist MR, Kenter GG, de Gruijl TD, Jordanova ES. Nodal metastasis in cervical cancer occurs in clearly delineated fields of immune suppression in the pelvic lymph catchment area. Oncotarget. 2015 Oct 20;6(32):32484–32493. doi:10.18632/oncotarget.5398.
28. Heeren AM, Koster BD, Samuels S, Ferns DM, Chondronasiou D, Kenter GG, Jordanova ES, de Gruijl TD. High and interrelated rates of PD-L1+CD14+ antigen-presenting cells and regulatory T cells mark the microenvironment of metastatic lymph nodes from patients with cervical cancer. Cancer Immunol Res. 2015 Jan;3(1):48–58. doi:10.1158/2326-6066.CIR-14-0149.
29. Ryden L, Jonsson PE, Chebil G, Dufmats M, Fernö M, Jirström K, Källström A-C, Landberg G, Stål O, Thorstenson S, et al. Two years of adjuvant tamoxifen in premenopausal patients with breast cancer: a randomised, controlled trial with long-term follow-up. Eur J Cancer. 2005 Jan;41(2):256–264. doi:10.1016/j.ejca.2004.06.030.
30. Swedish Breast Cancer Cooperative Group. Randomized trial of two versus five years of adjuvant tamoxifen for postmenopausal early stage breast cancer.. J Natl Cancer Inst. 1996 Nov 6;88 (21):1543–1549. doi:10.1093/jnci/88.21.1543.
31. Chebil G, Bendahl PO, Idvall I, Fernö M. Comparison of immunohistochemical and biochemical assay of steroid receptors in primary breast cancer–clinical associations and reasons for discrepancies. Acta Oncol. 2003;42(7):719–725. doi:10.1080/02841860310004724.
32. Bergenfelz C, Leandersson K. The generation and identity of human myeloid-derived suppressor cells. Front Oncol. 2020;10:109. doi:10.3389/fonc.2020.00109.
33. Lyford-Pike S, Peng S, Young GD, Taube JM, Westra WH, Akpeng B, Bruno TC, Richmon JD, Wang H, Bishop JA et al. Evidence for a role of the PD-1: PD-L1pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res. 2013 Mar 15;73 (6):1733–1741. doi:10.1158/0008-5472.CAN-12-2384.
34. Strand C, Bak M, Borgquist S, Chebil G, Falck A-K, Fjällskog M-L, Grabau D, Hedenfalk I, Jirström K, Klintman M, et al. The combination of Ki67, histological grade and estrogen receptor status identifies a low-risk group among 1,854 chemo-naive women with N0/N1 primary breast cancer. Springerplus. 2013 Dec;2 (1):111. doi:10.1186/2193-1801-2-111.
35. Gruvberger-Saal SK, Bendahl PO, Saal LH, Laakso M, Hegardt C, Eden P, Peterson C, Malmstrom P, Isola J, Borg A et al. Estrogen receptor beta expression is associated with tamoxifen response in ERalpha-negative breast carcinoma. Clin Cancer Res. 2007 Apr 1;13(7):1987–1994. doi:10.1158/1078-0432.CCR-06-1823.
36. Mehmeti M, Allaoui R, Bergenfelz C, Saal LH, Ethier SP, Johansson ME, Jirström K, Leandersson K. Expression of functional toll like receptor 4 in estrogen receptor/progesterone receptor-negative breast cancer. Breast Cancer Res. 2015 Sep 22;17(1):130. doi:10.1186/s13058-015-0640-x.
37. Benjamin DJ, Berger JO, Johannesson M, et al. Redefine statistical significance. Nat Hum Behav. 2018 Jan;2(1):6–10.
38. Sun S, Fei X, Mao Y, Wang X, Garfield DH, Huang O, Wang J, Yuan F, Sun L, Yu Q, et al. PD-1(+) immune cell infiltration inversely correlates with survival of operable breast cancer patients. Cancer Immunol Immunother. 2014 Apr;63(4):395–406. doi:10.1007/s00262-014-1519-x.
39. Muenst S, Soysal SD, Gao F, et al. The presence of programmed death 1 (PD-1)-positive tumor-infiltrating lymphocytes is associated with poor prognosis in human breast cancer. Breast Cancer Res Treat. 2013 Jun;139(3):667–676.
40. Jones D, Pereira ER, Padera TP. Growth and immune evasion of lymph node metastasis. Front Oncol. 2018;8:36. doi:10.3389/fonc.2018.00036.
41. Chang AY, Bhattacharya N, Mu J, Setiadi AF, Carcamo-Cavazos V, Lee GH, Simons DL, Yadegarynia S, Hemati K, Kapelner A, et al. Spatial organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients. J Transl Med. 2013 Oct;2(11):242. doi:10.1186/1479-5876-11-242.
42. Jing W, Guo X, Wang G, Bi Y, Han L, Zhu Q, Qiu C, Tanaka M, Zhao Y. Breast cancer cells promote CD169+ macrophage-associated immunosuppression through JAK2-mediated PD-L1 upregulation on macrophages. Int Immunopharmacol. 2020 Jan;78:106012. doi:10.1016/j.intimp.2019.106012.
43. Sabatier R, Finetti P, Mamessier E, Adelaide J, Chaffanet M, Ali HR, Viens P, Caldas C, Birnbaum D, Bertucci F et al. Prognostic and predictive value of PDL1 expression in breast cancer. Oncotarget. 2015 Mar 10;6(7):5449–5464. doi:10.18632/oncotarget.3216.
44. Herbst RS, Soria J-C, Kowanetz M, Fine GD, Hamid O, Gordon MS, Sosman JA, McDermott DF, Powderly JD, Gettinger SN et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature. 2014 Nov 27;515(7528):563–567. doi:10.1038/nature14011.
45. Wang X, Liu Y. PD-L1 expression in tumor infiltrated lymphocytes predicts survival in triple-negative breast cancer. Pathol Res Pract. 2020 Mar;216(3):152802. doi:10.1016/j.prp.2019.152802.

A


PD1 MLN
PD1 PT
Proportion distant recurrence free
1.0
0.8
0.6
0.4
0.2
0
P=0.64
P=0.023
low
high
0 1 2 3 4 5
Follow up (years)
Numbers at risk: - : 148 136 130 117 96 80
+ : 29 28 26 22 17 12
229 218 204 187 158 128
34 32 29 24 18 15

B


CD169+PDL1+ MLN
CD169+PDL1+ PT
Proportion distant recurrence free
1.0
0.8
0.6
0.4
0.2
0
P=0.11
P=0.17
-
+
0 1 2 3 4 5
Follow up (years)
Numbers at risk: - : 103 96 90 81 64 51
+ : 77 71 69 60 51 43
185 177 167 154 127 105
87 82 73 63 54 43

Supplementary Figure 1

A) Kaplan-Meier curves illustrating differences in 5-year distant recurrence-free interval (DRFi) according to a high ($PD1^{high}$) as compared to low ($PD1^{low}$) infiltration of PD1 expressing immune cells in metastatic lymph node (MLN) and primary tumors (PT) of breast cancer patients. PD1 presence in metastatic lymph node (PD1 MLN) (left) and primary tumor (PD1 PT) (right). P value by log-rank test. B) Kaplan-Meier curves illustrating differences in 5-year distant recurrence-free interval (DRFi) according to CD169 and PDL1 co-expression ($CD169^{+}PDL1^{+}$) in metastatic lymph node (MLN) and primary tumors (PT) of breast cancer patients. $CD169^{+}PDL1^{+}$ co-expression (-/+) on cells located in metastatic lymph node ($CD169^{+}PDL1^{+}$ MLN) (left) and primary tumor ($CD169^{+}PDL1^{+}$ PT) (right). P value by log-rank test.

Supplementary Table 1. Odds ratios of presence of a high number of $PD1^{+}$ infiltrating cells ($PD1^{high}$) in metastatic lymph node (MLN) and primary tumor (PT) by patient and tumor clinicopathological features.

Clinicopathological features		$PD1^{high}$ PT				$PD1^{high}$ MLN			
		OR	95%CI	*P* value[a]	N	OR	95%CI	*P* value[a]	N
Age		63[†]	26-81[‡]	0.77[b]	263	63[†]	26-81[‡]	0.29[b]	177
Menopausal status	Pre	1			54	1			40
	Post	0.57	0.26-1.28	0.176	209	1.49	0.53-4.19	0.63	137
Tumor size	≤20 mm	1			77	1			79
	>20 mm	0.99	0.45-2.19	1.00	186	0.60	0.27-1.34	0.23	98
Ki67 PT	Low (≤20%)	1			164	1			107
	High (>20%)	2.22	1.07-4.60	0.035	93	1.45	0.64-3.32	0.39	59
Ki67 MLN	Low (≤20%)	1			47	1			44
	High (>20%)	1.63	0.41-6.43	0.48	18	0.84	0.20-3.60	1.00	19
ER PT	Neg (<10%)	1			71	1			43
	Pos (≥10%)	0.44	0.20-0.97	0.048	179	0.43	0.18-1.02	0.059	124
ER MLN	Neg (<10%)	1			42	1			45
	Pos (≥10%)	1.00	0.34-2.96	1.00	118	0.56	0.23-1.33	0.23	124
PR PT	Neg (<10%)	1			113	1			75
	Pos (≥10%)	0.61	0.27-1.34	0.23	132	0.63	0.28-1.45	0.30	88
PR MLN	Neg (<10%)	1			79	1			84
	Pos (≥10%)	0.78	0.29-2.09	0.80	79	0.78	0.34-1.78	0.68	83
HER2 PT	Neg	1			198	1			121
	Pos	0.45	0.10-2.00	0.39	29	1.53	0.45-5.12	0.50	19
HER2 MLN	Neg	1			69	1			75
	Pos	0.91	0.18-4.65	1.00	15	0.75	0.15-3.73	1.00	16
TNBC	No	1			167	1			110
	Yes	3.64	1.52-8.74	0.007	44	2.57	0.86-7.67	0.10	22

Abbreviations: ER = estrogen receptor. PR = progesterone receptor. HER2 = human epidermal growth factor receptor 2. TNBC = triple negative breast cancer. PT = primary tumor. MLN = metastatic lymph node.
OR = Odds ratio. 95%CI = 95% confidence interval.
[†]Median age in years. [‡]Range in years.
[a]Fisher's exact test unless otherwise stated. [b]Mann-Whitney U test.

Paper III

CD169$^+$ macrophages present in primary tumors are monocyte derived type I IFN producers possessing immunosuppressive functions

Frida Björk Gunnarsdottir[1], Oscar Briem[§1], Aida Yifter Lindgren[§1], Eva Källberg[1], Cajsa Andersen[1], Robert Grenthe[1], Cassandra Rosenqvist[1], Mika Wallgren[1], Hannah Viklund[1], Camilla Rydberg Millrud[1], Daniel Bexell[2], Karin Jirström[3], Martin Johansson[4], Ingrid Hedenfalk[†5], Catharina Hagerling[†1, 6] and Karin Leandersson[1*]

[1] Cancer Immunology, Department of Translational Medicine, 214 28 Malmö, Lund University, Sweden. [2] Translational Cancer Research, TCR, Medicon Village, Lund University, 221 00 Lund, Sweden. [3] Division of Oncology and Therapeutic Pathology, Department of Clinical Sciences; Lund University, 221 85 Lund, Sweden. [4] Sahlgrenska Center for Cancer Research, Department of Biomedicine; University of Gothenburg, 405 30 Gothenburg, Sweden. [5] Division of Oncology, Department of Clinical Sciences; Lund University, 221 00 Lund, Sweden. [6] Division of Clinical Genetics, Department of Laboratory Medicine Lund; Lund University, 221 00 Lund, Sweden. [†] and [§] These authors contributed equally.

Keywords: Breast cancer, macrophage, CD169, tolerance, type I IFN

Abstract

Resident CD169$^+$ macrophages present in draining lymph nodes of cancer patients are associated with a beneficial prognosis for the patient. Their exact mechanism of action in tumor immunology is still unknown. In primary tumors, similar CD169$^+$ macrophage populations are found, but the role for these is unclear. In the search for novel tumoricidal macrophage populations, we here set out to investigate the origin and function of CD169$^+$ tumor associated macrophages (CD169 TAM) in primary tumors of breast cancer patients. We found that tumor infiltrating CD169 TAMs are monocyte derived and associated with a type I IFN environment and PDL1 expression. Just like resident CD169$^+$ macrophages, tumor infiltrating CD169 TAMs were able to produce type I IFNs themselves and displayed a unique pro-inflammatory surface phenotype and cytokine secretion profile. Importantly however, in contrast to lymph node resident CD169$^+$ macrophages, CD169 TAMs possessed an immunosuppressive function inhibiting T cell activity. Using a human breast cancer cohort, we could show a spatial association of CD169 TAMs with tertiary lymphoid structures (TLS) and, more importantly, with presence of T_{regs}. CD169 TAMs were also associated with worse prognosis. Our findings show that the origin, and subsequently, location of CD169$^+$ macrophages in breast cancer patients, has profound impact on their prognostic, functional and thus therapeutic perspectives, since only lymph node resident CD169$^+$ macrophages have a beneficial effect on survival.

Introduction

Macrophages are a heterogeneous population of innate immune cells, with the basic division of either being resident macrophages, originating from the yolk sac erythro-myeloid precursors or liver during the fetal stage (*1, 2*), or recruited macrophages that are bone marrow-derived, also referred to as monocyte derived (*2, 3*). The majority of tumor associated macrophages (TAMs) are monocyte derived recruited macrophages (*2-4*), representing a chronic inflammation and generally associated with worse prognosis for cancer patients (*3*). However, tumor infiltration of resident macrophages as an alternative source of TAMs is also discussed, and these may have a different function than the recruited monocyte derived TAMs (*2-4*). The tumor microenvironment may also affect the polarization of TAMs differently, thus making it important to consider origin (bone marrow derived or fetal), function (phenotype) and microenvironmental signals (localization or tumor type) when discussing TAM subpopulations in tumors.

As already mentioned, macrophages are generally associated with a worse prognosis in cancer patients. There is however one clear exception: the lymph node resident subcapsular sinus macrophages. Subcapsular sinus macrophages are resident CD169$^+$

macrophages present in lymph nodes surrounding the lymphoid follicles (5, 6). The presence of $CD169^+$ macrophages in lymph nodes has been correlated to an improved prognosis in patients with a variety of cancers (*7-10*). The role for $CD169^+$ subcapsular sinus macrophages is to act as gatekeepers for soluble, lymph-borne, particulate antigens (virus and bacteria), deliver antigens to B cells present in the lymphoid follicles, and they are considered to be crucial antigen presenting cells (APCs) for high-affinity B cell responses (*11*). $CD169^+$ macrophages have been associated with both activating (B, T and NK cell activation), and regulating (T_{regs}) immune responses in mice (*5, 12-15*). In tumor models, it has been shown that $CD169^+$ subcapsular sinus macrophages can phagocytose and bring lymph-borne tumor cell antigens to the lymph nodes (*16*), but the exact role of $CD169^+$ subcapsular sinus macrophages in cancer patients remains unknown. In viral infections $CD169^+$ subcapsular sinus macrophages have been shown to induce type I IFNs that in turn promotes PDL1 expression, resulting in a local T cell exhaustion (*15*). In line with this, we recently showed that $CD169^+$ macrophages co-localized with cells expressing PDL1 both in lymph node metastases and primary breast tumors (*17*).

Lymphoid structures formed in primary tumors and metastases, are called Tertiary Lymphoid Structures (TLS), and have recently been postulated to be the place where anti-tumor immune reactions actually are primed and maintained (*18*). They are also associated with a beneficial response to immunotherapy (*19*). The TLS architecture is similar to that of the follicles in the secondary lymphoid organs, including B cells, mature DCs, T_{fh} cells and memory $CD8^+$ T cells. It is not known whether macrophages analogous to $CD169^+$ subcapsular sinus macrophages are present in tumor TLSs, or whether they would have similar beneficial functions as the lymph node resident counterpart found in lymph nodes of cancer patients. We recently found $CD169^+$ macrophages to be present in primary breast tumors ($CD169^+$ TAMs) (*17*). We showed that while presence of $CD169^+$ subcapsular sinus macrophages in lymph nodes with breast cancer metastases was clearly associated with a beneficial prognosis, the $CD169^+$ TAMs were not (*17*). These findings led us to speculate on what functional relationships $CD169^+$ TAMs could have with resident $CD169^+$ subcapsular sinus macrophages, or if there were similarities that could be utilized to shift the $CD169^+$ TAMs towards more tumoricidal macrophages. We therefore also investigated whether the $CD169^+$ TAMs were present in association with tumor TLS formations in human breast tumors. Our findings in this study imply that the origin, location and thus function of $CD169^+$ macrophages in cancer patients, should be strictly partitioned when it comes to discussing them in prognostic, functional, and therapeutic perspectives.

Material and methods

Breast cancer patients and tumor tissue microarray

The small clinical breast cancer cohort presented in this study consists of 23 patients diagnosed with invasive primary breast cancer with lymph node and/or distal metastasis, in the South-Swedish Health Care Region between 1976-2005. The tumor material was collected retrospectively from paraffin embedded tissue. Ethical approval was obtained from the Regional Ethic committee Lund, Sweden (Dnr 2010/477), according to the Declaration of Helsinki. Estrogen receptor (ER) status was assessed by immunohistochemistry (IHC) and positivity was defined as >10% positive nuclei, according to current diagnostic routines in Sweden. Tissue cores (1 mm diameter) from primary tumors, lymph node metastases and/or distal metastases were collected and mounted in a tissue microarray (TMA). The larger breast cancer cohort consists of 304 patients diagnosed with locally advanced inoperable or metastatic breast cancer in Sweden between 2002 and 2007 and were included in the randomized phase III trial (TEX) (*20*). A detailed description regarding the trial and the patient cohort has been previously reported (*21-23*). Ethical approval was obtained from corresponding Regional Ethic committees in Sweden of each of the clinics involved in the trial, and written informed consent was given according to the Declaration of Helsinki (*20-23*). Primary tumor material (0.6 mm diameter) from 191 patients, ages ranging from 27 to 71 years, was included in the final analysis due to missing clinicopathological information or low quality or missing TMA cores for the remaining cases. All primary tumor material included was scored for CD169 and CD20, 175 samples were scored for FoxP3 and matched with CD169/CD20, and 174 samples were scored for CD3 and matched with CD169/CD20.

Immunohistochemistry

The cores were 1 mm Ø (test 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. 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). (v.12.4.3.5008). Staining of CD20, CD3, CD169, and CD56 in the small patient cohort was scored independently by three of the authors (CA, HV, KL). Staining of CD20, CD3, and CD169 in the larger patient cohort was scored independently by three of the authors (OB, EK, KL). Scoring of tertiary lymphoid structures was done using immune cell markers, as a cluster of B cells in association with T cells within the tumor, as either absent (0) or present (1). CD169 was scored as either absent (0) or present (1). For the larger cohort, separate staining and annotation for CD3 (T cells) and FoxP3 (T_{regs}) had been performed previously, with cases scored from 0 to 3 depending on immune cell density (*22*). For statistical analyses these categories were dichotomized into low (0-2) or high (3) (*22*). 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, a Double Stain Polymer Kit from Nordic Biosite (anti-mouse HRP (brown) and anti-rabbit AP (red)) 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 the Aperio slide scanner (Leica Biosystems), after which the material could be viewed in Aperio ImageScope. For immunofluorescence (IF) anti-mouse CD169 (Alexa488-conjugated; clone 3D6.112; Biolegend) and -F4/80 (Alexa647-conjugated; clone BM8; Biolegend) was used on frozen sections.

Animal procedures and the NSG co-xenograft model

Female 8-week-old NSG mice (NOD.Cg-Prkdc(scid)Il2rg(tm1Wji)/SzJ strain, The Jackson Laboraoty, USA) were housed in a controlled environment and all procedures were approved by the regional ethics committee for animal research at Lund University, Sweden (M11-15). Mice were anesthetized by isoflurane and injected with human breast cancer cells (SUM-159) at $1x10^6$ cells/mouse on the right flank, alone or in combination with primary human monocytes ($1x10^6$ cells/mouse) as previously described (*24*). 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. The NSG models (approval M11-15) were approved by the regional ethics committee for animal research at Lund University, Sweden. Frozen sections of stored 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 10^5 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.

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 2012/689), whereby written consent was given, as approved by the regional ethical committee at Lund University, according to the Declaration of Helsinki. Concentrated leukocytes were obtained from healthy donors. PBS containing 5 mM EDTA and 2.5% w/v sucrose was used to dilute the concentrated leukocytes, and Ficoll-Paque Plus (GE Healthcare Bio-sciences) gradient used to isolate peripheral blood mononuclear cells (PBMC). Monocytes, T cells and NK cells were isolated from PBMCs by magnetic cell sorting (MACS) using: Classical Monocyte Isolation kit, human; Naïve $CD4^+$ T cell isolation kit, human; and NK cell isolation kit, human (Miltenyi Biotec), according to manufacturer's protocol.

Cell culture

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. All cytokines used in differentiation cultures or stimulation cultures were from R&D Systems, except for IFNα, which was from PBL assay Science, USA. MDA-MB-231 (TNBC) breast cancer cells from ATCC were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Biosera), 1% sodium pyruvate (Hyclone), 1% HEPES (Hyclone) and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). SUM159 breast cancer cells,

produced by Professor S Ethier, were cultured in F-12 HAM'S medium supplemented with 5% FBS, 1 mM L-Glutamine, 1% HEPES (Hyclone) and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively). Necrotic SUM159 cell (necSUM159) was induced by performing three freeze-thaw cycles of cells in OptiMEM media. Condition media (CM) was harvested from cultures of breast cancer cell lines MDA-MB-231, MDA-MB-468, MCF7 and SUM159 and instead of polarization, macrophages were cultured in BC CM for 2 days. Primary human lymphoid fibroblasts (HLF) were purchased from 3hbiomedical (Uppsala, Sweden) and cultured in fibroblast medium supplemented with B27, epidermal growth factor (EGF), fibroblast growth factor (FGF) and FBS prior to seeding together with Mo-M cultures on day 5 instead of polarization. For CD169 induced expression on M2 macrophages, live SUM159 breast cancer cells (1:1 ratio with macrophages), necrotic SUM159 (necSUM159) breast cancer cells (1:1 ratio with macrophages) or TLR3 agonist Polyinosinic-polycytidylic acid sodium salt (Poly(I:C)) (20 µg/ml) (Sigma-Aldrich) was added to Mo-M cultures on day 5 instead of cytokine polarization.

Flow cytometry

Expression of surface markers was measured by flow cytometry. The cultured primary macrophages were harvested on day 7 or 8 of culture. Non-specific binding was blocked using FcR Blocking Reagent, human (Miltenyi Biotec) and cells were stained for 30 minutes for surface markers using antibodies found in **Supplementary table 2**. All antibodies used were purchased from BD Biosciences. Marker expression was measured using FACS Verse flow cytometer (BD Biosciences) and data analyzed using FlowJo (Tree Star).

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

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

Cytokine analysis

Supernatants from macrophage cultures were collected on day 7 or 8, and the levels of cytokines was screened using a V-PLEX Human Cytokine 36-Plex (Meso Scale Diagnostics) according to manufacturer's protocol. For further cytokines analysis, the amount of IL-10, IL-15 and TGF-β were measured using ELISA (R&D Systems), according to manufacturer's protocol and Human Inflammatory Cytokine bead array (BD Biosciences) was used for measuring levels of IL-1β, IL-6, IL-8, IL-12p70 and TNF.

Pinocytosis assay

Pinocytic activity of the primary human monocyte derived macrophages was analyzed using FITC-Dextran uptake. Cells were incubated with 0.25 mg/ml FITC-Dextran (Sigma-Aldrich) at 37°C for 20 minutes and subsequently analyzed using flow cytometry.

T cell suppression assay (TSA) and mixed lymphocyte reaction (MLR)

The cultured primary macrophages were harvested on day 7 of culture, reseeded in 96 well plates, and incubated with freshly isolated naïve $CD4^+$ T cells for T cell suppression assay (TSA); $CD4^+$ 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. Cells were incubated at 37°C for 5 days. 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. 18 hours before harvest, 1 µl Ci [methyl^{3}H] Thymidine (PerkinElmer) was added to each well. For analysis a Microbeta Filtermat-96 Cell Harvester (PerkinElmer) and 3H incorporation was determined with a Wallace 1450 MicroBeta TriLux Liquid Scintillation and Luminescence counter (PerkinElmer).

NK cell cytotoxicity assay

Cytotoxic effect of NK cells was determined by measuring lactate dehydrogenase (LDH) activity using a Cytotoxicity detection kit (Roche Diagnostics) according to manufacturer's protocol. Briefly, after polarization of macrophages, autologous or allogeneic NK cells were isolated from frozen PBMCs, and co-cultured with MDA-MB-231 breast cancer cells (10:1 ratio), with polarized macrophages for 5 hours in fresh

OptiMEM supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml), after which the LDH levels were measured. Inhibitors for HLA-G (10 µg/ml) (HLA-G monoclonal antibody, Thermo Fisher) and PDL1 (10 µg/ml) (Atezolizumab, Chemtronica AB) were added at start of the 5-hour incubation. Cytotoxicity of polarized macrophages alone was measured after co-culture with MDA-MB-231 breast cancer cells for 5 hours.

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. Kaplan-Meier analysis with log-rank test, as well as Pearson Chi Square and Linear by Linear association were performed using IBM SPSS Statistics version 26 (SPSS Inc), specifically correlations between $CD169^+$ macrophages and presence of tertiary lymphoid structures (TLS), T cells (CD3), T_{regs} (FoxP3), Ki67 and ER status and overall survival. Cox regression models were used in SPSS for estimation of hazard ratios (HR) with 95% confidence interval (CI) according to CD169 expression in primary tumors in the breast cancer cohorts, both in uni- and multivariable analysis. The correlation between CD169 mRNA expression and overall survival, and the correlations between CD169 mRNA and CXCL10, IFNA4 and IFNB1 mRNA in the human breast cancer 1097 TGCA database were performed via R2: microarray analysis and visualization platform http://2r.amc.nl. All P-values presented are two-sided and should in general be regarded as continuous measurement of evidence, but throughout this paper P values below 0.05 are considered significant evidence.

Results

$CD169^+$ TAMs originate from monocytes in a $PDL1^+$ breast cancer environment

We have recently shown that presence of $CD169^+$ macrophages in metastatic lymph nodes of breast cancer patients was significantly associated with a better prognosis, while presence of $CD169^+$ TAMs in primary human breast tumors showed an opposite, although not significant, trend (*17*). These previous results are herein supported by mRNA expression levels of CD169 in primary human breast tumors using the TCGA database, where CD169 mRNA expression in primary human breast tumors was significantly correlated with worse overall survival (**Figure 1A**). To understand the biological function of $CD169^+$ TAMs in primary human breast tumors, we initially set out to investigate the cellular origin of $CD169^+$ TAMs, with the aim to subsequently be able to characterize them in more detail *in vitro*. TAMs are generally associated with recruited monocyte derived macrophages of various alternative activation types (*2, 3*). The majority of TAMs in breast cancer express the typical M2 marker CD163 (*25*), and only a small minority express CD169 in primary human breast tumors (*17*). In mice, $CD169^+$ lymph node and spleen resident subcapsular sinus macrophages express high levels of mCD169 but lack the murine macrophage marker F4/80 (*6*). To investigate the potential cellular origin of $CD169^+$ TAMs in primary tumors, we first explored the mCD169 and F4/80 expression levels in normal Balb/c mouse spleen as control (**Figure 1B** left) and compared that to the levels expressed on TAMs present in the tumor microenvironment of a syngeneic murine breast cancer model 4T1 (**Figure 1B** middle and right). We could show that in the spleen of Balb/c mice, $CD169^+$ macrophages were mostly $F4/80^-$ as previously described (*6*) (yellow arrow; **Figure 1B** left), with some $F4/80^+$ cells in the outer margin of the follicle (white dashed arrows; **Figure 1B** left). In the 4T1-model (**Figure 1B** middle and right), the $CD169^+$ macrophages present were either positive for F4/80 ($F4/80^+$; white dashed arrow) indicating a monocyte derived recruited macrophage origin, or negative for F4/80 ($F4/80^-$; yellow arrow), indicating infiltrating macrophages of resident origin. Hence, a recruited monocyte derived origin of $CD169^+$ TAMs should not be excluded.

To investigate whether human monocytes could also generate $CD169^+$ TAMs, we next performed xenograft co-transplantations using primary human monocytes and a human triple negative breast cancer (TNBC) cell line, SUM159, in severely immunodeficient Nod scid gamma (NSG) mice (*26*) (**Figure 1C-D**). NSG mice lack functional lymphocytes, have defective macrophages and dendritic cells as a consequence of common gamma chain (γ_c) deletion, but produce some monocytes and neutrophils (*26*). TNBCs generally associate with TAM infiltration and PDL1 expression (*17, 27, 28*). As shown in **Figure 1C**, SUM159 breast cancer xenografts in NSG mice express PDL1 on the malignant cells (**Figure 1C** left). When co-transplanted with primary human monocytes for 21 days, these monocyte derived

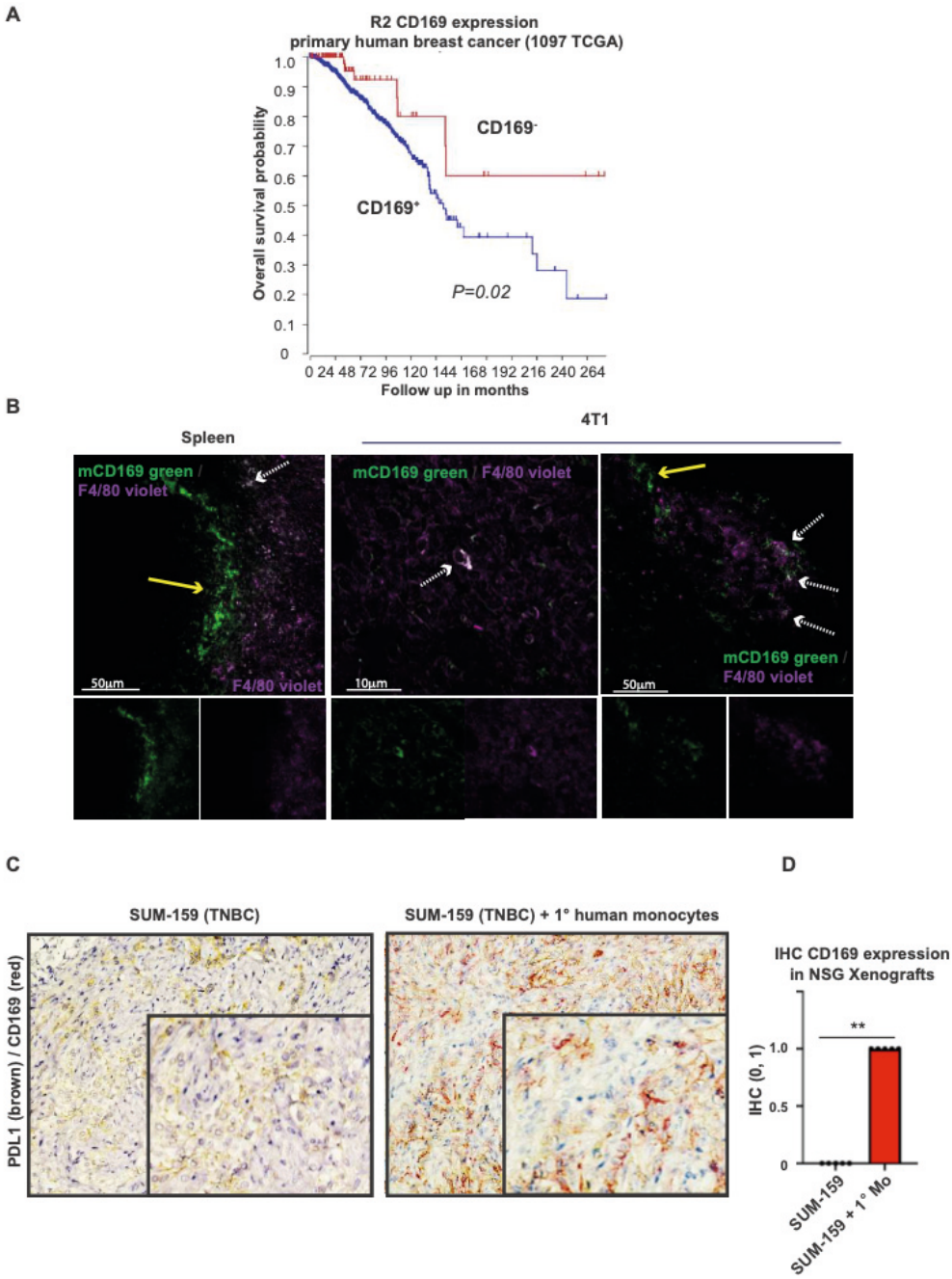


Figure 1. See legend on next page.

Figure 1. (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) Immunofluorescence (IF) staining of a Balb/c mouse spleen (left) and 4T1 tumor model (middle, right). Staining for mCD169 shown in green and staining for F4/80 in purple. Arrows point to macrophages only positive for mCD169 indicating resident origin (yellow), or double positive for mCD169 and F4/80 indicating monocyte derived origin (dashed white). (C) Tumor xenografts in NSG mice. Primary human monocytes were long term co-transplanted with SUM-159 breast cancer cell lines in NSG mice. Controls were transplanted with SUM-159 cells alone. Upregulation of CD169 (red) was seen in the SUM-159 + monocyte xenografts (right) while PDL1 (brown) was seen in both SUM-159 (left) and SUM-159 + monocyte (right) xenografts, with CD169/PDL1 co-expression observed in co-transplanted xenografts (right). (D) Immunohistochemistry statistics of (C) using Mann-Whitney t-test, N=5 in each group, ** $p < 0.01$.

TAMs upregulated CD169 and co-expressed PDL1 (**Figure 1C-D** right), indicating that human CD169$^+$ TAMs can be monocyte derived.

Type I IFN induce CD169 expression on monocyte derived macrophages

The majority of TAMs in human primary breast tumors lack CD169 expression (*17*). To understand what caused the CD169$^+$ phenotype on distinct TAM populations, we evaluated different inflammatory or tumor derived mediators on primary human monocyte derived macrophages in a relevant tumor microenvironment M2 setting *in vitro*. To narrow down specific mediators, we selected relevant cytokines that would be able to induce expression of CD169. Type I IFNs have previously been shown to induce CD169 on macrophages (*29*). CD169$^+$ subcapsular sinus macrophages are themselves also high producers of type I IFNs in viral immune responses (*15, 30*) and found responsible for the PDL1 expression on nearby cells (*15*). With this in mind, we first added IFNα to primary human monocyte derived macrophages in a tumor microenvironment M2-like setting *in vitro* (M2 / type I IFN). As expected, the monocyte derived macrophages upregulated CD169 specifically in the M2 / type I IFN setting (**Figure 2A**). PDL1 expression was already high in M2 (**Figure 2A** right, black) and the same could be seen in the M2 /type I IFN setting (**Supplementary Figure 1A**), supporting that the CD169$^+$ macrophages did co-express PDL1 and CD169 on their surface (**Figure 2A** and **Supplementary Figure 1A**) as also seen in the xenografts (**Figure 1C**). In primary human breast tumors, mRNA expression of the CD169 gene (*SIGLEC1*) significantly correlated to *IFNA4* (*P*=6.25e-04) and *IFNB1* (*P*=5.53e-41) (**Supplementary Fig 1B**) as shown using the TCGA database in R2. The source of natural IFN type I in tumors that could upregulate CD169 on the TAMs in breast tumors, is not obvious. We tried culturing human monocyte derived macrophages in breast cancer cell conditioned media (**Figure 2B**) and in co-culture with SUM159 breast cancer cells and primary lymph node associated human lymph node fibroblasts (**Figure 2C**), but none of these affected CD169 surface expression *in vitro*. Toll-like receptor 3 (TLR3) signaling has previously been shown to induce antitumoral function of macrophages and upregulation of inflammatory mediators (*31*) including type I IFN (*32*). Indeed, experiments with primary human monocyte derived macrophages co-cultured with the TLR3 ligand Polyinosinic:polytidylic acid (Poly(I:C)) upregulated CD169 expression on human monocyte derived macrophages (**Figure 2D**). Co-culture with necrotic SUM159 (nSUM159) breast cancer cells did however not upregulate CD169 expression on the macrophages (**Figure 2D**), which is surprising, since endogenous ligands for TLR3 have previously also been described to be derived from tumor cells (*33-35*). Finally, to judge whether the monocyte derived CD169$^+$ macrophages were themselves able to produce type I IFN, one hallmark for subcapsular sinus macrophages, qPCR was performed showing that indeed monocyte derived CD169$^+$ cells are capable of producing type I IFNs (*IFNA* and *IFNB*) themselves *in vitro* (**Figure 2E**). Together this indicates that CD169 can be induced on recruited monocyte derived macrophages in a type I IFN tumor microenvironment, and that this is associated with PDL1 expression and endogenous type I IFN production.

CD169$^+$ monocyte derived macrophages produce pro-inflammatory cytokines

Since resident CD169$^+$ subcapsular sinus macrophages have been shown to possess both immunogenic and tolerogenic functions (*5, 12-15*), we next asked which functional phenotype the monocyte derived CD169$^+$ macrophages

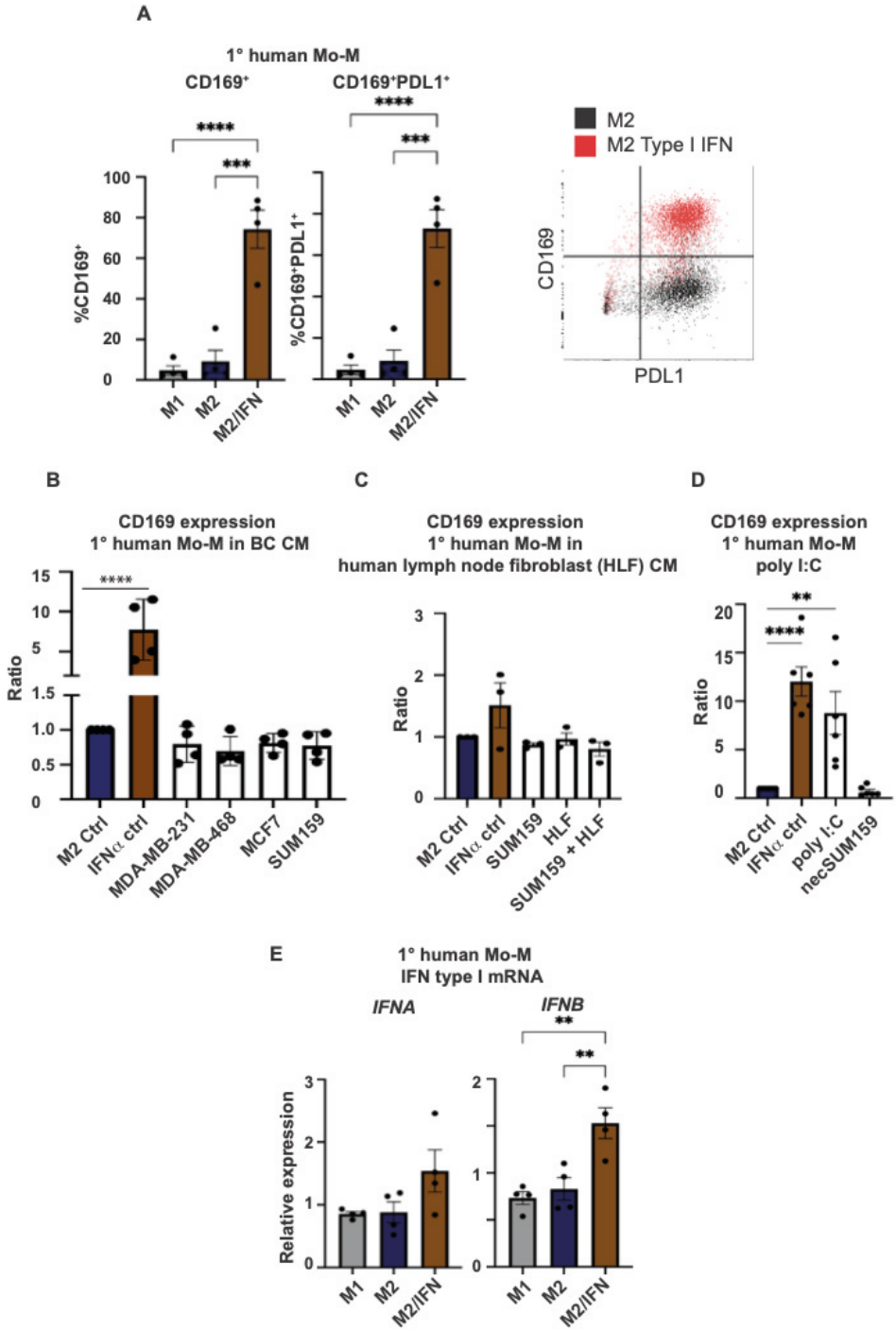


Figure 2. See legend on next page.

Figure 2. Expression of CD169 and PDL1 on primary human monocyte derived macrophages cultured under M1 and M2 conditions or as M2/ type I IFN treated. (A) Comparison of CD169 surface expression (left) and co-expression of CD169 and PDL1 (middle) 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. (B) Surface expression of CD169 on primary monocytes differentiated into macrophages cultured in breast cancer cell line condition media. IFN treated macrophages used as a positive control and M2 cultured macrophages as negative control for CD169 surface expression, N = 4. (C) Surface expression of CD169 on primary human monocytes differentiated into macrophages, cultured with human lymph node fibroblasts (HLF), SUM159 breast cancer cells, or both cell types. Compared to M2 cultured macrophages as negative control and IFN treated macrophages used as a positive control, N = 3. (D) Surface expression of CD169 on primary human monocyte derived macrophages with addition of Poly(I:C) (20 μg/ml) or necrotic SUM159 breast cancer cells (necSUM159) on day 5 of culture, compared to M2 cultured macrophages as negative control and IFN treated macrophages used as a positive control, N = 6. (E) Relative mRNA levels of *IFNA* and *IFNB* in primary human monocyte derived macrophages as measured by RT-qPCR, N = 4. For all figures: One-way ANOVA multiple comparison Dunnett's test. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

generated in a tumor microenvironment setting *in vitro* would have. We judged surface phenotype, cytokine profiles, pinocytosis and immune activation or suppression capacity in relation to T cells and NK cells. Surprisingly, the surface phenotype of the M2/ type I IFN induced $CD169^+$ macrophages was similar to M1 like macrophages, with a $CD14^+HLADR^+CD1a^-CD206^-$ cell surface phenotype (**Figure 3A-B** and **Supplementary Figure 1C**). The cytokine profile analyzed using the *V-Plex* system comparing M2 macrophages and M2/ type I IFN treated macrophages showed a unique cytokine profile (**Supplementary Table 1**). Of the 36 cytokines analyzed, CXCL10 and IL-15 were significantly upregulated (**Supplementary Figure 1D**), CCL2, CCL11 and IL-6 were secreted at notably higher levels for the M2/ type I IFN induced $CD169^+$ macrophages and CCL3, CCL4, CCL14 and CCL22 were secreted at a lower level (**Supplementary Table 1**). CXCL10 is a chemokine that attracts T cells to tumor sites and is induced by IFNγ and Type I IFNs (*36-38*). Using the TCGA database, we could further show that *CXCL10* was highly associated with *SIGLEC1* expression in breast cancer specimens (*P*=2.07e-122; **Supplementary Figure 1E**). 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*) (*39*). IL-15 is important for T and NK cell activity (*40*) and antitumor immunity (*41*). Using ELISA, we could see that M2/type I IFN treated macrophages secreted IL-15 in soluble form at higher levels than M2 macrophages, but at lower levels than M1 (**Figure 3C**). Both CXCL10 and IL15 findings were supported by analyses of mRNA levels, where the *CXCL10* and *IL15* gene expression was upregulated in the M2/ type I IFN treated, along with upregulation of the *IL15RA,* IL-15 receptor alpha (IL15Rα) gene (**Figure 3D**), and upregulation of the membrane bound protein form of IL15Rα on the surface of the $CD169^+$ cells (**Supplementary Figure 1F**). TNFα is an important effector molecule produced by macrophages, capable of killing tumors cells but also inducing tissue damage (*42*). Even though we did detect equal levels of TNFα in our *V-plex* assay in M2 like macrophages and M2/ type I IFN treated, this result was not consistent with results obtained using the CBA method (**Supplementary Figure 1G**), where M2/type I IFN $CD169^+$ macrophages did not secrete TNFα, while M1 macrophages secreted high levels (**Supplementary table 1** and **Supplementary Figure 1G**).

$CD169^+$ monocyte derived macrophages have immunosuppressive functions

We next investigated the functional polarization of the M2/ type I IFN induced $CD169^+$ macrophages in a cellular context. Despite having high levels of CXCL10 and IL-15, the M2/ type I IFN induced $CD169^+$ macrophages acted immunosuppressive in relation to T cells (**Figure 4A** and **4B**), a typical M2 like function. Surprisingly, the M2/ type I IFN induced $CD169^+$ macrophages still had a reduced pinocytic capacity compared to M2 like macrophages, more similar to the M1 like macrophages (**Figure 4C**). In relation to the effect of the macrophages on the tumoricidal capacity of NK cells, all three macrophage subtypes showed a slightly but non-significant increased effect on NK cell cytotoxicity using

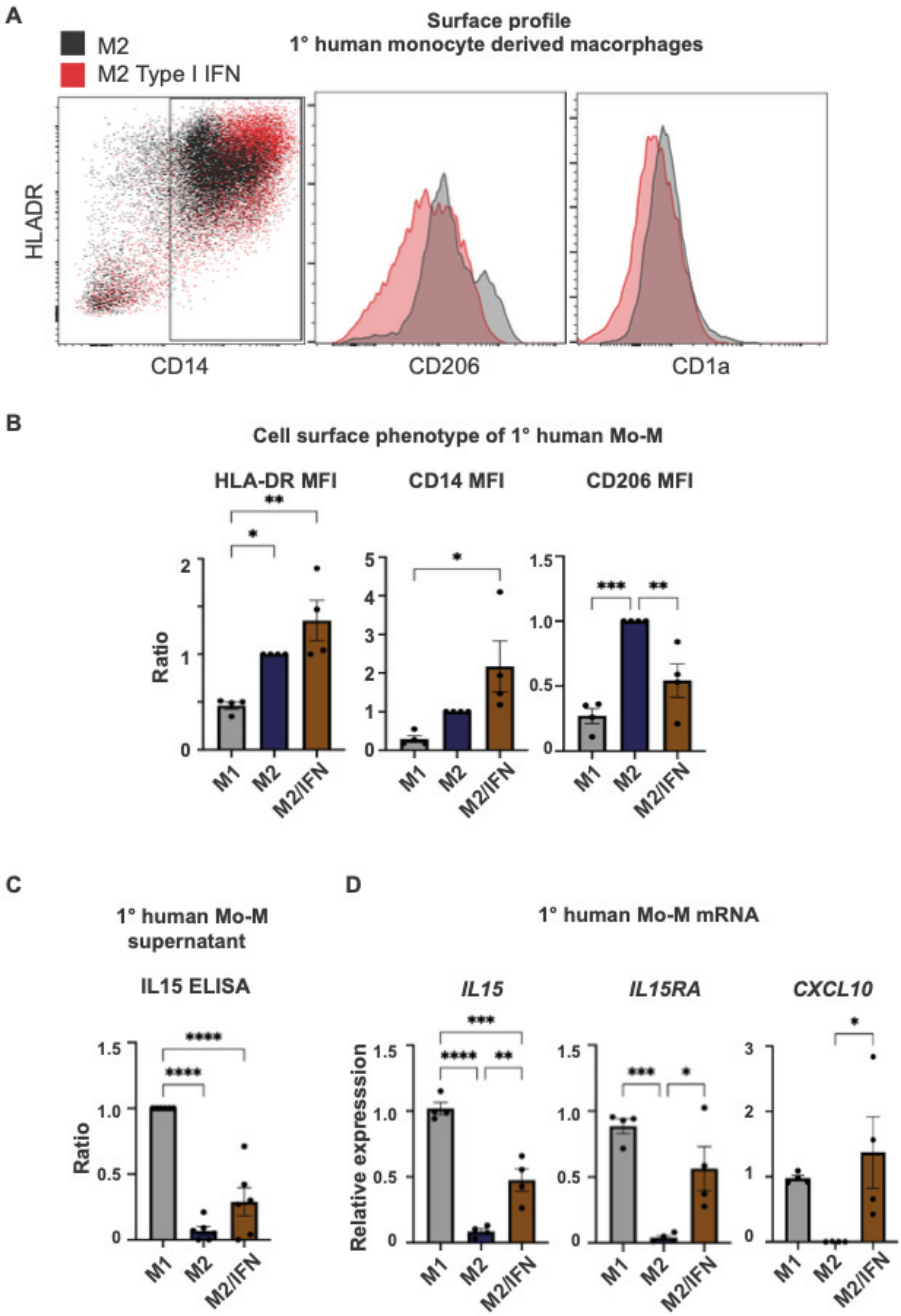


Figure 3. Phenotype of primary human monocyte derived macrophages cultured under M1 and M2 conditions or M2/ type I IFN treated. (A) Representative dot plot and histogram for HLA-DR/CD14, CD206 and CD1a surface expression on M2 treated control primary human macrophages (black) compared to IFN treated (red). (B) Ratio of MFI of cell surface markers HLA-DR, CD14 and CD206 on human primary macrophages with M2 as control, N = 4. (C) Cytokine secretion of IL15 as measured by ELISA, ratio of concentration with M1 as control, N = 6. (D) Relative mRNA expression of *IL15*, *IL15Ra and CXCL10* as measured by RT-qPCR, N = 4. For all figures: One-way ANOVA multiple comparison Dunnett's test. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

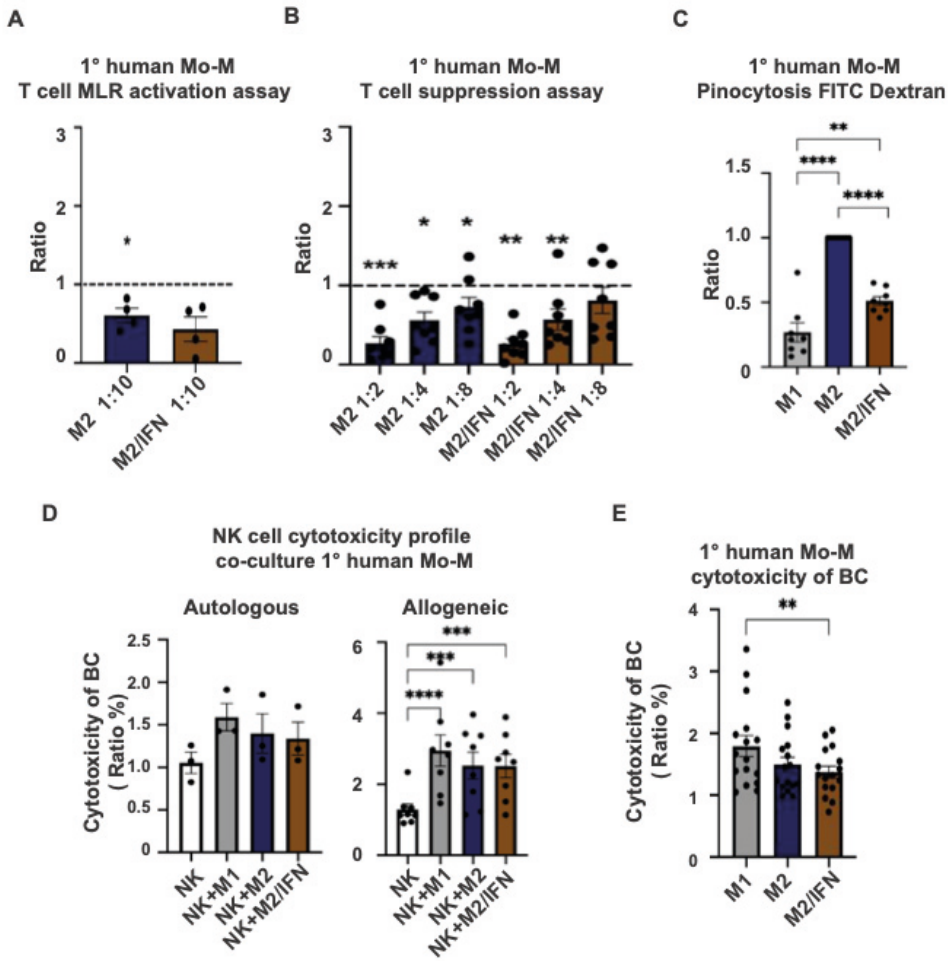


Figure 4. Immune suppressive functions of M2/ type I IFN treated primary human monocyte derived macrophages. (A) Allogeneic MLR of primary human monocyte derived macrophages and primary human $CD4^+$ T cells as measured by $[^3H]$ incorporation at ratio 1:10. Ratio with base T cell $[^3H]$ incorporation, represented by dashed line, N = 3. (B) 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 $[^3H]$ incorporation at stimulator-responder cell ratio 1:2, 1:4 and 1:8. Dashed line represents base activated T cell $[^3H]$ incorporation, N = 8. For (A-B): Ratio paired t-test. (C) Pinocytosis as measured by FITC-Dextran uptake with M2 macrophages as control, N = 8. (D-E) Cytotoxicity as measured by LDH activity released from cytosol of damaged cells. (D) Autologous (N = 3) and allogeneic (N = 8) co-culture of primary human NK cells, primary human monocyte derived macrophages and MDA-MB-231 breast cancer cell line. (E) Co-culture of primary human monocyte derived macrophages and MDA-MB-231 breast cancer cell line, N = 16. For (C-E): One-way ANOVA multiple comparison Dunnett's test. Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

co-cultures of autologous NK cells and macrophages with MDA-MB-231 breast cancer cells (**Figure 4D** left). In an allogeneic setting however, we observed a significant increase in cytotoxicity, but in this setting, we cannot exclude that increase in measured cytotoxicity is the result of allogeneic NK cells and macrophages killing each other (**Figure 4D** right). Finally, when we co-cultured MDA-MB-231 breast cancer cells and macrophages alone, we saw a significant decrease in cytotoxicity for M2/type I IFN $CD169^+$ macrophages as compared to M1 like macrophages (**Figure 4E**), again indicating that these $CD169^+$ macrophages would be more M2 like in function, and not tumoricidal.

CD169$^+$ monocyte derived macrophages express immunosuppressive mediators

To investigate possible immunosuppressive mediators in the M2/type I IFN CD169$^+$ macrophages other than PDL1 expression (**Figure 2A**), we next performed ELISA and qPCR analyses of various T and NK cell inhibitory effector molecules (**Figure 5**). In M2/ type I IFN induced CD169$^+$ macrophages, the level of secreted IL-10 was similar to M2-like macrophages, but TGF-β levels were surprisingly significantly lower than in M2-like macrophages (**Figure 5A**). Arginase (*ARG1*), Indoleamine 2,3-dioxygenase (*IDO1*) and inducible nitric oxide synthase (*iNOS*) were not significantly upregulated at the mRNA level in the M2/ type I IFN treated macrophages. The increased level of IDO1 in M1 macrophages can be explained by the fact that IFNγ upregulates IDO1 on M1 like macrophages (*43*). Importantly however, the T and NK cell inhibitory mediators PGE2 (*PTGES2)* (*44, 45*) and HLA-G (*HLA-G)* (*46*) were both specifically upregulated at the mRNA level in the M2/ type I IFN macrophages, compared to both M1- and M2-like macrophages (**Figure 5B**). Inhibition of HLA-G or PDL1 did however not alleviate the suppressive effect that M2/type I IFN CD169$^+$ macrophages had on T cells (**Supplementary Figure 2A**), nor affect NK cell cytotoxicity in co-cultures with macrophages and breast cancer cells (**Supplementary Figure 2B**). Together this indicates that CD169$^+$ monocyte derived macrophages generated in a tumor microenvironment *in vitro*, could theoretically inhibit both T cells and NK cells via specific signaling pathways like PDL1, PGE2 and HLA-G, although specific inhibitors did not show a clear alleviation of their immune suppressive effect.

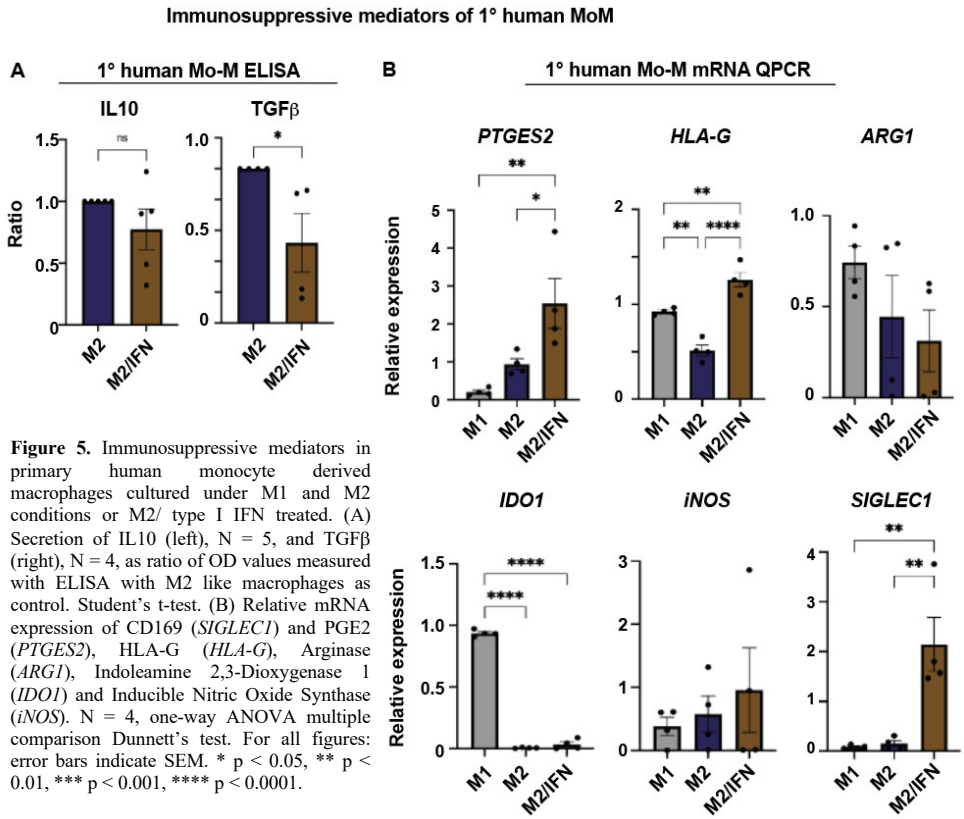


Figure 5. Immunosuppressive mediators in primary human monocyte derived macrophages cultured under M1 and M2 conditions or M2/ type I IFN treated. (A) Secretion of IL10 (left), N = 5, and TGFβ (right), N = 4, as ratio of OD values measured with ELISA with M2 like macrophages as control. Student's t-test. (B) Relative mRNA expression of CD169 (*SIGLEC1*) and PGE2 (*PTGES2*), HLA-G (*HLA-G*), Arginase (*ARG1*), Indoleamine 2,3-Dioxygenase 1 (*IDO1*) and Inducible Nitric Oxide Synthase (*iNOS*). N = 4, one-way ANOVA multiple comparison Dunnett's test. For all figures: error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CD169$^+$ TAMs are associated with TLS formation and a worse prognosis

The natural spatial location of resident CD169$^+$ lymph node macrophages surrounding B cell lymphoid follicles led us to next investigate the spatial organization of infiltrating CD169$^+$ TAMs in relation to tertiary lymphoid structure (TLS) and relevant immune cells, in primary human breast tumor specimens. For this purpose, we used two breast cancer patient cohorts, one small test cohort consisting of primary tumor material from 23 patients (**Supplementary Table 4**) and one large cohort consisting of tumor material from 304 patients (**Table 1**) (*20*). We stained the breast tumor TMAs using specific antibodies for: CD169, CD3 (T cells), CD20 (B cells) to investigate CD169$^+$ TAMs in relation to T cells and B cells (tertiary lymphoid structures (TLS)); or CD56 (NK cells) to investigate CD169$^+$ TAMs in relation to B cells and NK cells. A previous annotation of CD3 (T cells) and FoxP3 (T_{regs}) was also included in the analysis to investigate CD169$^+$ TAMs in relation to T_{regs} (*22*). Representative images of the IHC stainings are shown in **Figure 6A-C**. **Figure 6A** represents a lymph node, as a control for the CD169$^+$ cell / NK cells / B cells staining. **Figure 6B** represents a CD169$^+$ cell / T cells / B cells staining of a primary breast tumor with a tertiary lymphoid structure (TLS), where CD169$^+$ macrophages are present in the TLS (**Figure 6B**). **Figure 6C** represents a CD169$^+$ cell / T cells / B cells staining of a primary breast tumor with scattered cells positive for CD169 that were observed in close contact with T cells and only a few B cells (**Figure 6C**, black arrows). Analysis of the spatial association between NK cells and CD169$^+$ macrophages in breast tumors of the small cohort did not generate any significant results (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 analyses of NK cells in the large cohort.

A significant spatial association between CD169$^+$ TAMs in the primary tumors (CD169 PT) and presence of TLS formations in the primary tumors (TLS PT) was found both in the small (*p=0.048;* **Supplementary Table 4**) and large (*p=0.001;* **Table 1**) patient cohort. Continuing with the large cohort only, importantly, presence of CD169$^+$ TAMs (CD169 PT) was not associated with better overall survival but rather with worse OS, using Kaplan Meier Log Rank tests, (*p=0.047;* **Figure 6D**). Presence of CD169$^+$ TAMs in association with TLS (CD169 TLS PT) was further not significantly associated with better overall survival (*p=0.059;* **Figure 6E**), however showed the same trend towards worse outcome (*47*). We next performed multivariable analysis and cox regression analysis adjusting for tumor size, estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2, Ki67, and age of patient at diagnosis. In the unadjusted analysis, presence of CD169$^+$ TAMs in primary tumor was significantly associated with worse outcome (HR = 1.43, 95% CI: (1.01-2.04), *p=0.049*), but significance was lost in the multivariable analysis (HR = 1.09, 95% CI: (0.69-1.72), *p=0.711).*

Table 1. Cross-correlation CD169$^+$ tumor associated macrophages (CD169 PT) and tertiary lymphoid structures (TLS PT), Ki67 or ER-status in primary breast tumors

		CD169 PT		Total	*P*
		0	**1**		
TLS PT	***0***	126	39	165	
	1	12	14	26	
Total		138	53	191[a]	*P*=0.001
CD3 PT	***low***	116	38	154	
	high	12	8	20	
Total		128	46	174[a]	*P*=0.144
FoxP3 PT	***low***	55	13	68	
	high	72	35	107	
Total		127	48	175[a]	*P*=0.049
Ki67	***low***	91	24	115	
	high	39	24	63	
Total		130	48	178[a]	*P*=0.014
ER	***0***	18	18	36	
	1	115	32	147	
Total		133	50	183[a]	*P*=0.001

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

In line with this, a significant spatial association between CD169$^+$ TAMs in the primary tumors (CD169 PT) and presence of regulatory T cells (T_{regs}) in the primary tumors (T_{reg} PT) was found in the large patient cohort (*p=0.049;* **Table 1**) whereas for pan-T cells (CD3), there was no significant association (*p=0.145;* **Table 1**). Significant associations were also seen for TLS PT and T_{regs} (*p<0.001*) and for CD169 TLS PT and T_{regs} (*p=0.001*). T_{regs} have previously been shown to be an independent factor associated with a worse prognosis in this patient cohort (*22*). Dense infiltration of CD169$^+$ macrophages in the primary tumor *per se* (CD169$^+$ PT), was also

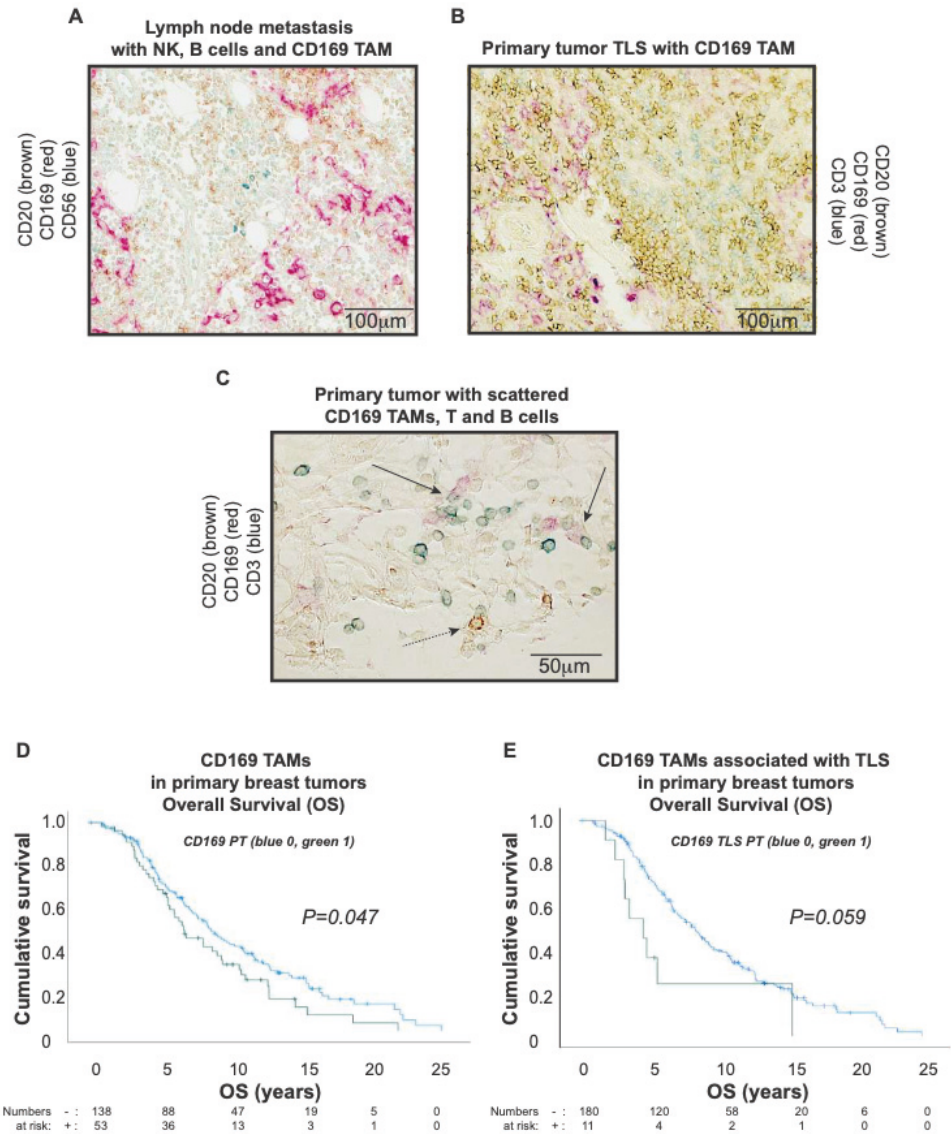


Figure 6. Immunohistochemical (IHC) triple staining of breast tumor tissue array cohort (TMA), using specific antibodies for CD169, CD3 (T cells), CD20 (B cells), and CD56 (NK cells). (A) Staining of lymph node metastasis for CD20 (brown), CD169 (red) and CD56 (blue). (B) Primary tumor tertiary lymphoid structure (TLS) with presence of $CD169^+$ tumor associated macrophages (TAMs) stained for CD20 (brown), CD169 (red) and CD3 (blue). (C) Same staining of primary tumor with TLS 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. (D-E) Kaplan-Meier curves illustrating differences in overall survival according to presence of $CD169^+$ TAMs (D) and $CD169^+$ TAMs in association with TLS (E) in primary breast tumors. *P* value by log-rank test.

correlated with a high proliferative index (Ki67) (*p=0.014;* **Table 1**) and ER negativity (ER-) (*p=0.001;* **Table 1**). One could speculate about whether the ER negative tumor microenvironment, and the high proliferative index with increased necrotic cell death and DAMPs as consequence, would be able to upregulate $CD169^+$ on monocyte derived macrophages. In summary, this indicates that, indeed, tumor infiltrating $CD169^+$ macrophages

are associated with TLS in primary breast tumors, however without having beneficial functions for the patient, rather the opposite. Instead, a clear association with immunosuppressive T_{regs} and poor prognosis was found. Hence, the unique beneficial functions that CD169$^+$ subcapsular sinus macrophages have when located in their resident place of lymph nodes are lost for peripheral monocyte derived CD169$^+$ TAMs present in the primary tumors.

Discussion

The importance of subcapsular sinus macrophages as beneficial immune cells in cancer patients has come into light lately. Although their role during viral infections is becoming clearer, there is still a large gap of knowledge regarding their mechanisms of action and biology in tumors and metastases. A number of studies for various solid tumor types have been published, showing a strong association between presence of CD169$^+$ subcapsular sinus macrophages in regional lymph nodes and a drastically improved clinical prognosis (*8, 48-51*). In cancer patients, CD169$^+$ subcapsular sinus macrophages present in regional lymph nodes correlate with a higher CTL and NK cell presence in tumor parenchyma (*52*), but also with poor prognosis in tumor models (*53*). Indeed, the first report on possible involvement of CD169$^+$ subcapsular sinus macrophages in tumor immunology came from mouse models where they were shown to pick up and cross-present lymph borne tumor antigens in draining lymph nodes (*54*). Since then, they have been shown to have both immunogenic and tolerogenic functions (*11, 12, 15, 29, 30, 53, 54*), thus more data is needed to understand their involvement also in cancer patients.

One confusing matter is the localization and origin of CD169$^+$ macrophages in tumors (CD169$^+$ TAMs) and metastases. Depending on which organ or tumor they are situated, or from where they derive, leads to diverging data. If they are defined by the CD169 surface molecule only, this will lead to contradictory findings (*6*). In this study, we try to define peripheral CD169$^+$ macrophages in primary tumors with regards to their origin, function, and prognostic impact. Our data indicate that CD169$^+$ TAMs in breast cancer patients are likely to be monocyte derived, just like other TAMs. This is in line with a recent preliminary study, where tumor infiltrating tissue macrophages were characterized using CyTOF, showing that the macrophages carrying CD169 also expressed the monocyte receptor CD14 (*55*). Undoubtedly, to be able to visualize whether tissue resident macrophages would be able to infiltrate tumors, specific markers for resident phagocytes are needed. A recent study using the syngeneic 4T1 tumor model, argued that resident CD169$^+$ macrophages indeed infiltrate tumors (*53*). However, this study did not take into account whether these CD169$^+$ macrophages were monocyte derived or not. We here show that a proportion of these may indeed be monocyte derived as judged by co-expression of F4/80, also supported by human primary monocytes upregulating CD169 in breast cancer cell xenografts.

Our *in vitro* data indicate that in contrast to having an anti-tumoral function, the *in vitro* cultured M2/ type I IFN primary human monocyte derived CD169$^+$ macrophages lack tumoricidal and immunogenic capacity. The CD169$^+$ macrophages cannot activate NK cells despite having functional IL-15/IL15Rα signaling previously proposed to stimulate antitumoral NK cell activity (*56*), but rather possess T cell suppression ability. The suppressive capacity of the CD169$^+$ macrophages was neither caused by typical TAM cytokines (IL10 or TGFβ) nor by soluble mediators iNOS, IDO1 or Arginase. Although having a high expression level of the membrane bound immune regulators PDL1 and HLA-G, addition of specific inhibitors did not alleviate the T cell suppressive effect the CD169$^+$ macrophages had. We therefore conclude that despite showing a pro-inflammatory M1-like phenotype and cytokine profile, CD169$^+$ macrophages act in an immunosuppressive M2-like and TAM-like way, with the two most likely inhibitory mechanisms being PGE2 or ROS. The immunosuppressive mechanism of CD169$^+$ TAMs is supported by a study using the CD169-DTA 4T1 tumor model, showing that CD169$^+$ macrophages indeed induce tumor progression (*53*).

When we analyzed human breast cancer tissue to investigate the spatial location of CD169$^+$ macrophages in primary tumors and their relation to other immune cells present, we

found that CD169$^+$ macrophages were not spatially associated with NK cells. They were on the other hand spatially associated with tertiary lymphoid structures (TLS) in the primary tumor, and lastly were associated with presence of regulatory immunosuppressive T_{regs}. TLS formation has previously been postulated to be important for anti-tumor immune reactions (*18, 19*). In breast cancer, the prognostic impact of TLSs in primary tumors is generally associated with a better prognosis, however dependent on the breast cancer subtype. Interestingly, presence of TLSs in primary tumors of TNBC and HER2 positive breast cancer subtypes in particular, is associated with a beneficial prognosis (*18, 57-64*). This is interesting and in sharp contrast to the present study, where presence of CD169$^+$ cells in relation with TLS in the primary breast tumor was not associated with better prognosis for the patients, but rather with a non-significant trend towards a worse outcome. The CD169 TLSs were however also associated to presence of T_{regs}. Infiltration of T_{regs} in TLSs has previously been described to be associated with worse outcome in several types of cancer, including breast cancer (*18, 63, 65*).

CXCL10, which was secreted at high levels in our *in vitro* cultured M2/ type I IFN CD169$^+$ primary human monocyte derived macrophages, has previously been shown to induce cell proliferation, migration and epithelial-mesenchymal transition of breast cancer cell lines MCF7 and MDA-MB-231, and mRNA expression of CXCL10 correlated positively with infiltration of both CD8$^+$ and FOXP3$^+$ TILs, as well as PDL1$^+$ immune cells in breast cancer (*66*). CXCL10 has also been linked to increased metastases and, further, tumor driven macrophage expression of CXCL10 in osteolytic bone metastasis (*67*). This could explain the correlation seen in the herein investigated patient cohort, with presence of CD169$^+$ cells correlating with presence of T_{regs} and worse prognosis. However, CXCL10 has also been shown to be associated with anti-tumor immunity and T cell infiltration in melanoma patients following immune checkpoint blockade (*68*) and in patients with ovarian cancer (*69*). 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, but CXCL10 has been shown to promote re-awakening of dormant breast cancer cells in the metastatic liver environment (*70*). We suggest that CD169$^+$ macrophages in breast cancer tumors are immunosuppressive TAMs that associate with T_{regs} in TLSs, with possible detrimental effects for breast cancer patients. This rhymes with our previous study where it was shown that CD169$^+$ TAMs located in primary tumor do not correlate with a better prognosis, as well as correlating with more unfavorable tumor characteristics, such as a high proliferative index (Ki67), and ER negativity (*17*).

Why TAMs would adapt the CD169$^+$ phenotype is still unclear. Type I IFNs and signaling molecules leading to type I IFN production may be one cause. As mentioned, TLR3 signaling has previously been shown to induce antitumoral function of macrophages and to upregulate secretion of inflammatory cytokines and chemokines such as CXCL10 (*31*) and type I IFN (*32*). Tumor specific ligands for TLR3 in the form of DAMPs released from tumor and necrotic cells have also been shown previously (*33-35*). This might indicate that the high proliferative index in primary human breast tumors associated with CD169$^+$ TAM infiltration, may be a consequence of increased necrotic cell death, causing an upregulation of CD169$^+$ on monocyte derived macrophages. In this present study we did observe an upregulation of CD169 on M2/ Type I IFN treated primary human macrophages when treated with Poly(I:C), a TLR3 agonist. Importantly, inflammatory breast cancer has lately been associated with an elevated Type I IFN signalling pathway signature and associated with a poor prognosis (*71*). This indicates that presence of CD169$^+$ TAMs may vary depending on tumor types and further should be evaluated as a biomarker for IBCs.

In conclusion, we propose that CD169$^+$ macrophages present in primary human breast cancer (CD169$^+$ TAMs), are monocyte derived macrophages generated in a type I IFN stimulating tumor microenvironment, that possess immunosuppressive functions and a regulatory gene expression profile, despite showing a pro-inflammatory M1 like surface phenotype *in vitro*. In human breast cancer specimens, the CD169$^+$ TAMs associate with formation of TLS and presence of T_{regs}, and

with worse prognosis for the patients. The anti-tumorigenic function that lymph node resident $CD169^+$ macrophages possess is hence unique in tumor immunology and yet to be explored. We propose that lymph node resident $CD169^+$ macrophages should be considered as a therapeutic target, while considering the negative side effects of $CD169^+$ TAMs.

References

1. F. Ginhoux, M. Guilliams, Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* **44**, 439-449 (2016).
2. S. Y. Zhang *et al.*, Tumor-associated macrophages: A promising target for a cancer immunotherapeutic strategy. *Pharmacol Res* **161**, 105111 (2020).
3. A. Mantovani, F. Marchesi, S. Jaillon, C. Garlanda, P. Allavena, Tumor-associated myeloid cells: diversity and therapeutic targeting. *Cell Mol Immunol*, (2021).
4. S. Watanabe, M. Alexander, A. V. Misharin, G. R. S. Budinger, The role of macrophages in the resolution of inflammation. *J Clin Invest* **129**, 2619-2628 (2019).
5. K. Asano, K. Kikuchi, M. Tanaka, CD169 macrophages regulate immune responses toward particulate materials in the circulating fluid. *J Biochem* **164**, 77-85 (2018).
6. D. A. P. Louie, S. Liao, Lymph Node Subcapsular Sinus Macrophages as the Frontline of Lymphatic Immune Defense. *Front Immunol* **10**, 347 (2019).
7. K. Ohnishi *et al.*, Prognostic significance of CD169-positive lymph node sinus macrophages in patients with endometrial carcinoma. *Cancer science* **107**, 846-852 (2016).
8. K. Ohnishi *et al.*, CD169-positive macrophages in regional lymph nodes are associated with a favorable prognosis in patients with colorectal carcinoma. *Cancer Sci* **104**, 1237-1244 (2013).
9. T. Asano *et al.*, CD169-positive sinus macrophages in the lymph nodes determine bladder cancer prognosis. *Cancer Sci* **109**, 1723-1730 (2018).
10. K. Stromvall, K. Sundkvist, B. Ljungberg, S. Halin Bergstrom, A. Bergh, Reduced number of CD169(+) macrophages in pre-metastatic regional lymph nodes is associated with subsequent metastatic disease in an animal model and with poor outcome in prostate cancer patients. *Prostate* **77**, 1468-1477 (2017).
11. H. Veninga *et al.*, Antigen targeting reveals splenic CD169+ macrophages as promoters of germinal center B-cell responses. *Eur J Immunol* **45**, 747-757 (2015).
12. Z. Garcia *et al.*, Subcapsular sinus macrophages promote NK cell accumulation and activation in response to lymph-borne viral particles. *Blood* **120**, 4744-4750 (2012).
13. L. Martinez-Pomares, S. Gordon, CD169+ macrophages at the crossroads of antigen presentation. *Trends Immunol* **33**, 66-70 (2012).
14. S. Muerkoster, M. Rocha, P. R. Crocker, V. Schirrmacher, V. Umansky, Sialoadhesin-positive host macrophages play an essential role in graft-versus-leukemia reactivity in mice. *Blood* **93**, 4375-4386 (1999).
15. N. Shaabani *et al.*, CD169+ macrophages regulate PD-L1 expression via type I interferon and thereby prevent severe immunopathology after LCMV infection. *Cell Death Dis* **7**, e2446 (2016).
16. J. Dejmek *et al.*, Expression and signaling activity of Wnt-5a/discoidin domain receptor-1 and Syk plays distinct but decisive roles in breast cancer patient survival. *Clin Cancer Res* **11**, 520-528 (2005).
17. F. Bjork Gunnarsdottir *et al.*, Co-localization of CD169(+) macrophages and cancer cells in lymph node metastases of breast cancer patients is linked to improved prognosis and PDL1 expression. *Oncoimmunology* **9**, 1848067 (2020).
18. C. Sautes-Fridman *et al.*, Tertiary Lymphoid Structures in Cancers: Prognostic Value, Regulation, and Manipulation for Therapeutic Intervention. *Front Immunol* **7**, 407 (2016).
19. T. C. Bruno, New predictors for immunotherapy responses sharpen our view of the tumour microenvironment. *Nature* **577**, 474-476 (2020).
20. S. Kimbung *et al.*, Transcriptional Profiling of Breast Cancer Metastases Identifies Liver Metastasis-Selective Genes Associated with Adverse Outcome in Luminal A Primary Breast Cancer. *Clin Cancer Res* **22**, 146-157 (2016).
21. T. Hatschek *et al.*, Individually tailored treatment with epirubicin and paclitaxel with or without capecitabine as first-line chemotherapy in metastatic breast cancer: a randomized multicenter trial. *Breast Cancer Res Treat* **131**, 939-947 (2012).
22. J. Stenstrom, I. Hedenfalk, C. Hagerling, Regulatory T lymphocyte infiltration in metastatic breast cancer-an independent prognostic factor that changes with tumor progression. *Breast Cancer Res* **23**, 27 (2021).
23. S. Kimbung *et al.*, Contrasting breast cancer molecular subtypes across serial tumor progression stages: biological and prognostic implications. *Oncotarget* **6**, 33306-33318 (2015).
24. R. Allaoui *et al.*, Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers. *Nat Commun* **7**, 13050 (2016).
25. C. Medrek, F. Ponten, K. Jirstrom, K. Leandersson, The presence of tumor associated macrophages in tumor stroma as a prognostic marker for breast cancer patients. *BMC Cancer* **12**, 306 (2012).

26. L. D. Shultz *et al.*, Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J Immunol* **174**, 6477-6489 (2005).
27. R. Sabatier *et al.*, Prognostic and predictive value of PDL1 expression in breast cancer. *Oncotarget* **6**, 5449-5464 (2015).
28. X. Lu *et al.*, Macrophage Colony-stimulating Factor Mediates the Recruitment of Macrophages in Triple negative Breast Cancer. *Int J Biol Sci* **15**, 2859-2871 (2019).
29. H. Akiyama *et al.*, Interferon-Inducible CD169/Siglec1 Attenuates Anti-HIV-1 Effects of Alpha Interferon. *J Virol* **91**, (2017).
30. J. Grabowska, M. A. Lopez-Venegas, A. J. Affandi, J. M. M. den Haan, CD169(+) Macrophages Capture and Dendritic Cells Instruct: The Interplay of the Gatekeeper and the General of the Immune System. *Front Immunol* **9**, 2472 (2018).
31. A. Maeda, E. Digifico, F. T. Andon, A. Mantovani, P. Allavena, Poly(I:C) stimulation is superior than Imiquimod to induce the antitumoral functional profile of tumor-conditioned macrophages. *Eur J Immunol* **49**, 801-811 (2019).
32. L. Mazaleuskaya, R. Veltrop, N. Ikpeze, J. Martin-Garcia, S. Navas-Martin, Protective role of Toll-like Receptor 3-induced type I interferon in murine coronavirus infection of macrophages. *Viruses* **4**, 901-923 (2012).
33. Y. Liu *et al.*, Tumor Exosomal RNAs Promote Lung Pre-metastatic Niche Formation by Activating Alveolar Epithelial TLR3 to Recruit Neutrophils. *Cancer Cell* **30**, 243-256 (2016).
34. D. M. Lim, M. L. Wang, Toll-like receptor 3 signaling enables human esophageal epithelial cells to sense endogenous danger signals released by necrotic cells. *Am J Physiol Gastrointest Liver Physiol* **301**, G91-99 (2011).
35. K. A. Cavassani *et al.*, TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med* **205**, 2609-2621 (2008).
36. N. Karin, H. Razon, Chemokines beyond chemo-attraction: CXCL10 and its significant role in cancer and autoimmunity. *Cytokine* **109**, 24-28 (2018).
37. V. Pandey *et al.*, CXCL10/CXCR3 signaling contributes to an inflammatory microenvironment and its blockade enhances progression of murine pancreatic precancerous lesions. *Elife* **10**, (2021).
38. G. Trinchieri, Type I interferon: friend or foe? *J Exp Med* **207**, 2053-2063 (2010).
39. Y. S. Niknafs *et al.*, MiPanda: A Resource for Analyzing and Visualizing Next-Generation Sequencing Transcriptomics Data. *Neoplasia* **20**, 1144-1149 (2018).
40. T. A. Fehniger, Mystery Solved: IL-15. *J Immunol* **202**, 3125-3126 (2019).
41. R. M. Santana Carrero *et al.*, IL-15 is a component of the inflammatory milieu in the tumor microenvironment promoting antitumor responses. *Proc Natl Acad Sci U S A* **116**, 599-608 (2019).
42. J. Shen *et al.*, Anti-cancer therapy with TNFalpha and IFNgamma: A comprehensive review. *Cell Prolif* **51**, e12441 (2018).
43. X. F. Wang *et al.*, The role of indoleamine 2,3-dioxygenase (IDO) in immune tolerance: focus on macrophage polarization of THP-1 cells. *Cell Immunol* **289**, 42-48 (2014).
44. V. Sreeramkumar, M. Fresno, N. Cuesta, Prostaglandin E2 and T cells: friends or foes? *Immunol Cell Biol* **90**, 579-586 (2012).
45. N. Kundu *et al.*, Antagonism of the prostaglandin E receptor EP4 inhibits metastasis and enhances NK function. *Breast Cancer Res Treat* **117**, 235-242 (2009).
46. P. Contini, G. Murdaca, F. Puppo, S. Negrini, HLA-G Expressing Immune Cells in Immune Mediated Diseases. *Front Immunol* **11**, 1613 (2020).
47. F. Björk Gunnarsdottir *et al.*, Co-localization of CD169+ macrophages and cancer cells in lymph node metastases of breast cancer patients is linked to improved prognosis and PDL1 expression. *OncoImmunology* **9**, 1848067 (2020).
48. Y. Saito *et al.*, Prognostic Significance of CD169+ Lymph Node Sinus Macrophages in Patients with Malignant Melanoma. *Cancer Immunol Res* **3**, 1356-1363 (2015).
49. T. Shiota *et al.*, The clinical significance of CD169-positive lymph node macrophage in patients with breast cancer. *PLoS One* **11**, (2016).
50. K. Ohnishi *et al.*, Prognostic significance of CD169-positive lymph node sinus macrophages in patients with endometrial carcinoma. *Cancer Science* **107**, 846-852 (2016).
51. K. Ohnishi *et al.*, Clinical significance of CD169-positive lymph node macrophages in human malignant tumors. *Journal of Clinical Oncology* **32**, (2014).
52. Y. Komohara, K. Ohnishi, M. Takeya, Possible functions of CD169-positive sinus macrophages in lymph nodes in anti-tumor immune responses. *Cancer Science* **108**, 290-295 (2017).
53. W. Jing *et al.*, Breast cancer cells promote CD169(+) macrophage-associated immunosuppression through JAK2-mediated PD-L1 upregulation on macrophages. *Int Immunopharmacol* **78**, 106012 (2020).
54. K. Asano *et al.*, CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity* **34**, 85-95 (2011).
55. Wogsland *et al.*, High dimensional immunotyping of the obese tumor microenvironment reveals model specific adaptation. *bioRxiv - The preprint server for*

biology **This article is a preprint and has not been certified by peer review** (2020).

56. Y. Guo, L. Luan, N. K. Patil, E. R. Sherwood, Immunobiology of the IL-15/IL-15Ralpha complex as an antitumor and antiviral agent. *Cytokine Growth Factor Rev* **38**, 10-21 (2017).
57. I. H. Song *et al.*, Predictive Value of Tertiary Lymphoid Structures Assessed by High Endothelial Venule Counts in the Neoadjuvant Setting of Triple-Negative Breast Cancer. *Cancer Res Treat* **49**, 399-407 (2017).
58. X. Liu *et al.*, Distinct Tertiary Lymphoid Structure Associations and Their Prognostic Relevance in HER2 Positive and Negative Breast Cancers. *Oncologist* **22**, 1316-1324 (2017).
59. M. Lee *et al.*, Presence of tertiary lymphoid structures determines the level of tumor-infiltrating lymphocytes in primary breast cancer and metastasis. *Mod Pathol* **32**, 70-80 (2019).
60. H. J. Lee *et al.*, Prognostic Significance of Tumor-Infiltrating Lymphocytes and the Tertiary Lymphoid Structures in HER2-Positive Breast Cancer Treated With Adjuvant Trastuzumab. *Am J Clin Pathol* **144**, 278-288 (2015).
61. C. Gu-Trantien *et al.*, CD4(+) follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest* **123**, 2873-2892 (2013).
62. S. L. Figenschau, S. Fismen, K. A. Fenton, C. Fenton, E. S. Mortensen, Tertiary lymphoid structures are associated with higher tumor grade in primary operable breast cancer patients. *BMC Cancer* **15**, 101 (2015).
63. M. C. Dieu-Nosjean *et al.*, Tertiary lymphoid structures, drivers of the anti-tumor responses in human cancers. *Immunol Rev* **271**, 260-275 (2016).
64. L. Buisseret *et al.*, Tumor-infiltrating lymphocyte composition, organization and PD-1/ PD-L1 expression are linked in breast cancer. *Oncoimmunology* **6**, e1257452 (2017).
65. M. Gobert *et al.*, Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome. *Cancer Res* **69**, 2000-2009 (2009).
66. M. Kim, H. Y. Choi, J. W. Woo, Y. R. Chung, S. Y. Park, Role of CXCL10 in the progression of in situ to invasive carcinoma of the breast. *Sci Rep* **11**, 18007 (2021).
67. J. H. Lee *et al.*, CXCL10 promotes osteolytic bone metastasis by enhancing cancer outgrowth and osteoclastogenesis. *Cancer Res* **72**, 3175-3186 (2012).
68. I. G. House *et al.*, Macrophage-Derived CXCL9 and CXCL10 Are Required for Antitumor Immune Responses Following Immune Checkpoint Blockade. *Clin Cancer Res* **26**, 487-504 (2020).
69. L. Ardighieri *et al.*, Infiltration by CXCL10 Secreting Macrophages Is Associated With Antitumor Immunity and Response to Therapy in Ovarian Cancer Subtypes. *Front Immunol* **12**, 690201 (2021).
70. A. M. Clark, H. L. Heusey, L. G. Griffith, D. A. Lauffenburger, A. Wells, IP-10 (CXCL10) Can Trigger Emergence of Dormant Breast Cancer Cells in a Metastatic Liver Microenvironment. *Front Oncol* **11**, 676135 (2021).
71. F. Bertucci *et al.*, Genomic profiling of inflammatory breast cancer: a review. *Breast* **23**, 538-545 (2014).

Supplementary tables:

Supplementary Table 1. V Plex assay with cytokines secreted at higher levels in M2/type I IFN marked in red and cytokines secreted at lower levels marked in blue.

Cytokine/ Chemokine	M2 (pg/ml)	IFN (pg/ml)	P value
CCL2	18058,81	26258,18	0,45
CCL3	4408,45	544,15	0,31
CCL4	14568,58	2313,65	0,092
CCL11	31,36	52,70	0,082
CCL13	8110,83	2588,21	0,21
CCL17	1266,14	1800,93	0,43
CCL20	16,71	16,55	0,98
CCL22	74285,06	26530,56	0,33
CCL26	28,38	41,09	0,72
CXCL10	-	34823,64	0,0007
IFNg	0,78	3,83	0,45
IL1a	164,41	178,74	0,92
IL1b	193,23	256,41	0,59
IL2	-	-	-
IL5	-	-	-
IL6	1007,46	1332,13	0,60
IL7	4,83	5,34	0,92
IL8	5475,78	5507,61	0,55
IL10	-	-	-
IL12p70	0,37	0,01	0,36
IL-12/IL-23p40	82,81	78,31	0,93
IL13	4,56	6,23	0,59
IL15	9,19	10,43	0,017
IL16	87,54	84,54	0,84
IL17A	10,50	8,81	0,64
IL21	15,54	13,20	0,41
IL22	-	-	-
IL23	-	-	-
IL27	81,80	87,73	0,82
IL31	-	-	-
TNFa	123,37	152,31	0,57
TNFb	0,83	1,42	0,71
VEGF	0,30	-	-

- : Not detected

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

Supplementary Table 3. Primer sequences for RT-qPCR

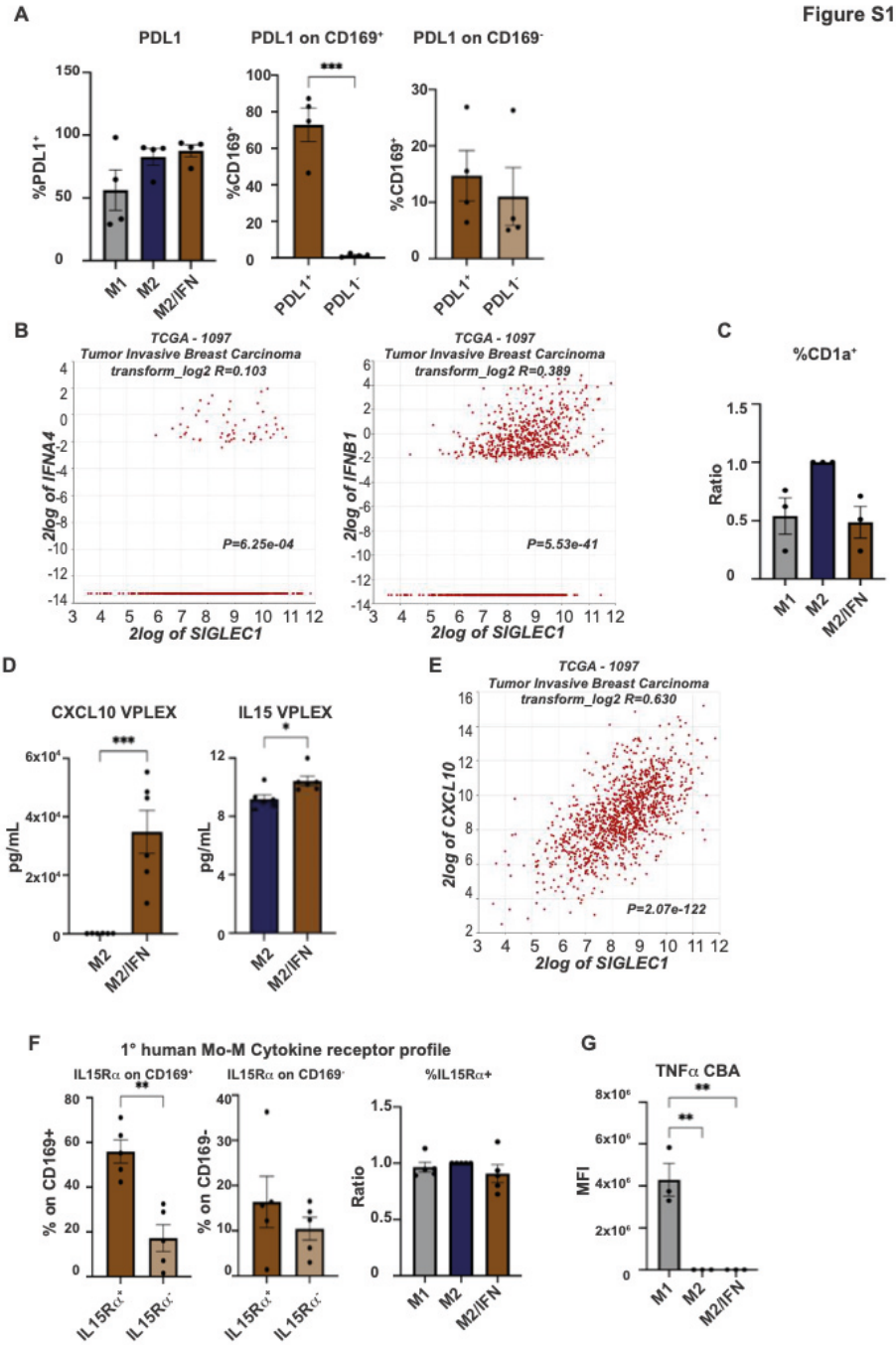
Gene	Sequence
SIGLEC1	F: 5'-GGCTGTTACGATGGTTTATGATGT-3' R: 5'-AATCAAAGGCATCATTTTAGGGATA-3'
IL15	F: 5'-GGAGGCATCGTGGATGGAT -3' R: 5'-AACACAAGTAGCACTGGATGGAAA-3'
IL15RA	F: 5'-GTCAAGAGCTACAGCTTGTA-3' R: 5'-CTTGTTCAACACGCACTC-3'
IFNA	F: 5'-GACTCCATCTTGGCTGTGA-3' R: 5'-TGATTTCTGCTCTGACAACCT-3'
IFNB	F: 5'-TTGACATCCCTGAGGAGATTAAGC-3' 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'
YWHAZ	F: 5'-ACTTTTGGTACATTGTGGCTTCAA-3' R: 5'-CCGCCAGGACAAACCAGTAT-3'

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

		CD169 PT				Total	*P*
		0	**1**	**2**	**3**		
TLS 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

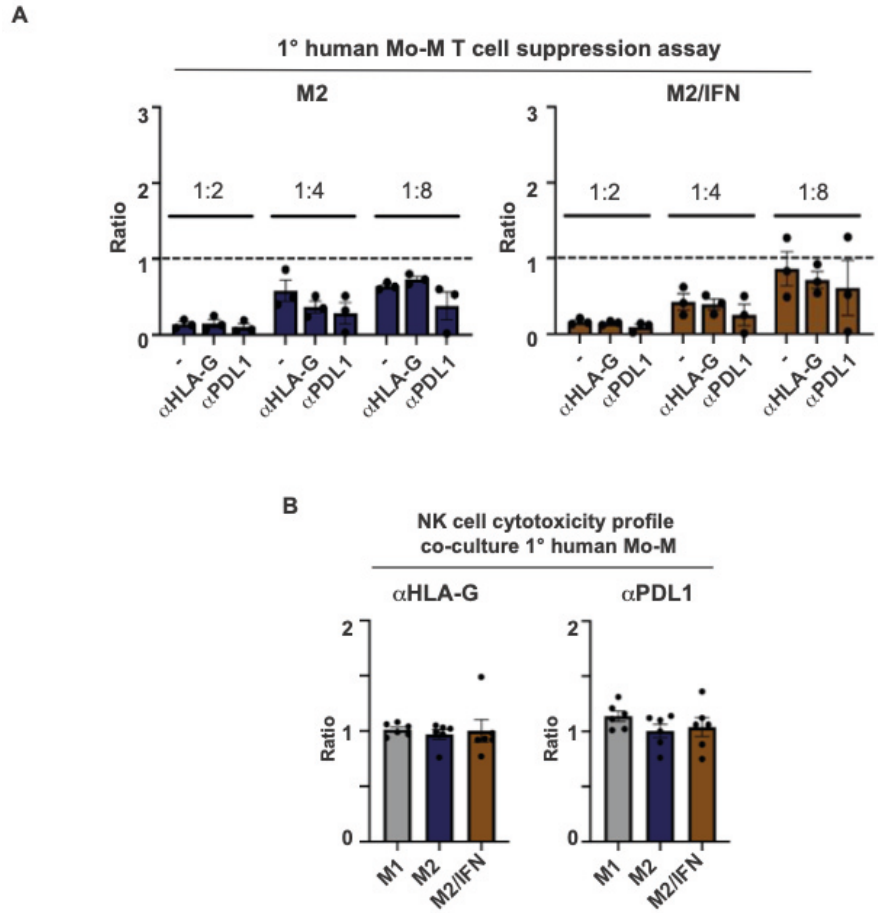
[a] Pearson Chi-Square, Linear by Linear association

Supplementary figures



Supplementary figure 1. See legend on next page.

Supplementary figure 1. Profile of primary human monocyte derived macrophages. (A) PDL1 surface expression of primary human monocyte derived macrophages (left), N = 4, one-way ANOVA multiple comparison Dunnett's test. PDL1 expression on $CD169^+$ cells (middle) and $CD169^-$ cells (right), N = 4, student's t-test. (B) *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). (C) Ratio of median MFI of CD1a surface expression, N = 3, one-way ANOVA multiple comparison Dunnett's test. (D) Cytokine secretion measured with VPLEX of CXCL10 and IL-15, N = 6, student's t-test. (E) *SIGLEC1* association to *CXCL10* mRNA expression in primary tumors of breast cancer patients using the TCGA data base in R2 (r2.amc.nl). (F) Surface expression of IL15Rα on $CD169^+$ macrophages (left) and $CD169^-$ macrophages (middle), N = 5, student's t-test. Ratio of $IL15R\alpha^+$ surface expression on primary human monocyte derived macrophages (right), N = 5, one-way ANOVA multiple comparison Dunnett's test. (G) TNFα cytokine secretion measured with CBA, N = 3, one-way ANOVA multiple comparison Dunnett's test. For all figures: Error bars indicate SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplementary Figure 2. (A) Allogeneic MLR of primary human monocyte derived macrophages and primary human $CD4^+$ T cells as measured by $[^3H]$ incorporation at ratio 1:1, 1:10 and 1:100 with M2 macrophages (left) and M2/IFN macrophages (right), with inhibitors for HLA-G (10 μg/ml) and PDL1 (Atezolizumab, 10 μg/ml). Ratio with base activated T cell $[^3H]$ incorporation, represented by dashed line, N = 3, Paired ratio t-test. (B) 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, One way ANOVA. For all figures: Error bars indicate SEM. * $p < 0.05$.