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Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer

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2017

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

Link to publication

Citation for published version (APA):
Allaoui, R. (2017). *Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer*. [Doctoral Thesis (compilation), Department of Translational Medicine]. Lund University: Faculty of Medicine.

Total number of authors:
1

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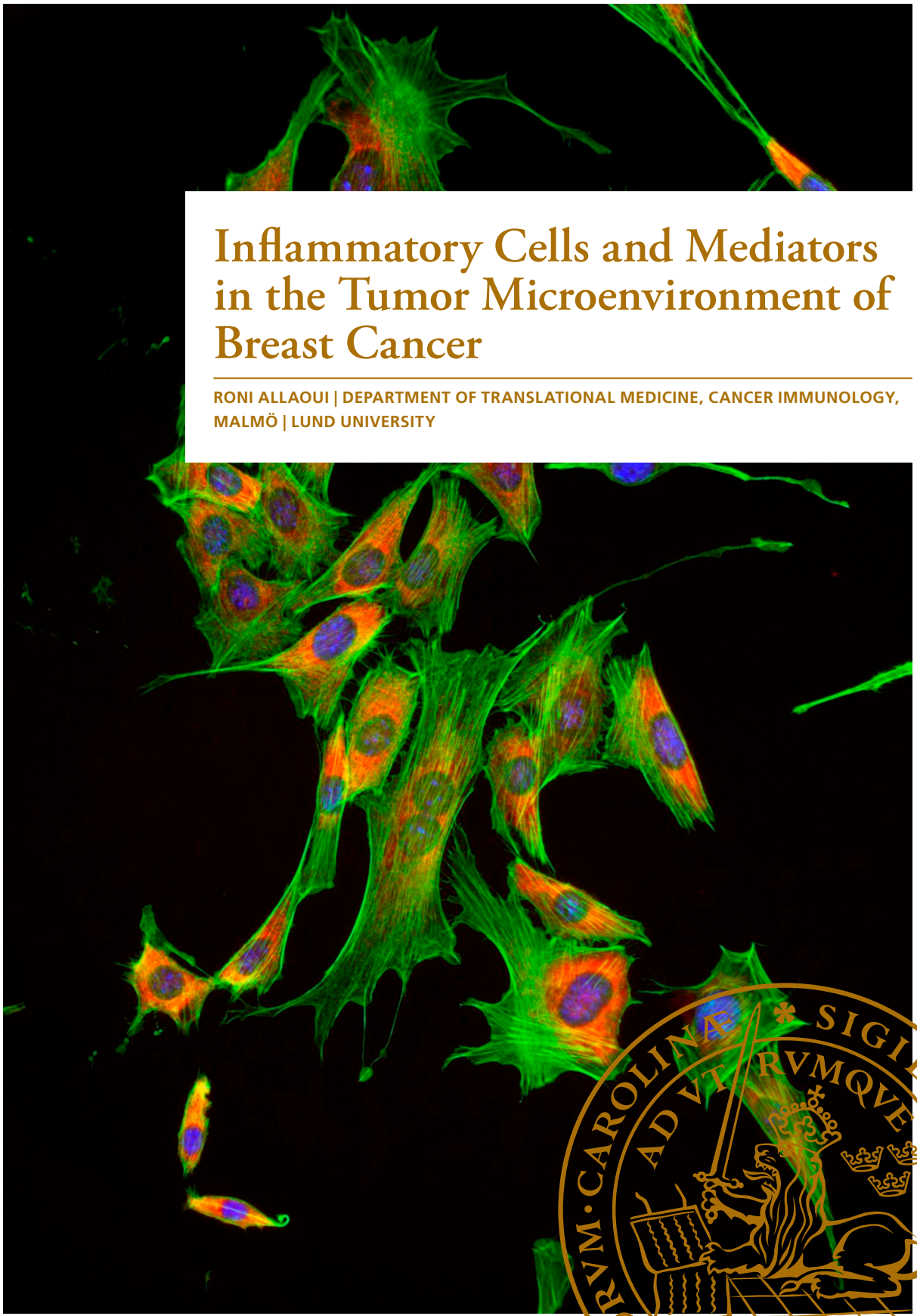

Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer

RONI ALLAOUI | DEPARTMENT OF TRANSLATIONAL MEDICINE, CANCER IMMUNOLOGY, MALMÖ | LUND UNIVERSITY

Errata

IV. Infiltration of γδ T cells, IL-17$^+$ T cells and FoxP3$^+$ T cells in human breast cancer.

Roni Allaoui, Catharina Hagerling, Eva Desmond, Carl-Fredrik Warfvinge, Karin Jirström and Karin Leandersson
Accepted manuscript 2017

Table 1.
The different molecular classifications and their features [22,24-26].

Molecular subtype	Molecular profile	Frequency	Histological grade	Human cell lines	Clinical outcome
Luminal A	ER$^+$, PR$^+$, HER2$^-$, low Ki67	50-60%	Low	MCF-7 T47D	Good
Luminal B	ER$^+$, PR$^+$, HER2$^+$, high Ki67	10-20%	Moderate/high	BT474	Moderate/poor
HER2	ER$^-$, PR$^-$, HER2$^+$,	10-15%	High	SKBR-3	Poor
Basal-like	ER$^-$, PR$^-$, HER2$^-$, CK5/6^{+a} and/or EGFR^{+b}	10-20%	High	MDA-MB-468 SUM149	Poor
Claudine-low	ER$^-$, PR$^-$, HER2$^-$, CK5/6$^+$, EGFR$^-$	12-14%	High	MDA-MB-231 SUM159	Poor
Normal-like	ER$^{+/-}$, HER2$^-$	5-10%	Low	-	Moderate

[a]CK5/6; Cytokeratin 5/6
[b]EGFR; Epidermal growth factor receptor

Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer

Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer

Roni Allaoui

LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at the main lecture hall, Pathology building, Skåne University Hospital, Malmö on Thursday 1st of June, 2017 at 9.00 a.m.

Faculty opponent
Professor Arne Östman
Department of Oncology-Pathology at Cancer Center Karolinska
Karolinska Institute, Stockholm, Sweden

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
Faculty of Medicine Department of Translational Medicin Cancer Immunology Malmö	Date of issue 2017-06-01
Author(s): Roni Allaoui	Sponsoring organization

Title and subtitle: Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer

Abstract

Breast cancer is the most common cancer type among women. There are different subtypes of breast cancer, all with different prognosis. They are defined by their receptor status of Estrogen-, Progesterone-, and HER2-receptor expression (ER, PR, HER2). The most aggressive subtype is the Triple Negative ($ER^-PR^-HER2^-$; TNBC) subtype. The tumor microenvironment also consists of non-malignant cells of various origin. Immune cells infiltrate tumors as a natural host protective reaction. Various immune cells infiltrate breast tumors depending on the breast cancer subtype. In this thesis, the interplay between immune cells, breast cancer cells and other non-malignant cells in the tumor microenvironment are investigated.

In the first part of the thesis, we showed that the innate immune receptor, toll-like receptor 4 (TLR4), was expressed on the malignant cells of ER^-PR^- breast cancers. TLR4-expression correlated to a negative clinical prognosis in breast cancer patients. We also showed that the expressed TLR4 is functional since it responds to ligands belonging the danger- and pathogen associated molecular pattern families (DAMP and PAMP). In the second part of this thesis we show that the DAMP and TLR4 ligand, S100A9, was expressed by both malignant cells in ER^-PR^- breast cancer subtype and by infiltrating $CD163^+$ myeloid cells. Moreover, S100A9 expression in myeloid cells correlated with decreased overall survival in breast cancer patients.

In the third part of this thesis, we investigated the interplay of primary human myeloid cells and breast cancer cells in vivo. We performed a co-transplantation model of primary human monocytes from healthy donors with either luminal A or TNBC breast cancer cell lines in immunodeficient NSG mice. We observed that the human monocytes survived, proliferated and differentiated into anti-inflammatory $CD163^+$ myeloid cells in TNBCs in these mice. The transplanted monocytes promoted stroma formation in both tumor types, but were able to activate fibroblasts in the TNBC microenvironment only. This indicates that monocytes may influence tumor progression differently in distinct breast cancer subtypes. This finding could be of importance for the targeted therapy against myeloid cells in the treatment of breast cancer.

Finally, using immunohistochemistry on a large breast cancer cohort, we investigated the impact of different tumor infiltrating T cell subtypes with the aim to understand whether they differ between breast tumor subtypes. Using selected T cell subset markers against; $CD3^+$ T cells, $CD8\alpha^+$ T cells, $\gamma\delta$ T cells, $FoxP3^+$ T cells (T_{reg}) and $IL\text{-}17^+$ T cells, we observed different prognostic outcomes in various breast cancer subtypes. Infiltration of $\gamma\delta$ T cells was associated with a better clinical outcome in all breast cancer subtype except the TNBC subtype, while the opposite was observed for Tregs. However, infiltration of $CD3^+$ T cells and $CD8\alpha^+$ T cells was independently associated with an improved prognosis for all breast cancer patients. The results indicate that different T cell subtypes have different functions and outcome depending on the breast cancer subtype.

In conclusion, this thesis investigates the role of inflammatory cells and mediators in breast cancer progression. The findings from this thesis could contribute to the understanding of which breast cancer subtypes that will profit from novel therapies against specific immune cell populations and mediators.

Key words: Breast cancer, cancer-associated fibroblasts, DAMP, inflammatory mediators, myeloid-derived supressor cells, toll-like receptors, triple-negative breast cancer, tumor infiltrating lymphocytes, tumor microenvironment, tumor stroma

Classification system and/or index terms (if any)

Supplementary bibliographical information		Language
ISSN and key title: 1652-8220		ISBN: 978-91-7619-452-2
Recipient's notes	Number of pages 74	Price
	Security classification	

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Inflammatory Cells and Mediators in the Tumor Microenvironment of Breast Cancer

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Coverphoto by Roni Allaoui

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ISBN 978-91-7619-452-2
ISSN 1652-8220
Lund University, Faculty of Medicine Doctoral Dissertation Series 2017:72

Printed in Sweden by Media-Tryck, Lund University
Lund 2017

Till Mamma och Pappa

Content

Original papers

This thesis is based on the following papers:

I. Expression of functional toll like receptor 4 in estrogen receptor/progesterone receptor-negative breast cancer.

Meliha Mehmeti, **Roni Allaoui**, Caroline Bergenfelz, Lao H. Saal, Stephen P. Ethier, Martin E. Johansson, Karin Jirström and Karin Leandersson
Breast Cancer Res 2015, 17:130

II. S100A9 expressed in ER(-)PgR(-) breast cancers induces inflammatory cytokines and is associated with an impaired overall survival.

Caroline Bergenfelz, Alexander Gaber, **Roni Allaoui**[#], Meliha Mehmeti[#], Karin Jirström, Tomas Leanderson and Karin Leandersson
Br J Cancer 2015, 113(8):1234-1243
[#] Equal contribution

III. Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers.

Roni Allaoui, Caroline Bergenfelz[#], Sofie Mohlin[#], Catharina Hagerling[#], Kiarash Salari, Zena Werb, Robin L. Anderson, Stephen P. Ethier, Karin Jirström, Sven Påhlman, Daniel Bexell, Balázs Tahin, Martin E. Johansson, Christer Larsson and Karin Leandersson
Nature Communications 2016, Oct 11; 7:13050
[#] Equal contribution

IV. Infiltration of γδ T cells, IL-17$^+$ T cells and FoxP3$^+$ T cells in human breast cancer.

Roni Allaoui, Catharina Hagerling, Eva Desmond, Carl-Fredrik Warfvinge, Karin Jirström and Karin Leandersson
Submitted manuscript 2017

Papers not included in this thesis

A high frequency of MDSCs in sepsis patients, with the granulocytic subtype dominating in gram-positive cases.

Helena Janols, Caroline Bergenfelz, **Roni Allaoui**, Anna-Maria Larsson, Lisa Rydén, Sven Björnsson, Sabina Janciauskiene, Marlene Wullt, Anders Bredberg and Karin Leandersson
J Leukoc Biol 2014, 96(5):685-693

List of abbreviations

ADCC	Antibody dependent cytotoxicity
APC	Antigen presenting cells
αSMA	Alpha-smooth muscle actin
ATP	Adenosine triphosphate
BCR	B cell receptor
BRCA 1/2	Breast cancer 1/2
CAF	Cancer-associated fibroblast
CD	Cluster of differentiation
CK	Cytokeratin
COX2	Cytochrome c oxidase subunit 2
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	Chemokine (C-X-C motif) ligand
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FAP	Fibroblast activating protein
FAS	Death receptor
FASL	Death receptor ligand
FISH	Fluorescence in situ hybridization
FOXP3	Forkhead box P3
FSP-1	Fibroblast-specific protein 1
GATA3	GATA3 binding protein (nuclear protein that recognizes G-A-T-A sequence)
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRN	Granulin
HER2	Human epidermal growth factor receptor 2
HMGB1	High mobility group box 1
HSP	Heat shock protein
ICI	Immune checkpoint inhibitor
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IHC	Immuno-histochemistry
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF-3	Interferon regulatory factor 3
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MICA/B	MHC class I polypeptide-related sequence A/B
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa B
NG2	Neuron-glial antigen 2
NHG	Nottingham histologic grade
NK	Natural killer
NKG2D	Killer cell receptor
NKT	Natural killer T
PAMP	Pathogen-associated molecular patterns
PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
PDGF	Platelet-derived growth factor
PDGR α/β	Platelet-derived growth factor receptor alpha/beta
PGE2	Prostaglandin E2
PR	Progesterone receptor
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation end-products
RORγ	Retinoic acid-receptor-related orphan receptor gamma
T-bet	T-box transcription factor
TAM	Tumor-associated macrophages
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TIL	Tumor infiltrating lymphocyte
TLR	Toll-like receptor
TMA	Tissue microarray
TNBC	Triple-negative breast cancer
TNM	Tumor size (T), number of axillary nodes (N), distant metastasis (M)
VEGF	Vascular endothelial growth factor

Populärvetenskaplig sammanfattning

Våra kroppar består av ofantligt många celler. Under normala förhållanden ersätts gamla och döende celler av en process som kallas ”celldelning”. Alla celler vet när de ska börja eller sluta dela sig eftersom instruktionerna står skrivet i deras gener. Cancercellen saknar denna självkontroll eftersom delar av instruktionerna saknas, vilket ger dem fördelen att dela sig när de vill, hur mycket de vill. Konsekvensen är att det bildas fler och fler cancerceller som tillslut bildar en ”cellklump” – en tumör. I takt med att tumören växer bildar den nya egna blodkärl som försörjer den med syre och näring.

Det är lätt att tro att tumören bara består av cancerceller men den består också av andra slags celler, till exempel vita blodkroppar, bindvävsceller och blodkärl. Dessa andra celler är normalt fungerande celler som hamnat under cancercellens kontroll för att hjälpa tumörutvecklingen. I den här avhandlingen försöker jag förklara hur och varför cancerceller lyckas tämja och manipulera vissa av dessa fullt normala celler, och hur cancerceller får dem att hjälpa tumören växa och frodas.

Cancer är ett samlingsnamn för ungefär 200 olika cancertyper. Bröstcancer är en av dessa och även den vanligaste typen bland kvinnor där cirka 20 kvinnor insjuknar varje dag i Sverige. Andelen som botas från sin bröstcancer är stor och ökar för varje år. Väl vid ett läkarbesök så brukar man tala om godartad eller elakartad tumör. De godartade tumörerna kan bli stora men de kan inte bryta sig loss och sprida sig till andra delar av kroppen. De elakartade tumörerna har däremot förmågan att växa ut och sprida sig till andra delar i kroppen och bilda nya tumörer – dottertumörer. Denna spridning av cancerceller i kroppen kan vara dödligt för patienten. Den mest elakartade formen av bröstcancer går under samlingsnamnet trippel-negativ bröstcancer. Trippel-negativ bröstcancer drabbar nästan en av tio och är vanligare hos yngre kvinnor. Dessvärre är behandlingsmöjligheterna få då trippel-negativ bröstcancer inte svarar så bra på de vanliga behandlingarna. Dessutom är spridningsrisken av denna elakartade bröstcancern stor.

Den här avhandlingen fokuserar på kroppens immunförsvar, som består av vita blodkroppar och dess relation med bröstcancer. Immunförsvarets vita blodkroppar har en rad viktiga uppgifter. De vita blodkropparna ska t.ex. skydda oss mot virus och bakterier, ta bort skadade eller döda celler i vår kropp och inte anfalla normala celler. För immunförsvaret är det svårt att känna igen en cancercell eftersom den härstammar från kroppens egna celler, och kommer således inte betraktas som ett hot. Om cancercellen avviker alltför mycket i sitt beteende och utseende jämfört mot de normala cellerna, så kommer den att bli upptäckt och dödad av immunförsvaret, men så är inte alltid fallet. Vid en väldigt elakartad tumör, så som

trippel-negativ bröstcancer, avviker cancercellerna kraftigt från de friska celler men blir ändå inte dödade av immunförsvaret. Förklaringen till detta fenomen är att cancercellerna skapar en miljö runtomkring sig som hindrar immunförsvaret från att förgöra cancercellerna.

I delarbete I och II visar vi hur bröstcancer, av den mer aggressiva formen, använder sig av samma medel som immunförsvaret för att förstärka den rådande inflammatoriska miljön i tumören. Inflammation innebär ökad blodtillförsel, större närvaro av immunförsvaret och ”lösare” vävnad som underlättar för cancercellerna att sprida sig ut till nya destinationer i kroppen. De vita blodkropparna som lockas in till tumören genom rådande inflammationen hamnar under cancercellernas kontroll för att hjälpa sjukdomen att utvecklas. De vita blodkropparna fungerar på så vis som cancercellernas förlängda arm.

Immunförsvaret är inte bara ett försvar utan också ett exemplariskt lagspel. Liksom ett fotbollslag, består av olika spelare som fyller en viss funktion. Vissa har en mer specifik och smalare roll medan andra spelare har en bredare och mer omfattande roll. I delarbete III identifierar vi stjärnspelaren som har förmågan att få med sig hela laget. Stjärnspelaren är en *monocyt*, en slags vit blodkropp. *Monocyter* har förmågan att ta på sig många olika roller som kan vara bra eller dåligt för tumören. Vid trippel-negativ bröstcancer är antar *monocyterna* en form som bl.a. stoppar andra vita blodkroppar från att attackera cancerceller och som ser till att fler blodkärl bildas som driver på tumörutvecklingen. I delarbetet fann vi också att dessa *monocyter* har förmågan att påverka omkringliggande celler, som inte är en del av immunförsvaret, för att sin tur bidra till tumörutvecklingen. När *monocyterna* är utvisade från spelplanen så ser vi inte samma aggressiva tumörutveckling i trippel-negativ bröstcancer.

Som tidigare nämnt, finns det skillnader mellan en godartad och elakartad tumör. Skillnaden är dock inte enbart baserat på cancercellen utan på vilken uppsättning andra icke-cancerceller som är närvarande. I delarbete IV så studerar vi en annan vit blodkropp som finns i många tumörer, nämligen *T-cellen*. *T-cellen* är en mycket specialiserad lagspelare av immunförsvaret. Det finns olika typer av *T-celler*. Där finns *T-celler* med förmågan att skydda oss mot virus och bakterier. Motsatsen är typen av *T-celler* som är experter på att bromsa och stoppa immunförsvaret. Men vilken typ av *T-cell* är vanligast i tumören? Är det *T-cellen* som är expert på att döda cancerceller eller är det typen som sätter stopp för dödandet av cancerceller? Att utforska vilken typ av *T-cell* som härjar i tumörerna kan leda till ökad förståelse till varför vissa tumörer är mer aggressiva än andra. Bröstcancerpatienter med tumörer som innehåller cancerdödande *T-celler* har en ökad chans att överleva. Patienter med tumörer som innehåller *T-celler* som sätter stopp för dödandet av cancerceller har en ökad risk för dödligt utfall.

Den här avhandlingen bidrar till kartläggningen av celler som finns i bröstcancertumörer, med fokus på de vita blodkropparna. Våra resultat visar att trippel-negativ bröstcancer medvetet rekryterar vita blodkroppar som hamnar under cancercellernas kontroll. De vita blodkropparna, bl.a. *monocyterna* hjälper sedan tumören att växa och sprida sig. Våra resultat kommer förhoppningsvis leda till ökad förståelse till vad det är som driver utvecklingen i tumören, och således öppna upp möjligheten för utveckling av nya behandlingar. Det börjar bli mer aktuellt för läkemedelsbolagen att utveckla behandlingar som riktar sig mot icke-cancerceller för att kapa cancercellens förlängda arm och därmed bromsa tumörens möjligheter att växa och sprida sig.

Cancer – perpetrator without restraint

> "Any living cell carries with it the experience of a billion years of experimentation by its ancestors. You cannot expect to explain so wise an old bird in a few simple words." – Max Delbrück, geneticist, 1966.

A brief introduction

Our body is comprised of more than 10^{14} cells all of which collaborate selflessly to maintain tissue and cellular functions across the organism. As opposed to the natural selection of organisms and the survival of the fittest, the only rule that applies among the cells in the organism is self-sacrifice. Cancer cells break that one rule. Cancer is a term that is well known among the general population in our society, however, it is a term that comprises over 200 different forms of cancer. They all have one common denominator and that is genetic alterations.

When a genetic alteration, or mutation, gives a cell survival advantage over their fellow neighbouring cells they ultimately may become a cancer cell, or neoplastic, which means new tissue. Hanahan and Weinberg proposed that all cancer cells share six common biological capabilities known as the hallmarks of cancer. These hallmarks of cancer include sustaining proliferation, evading growth suppression, inducing angiogenesis, become immortal, resisting cell death and breaking free from the original tissue to invade and metastasis [1]. In 2011, Hanahan and Wienberg published an updated version, now with ten hallmarks of cancer, including tumor promoting inflammation and avoiding immune destruction, which is the focus of this thesis [2].

In Sweden during 2015, 61 100 patients were diagnosed with cancer and 22 422 died of the disease. The five most common cancer diagnosis that year was prostate, breast, skin, colorectal and lung cancer. Although the rate of incidence is rapidly increasing the mortality rate is decreasing due to better therapies and improved methods of diagnosis [3].

Breast cancer

Epidemiology and risk factors

Among women in Sweden, breast cancer r epresents 30 percent of all newly diagnosed cancers, making it the most common type of cancer [3]. Although breast cancer encompasses the majority of the newly diagnosed cancer types, lung cancer is the leading cause of death among women with cancer [3]. However, the breast cancer incidence is increasing. One explanation is the systematic screening procedures among women provided by the Swedish health care. Another explanation is that awareness among the general population has increased. Although the incidence is increasing, the survival rate has greatly improved [3,4].

The etiology of breast cancer comprises of multiple risk factors that include age, diet, smoking, alcohol consumption, radiation and genetic predisposition, early menarche, late menopause, use of oral contraceptives, use of hormonal therapies and pregnancy at late age [5-8]. In addition, there are factors that can decrease the risk of breast cancer, including early pregnancies, the number of childbirths and late menarche [6-8].

There are also heritable, germline mutations including BRCA1 and BRCA2 that predispose women to breast cancer and make up approximately 5% of all breast cancers [7,9-11]. The mutations of BRCA1 and BRCA2 account for 40% of all familial breast cancer cases, therefore, suggesting the existence of additional genetic mutation that predispose women to breast cancer [10]. BRCA1 and BRCA2 are tumor suppressor genes that are involved in maintaining genetic integrity through the management of DNA repair [10,12], which when impaired increase the risk of accumulating additional mutations that causes breast cancer. In addition, impaired BRCA1 is associated with triple-negative breast cancer (TNBC; breast cancer subtype with poor survival outcome) while BRCA2 is associated with a wider range of breast cancer subtypes [13].

Breast cancer diagnosis and classification

The primary source of detecting and diagnosing breast cancer is either by the patients feeling a solid lump in their breast or during a routine mammography screening. To verify and confirm the diagnosis a magnetic resonance imaging (MRI) is performed, as well as needle biopsy for both cytological and histological evaluation of the tumor [14].

Histological grading in breast cancer

Histopathological assessment of breast cancer includes determination of morphological subtypes such as lobular or ductal carcinoma. This does not indicate the origin of the cancer but rather describes the cytological features and immunohistochemical (IHC) characteristics [15]. Furthermore, two grading methods have been established that are of prognostic/predictive relevance; (i) the Nottingham histological grading (NHG) and (ii) the tumor size, nodal status and distant metastatic spread (TNM). The NHG method evaluates the level of differentiation and the proliferative grade of the tumor cells. This grading method is divided in three histological features – (i) how well the tumor cells forms tubular structures, (ii) the heterogeneity of the nuclear size among tumor cells and (iii) the mitotic frequency. The NHG grades between I-III where I is a well differentiated tumor and III is poorly differentiated [16]. The TNM classification refers to (i) the size of the tumor, (ii) if and how many nearby lymph nodes that contains tumor cells and (iii) if there are any distant metastatic lesions [17].

Receptor status in breast cancer

Further classification of breast cancers is performed through the assessment of the receptor status including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [18]. These are assessed using IHC staining or for HER2 amplification the fluorescence in situ hybridization (FISH) technique. When all of the receptors are negative then the tumor is classified as triple-negative breast cancer (TNBC). TNBC is associated with poor prognosis [19].

Molecular classification of breast cancer

In 2000, *Perou et al* published a novel way of breast cancer classification where they furthered the molecular portrait of breast cancer based on analyzing the gene expression patterns [20]. This provided five molecular subtypes all of which can

roughly be sorted into either ER^+ or ER^- breast cancers. The following molecular subtypes were identified; luminal A, luminal B, HER2 amplified, basal-like and normal breast-like [20-22] all of which have different prognostic outcome [22]. Moreover, as the gene expression analysis develops, more subcategories of the molecular subtypes have surfaced; such as claudine-low breast cancer, which is even more mesenchymal then the basal-like molecular subtype [23].

Table 1.
The different molecular classifications and their features [22,24-26].

Molecular subtype	Molecular profile	Frequency	Histological grade	Human cell lines	Clinical outcome
Luminal A	ER^+, PR^+, $HER2^-$, low Ki67	50-60%	Low	MCF-7 T47D	Good
Luminal B	ER^+, PR^+, $HER2^+$, high Ki67	10-20%	Moderate/high	BT474	Moderate/poor
HER2	ER^-, PR^-, $HER2^+$,	10-15%	High	SKBR-3	Poor
Basal-like	ER^+, PR^+, $HER2^-$, $CK5/6^{+a}$ and/or $EGFR^{+b}$	10-20%	High	MDA-MB-468 SUM149	Poor
Claudine-low	ER^+, PR^+, $HER2^-$, $CK5/6^+$, $EGFR^-$	12-14%	High	MDA-MB-231 SUM159	Poor
Normal-like	$ER^{+/-}$, $HER2^-$	5-10%	Low	-	Moderate

[a]CK5/6; Cytokeratin 5/6
[b]EGFR; Epidermal growth factor receptor

Available treatments

The first line treatment of breast cancer according to the Swedish guidelines (Socialstyrelsen) is surgery, which includes the removal of tumors as well as regional lymph nodes. In some cases, chemotherapy and radiotherapy is required to shrink the tumor mass prior to surgery [14]. Further tumors classified as ER^+ breast cancers commonly undergo tamoxifen and aromatase inhibitor treatments. HER2 amplified breast cancers meanwhile are treated with the monoclonal antibody; trastuzumab (Herceptin) [26]. TNBCs are treated with limited options of chemotherapies [27].

Cancer-associated fibroblasts

Fibroblasts are commonly described as large elongated mesenchymal cells residing within the connective tissue. Therefore, it is likely that fibroblasts are responsible in upholding the integrity of the extra cellular matrix (ECM) [28]. Fibroblasts in their active form, also known as myofibroblasts, can regulate inflammatory processes [29] and play a crucial role in wound healing [30]. Cancer-associated fibroblasts (CAFs) resemble activated myofibroblasts due to their ability to drive similar processes such as, wound healing, ECM remodeling and angiogenesis [31]. The recruitment and activation of CAFs, is possibly mediated by tumor cells. During tumor progression, CAFs can originate from different progenitor cells including resident fibroblasts, mesenchymal stem cells and endothelial cells. Tumor derived factors such as transforming growth factor-β (TGF-β) and PDGF are potent inducers of CAFs [28,32-34]. Moreover, CAFs have been proposed as drivers of proliferation, angiogenesis and invasion of cancer cells [35-37].

CAFs are a very heterogeneous group of cells. Thus, no specific molecular definition of CAFs exist yet [38]. Therefore, CAFs are mostly identified through several molecular markers in combination with morphological properties. CAFs appears as large, elongated and spindle-shaped [38]. However, the most commonly used molecular markers are; α-smooth-muscle actin (αSMA), fibroblast specific protein-1 (FSP-1), fibroblast activating protein (FAP), vimentin, platelet-derived growth factor receptor-α (PDGFR-α), PDGFR-β and neuron-glial 2 (NG2) [38-41]. Furthermore, none of the mentioned molecular markers are exclusively expressed by CAFs [38].

In breast cancer, high tumor-stroma ratio has been associated with both improved and poor clinical outcome depending on the breast cancer subtype [42-44]. Another study showed that PDGFR-β was associated with high histopathological grade, ER^- breast cancers and HER2 expression. Furthermore, they also showed that this correlated with decreased survival outcome and higher recurrence rate [45]. A follow-up study also revealed that the expression of PDGFR-β reduced the benefit of tamoxifen in two other cohorts with early breast cancer. Thus, suggesting that PDGFR-β might be of clinical relevance when predicting tamoxifen response [46]. Few studies have evaluated the clinical relevance of αSMA in breast cancer. Two reports, using small breast cancer cohorts, stained for αSMA concluded that high expression of αSMA was associated with poor survival outcome [47,48]. This highlights the demand of characterizing the CAF so that we can truly evaluate the clinical relevance [49].

Tumor immunology

> "In the end, we will remember not the words of our enemies, but the silence of our friends." – Martin Luther King Jr

Basic overview of the immune system

The immune system is evolved and fine-tuned to protect the host from external and internal threats. The external threats may be pathogens or toxins. The internal threats may be damaged, or dead cells of host origin, but also tumor cells may be recognized as an internal threat. Distinguishing self from non-self, as well as managing the process of wound-healing and tissue remodeling, are two major functions of the immune system which will be discussed further in the context of tumor progression. To be able to fulfill these functions, the immune system is divided into an immediate innate response and a delayed adaptive response.

The innate immune system

The innate immune system is comprised of physical barriers, the complement system and innate immune associated leukocytes. The innate immune cells are the monocytes, macrophages, dendritic cells (DCs), granulocytes (neutrophils, eosinophils, basophils and mast cells) [50], and natural killer (NK) cells [51]. The innate immune cells may be resident, thus embedded within the physical barriers (mainly macrophages, DCs and mast cells), or patrolling between the blood circulation, lymph and tissues [52]. All innate immune cells are derived from the myeloid cell lineage [50,52], except for NK cells and a certain DC subpopulation that are derived from the lymphoid lineage [51]. Some of the functions of the innate immune system are to act as a barrier, to quickly react upon encounter of external pathogens with the aim to recruit more immune cells, to activate the complement system, to remove the foreign substances and the damaged or dead cells though phagocytosis and finally to activate the adaptive immune system through a process known as antigen presentation [53].

To be able to promptly react against foreign substances, the innate immune cells carry a set of receptors that recognize pathogen- as well as damage- associated molecular patterns. The molecular structures are called pathogen- or damage-associated molecular patterns (PAMPs and DAMPs respectively) and the responsible receptors are called pattern recognition receptors (PRRs). PAMPs are conserved non-self molecular patterns originating from external pathogens including bacteria (i.e. lipopolysaccharides; LPS) and viral RNA/DNA. On the contrary, DAMPs are molecules that originates from host cells when injured or in distresses (i.e. high-mobility group protein 1; HMGB1) that serves as a "danger-signal" [54]. When PRRs are activated, they will trigger a signaling pathway that ultimately leads to activation of the transcription factor nuclear factor kappa B (NFκB), a key player in early host defense. The genetic program that is enabled by NFκB includes the production of cytokines (e.g. IL-1β, IL-6, IFN-γ and TNF-α) and chemokines (e.g. IL-8) all of which are crucial for attracting and activating more leukocytes [55]. The factors that are secreted not only attract additional leukocytes, but also influence surrounding stromal cells and endothelial cells to remodel ECM and dilate blood vessels. This helps facilitate the homing and transmigration of additional leukocytes from the blood stream into the tissue. The processes described above are associated with inflammation [56].

The innate immune cells clear the infected or damaged tissue through a mechanism called phagocytosis. Some phagocytes (monocytes, macrophages and DCs) can also act as professional antigen presenting cells (APCs), and have the ability to migrate to a regional lymph node to present the processed foreign substance (antigens) to cells of the adaptive immune system (naïve T and B lymphocytes). This way, the innate immune cells initiate activation of the highly specific adaptive immunity. DCs are professional APCs that play an important role in connecting the innate and the adaptive immune response [57-59]. The molecules that are responsible for antigen presentation are known as major histocompatibility complexes (MHC). There are two classes of MHC molecules; (i) the MHC class I that is expressed by all cells and presents intracellular peptides and (ii) the MHC class II that is expressed by professional APCs presenting antigens derived from extracellular substances.

The adaptive immune system

The ability to resist infection upon encounter with a pathogen is termed immunity. Although the acute actions of the innate immune system can efficiently isolate and destroys pathogens, it usually does not clear the infection completely and does not lead to long-term immunity. For this purpose, the adaptive immune system is thought to have evolved, with its ability to orchestrate a specific, long-lasting attack. The adaptive immune system is comprised of cells of the lymphoid lineage; T and B lymphocytes. T lymphocytes develop in the thymus, whereas B lymphocytes

develop in the bone marrow [60]. There are two classical hallmarks of the adaptive immune system that defines the major difference compared to the innate immune system. Firstly, the adaptive immune system is completely dependent on their antigen-specific receptors (T cell receptor [TCR] and B cell receptor [BCR; membrane bound Ig]) [52]. Secondly, the adaptive immune system has the ability to generate immunological memory that is long lasting and allows the immune system to act faster when the host is re-infected with the same pathogen. In contrast to the PRRs of the innate immune system, the antigen-specific receptors of the adaptive immune system are products of a gene assembly that is rearranged in infinite combinations to build a specific receptor for each foreign substance that could challenge our body. The foreign structures that are specifically recognized are called antigens. Antigens are non-self small peptides or native structural parts of proteins or carbohydrates. This results in a vast repertoire of adaptive immune cells, each with a unique antigen-specific receptor [52,57,60].

T lymphocytes

The classical T lymphocytes are αβTCR expressing cells, grossly divided into two main subsets; $CD4^+$ T helper (T_h) cells and $CD8^+$ T cells. T_h cells modulate and facilitate immune responses and are further divided into several subtypes; for example T_{h1}, T_{h2}, T_{h9}, T follicular helper cells, T_{h17} and regulatory T cells (T_{regs}), all of which have different functions and effects on the immune response[61]. The second subset of T cells is the $CD8^+$ cytotoxic T lymphocyte subset (CTLs) that target and destroy individual somatic cells that are infected, damaged or transformed. Both Th cells and CTLs requires recognition of their specific antigens presented on MHC molecules [62]. The difference between T_h and CTL activation is that T_h cells recognizes their antigen through an antigen/MHC class II molecule. CTLs recognizes their antigen through antigen/MHC class I molecule. Also, DCs has the ability to engulf extracellular antigens and present it in a MHC class I manner – a process known as cross-presentation [62]. Cross-presentation is a crucial function in tumor immunology, which will be mentioned in detail later on in this thesis. In contrast to the classical αβTCR expressing lymphocytes, there are T cells with invariable TCRs that recognize their antigen on non-classical MHC molecules. These are the γδ T cells and the natural killer T cells (NKT cells) [60]. In this thesis, I will further discuss CTLs, γδ T cells, Th17 and T_{regs} in the context of breast cancer.

Regulation of the immune response

To be able to distinguish self from non-self, the immune system has evolved important regulatory strategies: Central and Peripheral Tolerance. Central tolerance is mediated by selection of lymphocytes that only recognizes non-self antigens by deletion of lymphocytes capable of recognizing self-antigens. If the central

tolerance fails, peripheral tolerance takes over. Peripheral tolerance can be induced among lymphocytes via the following mechanisms; (i) peripheral deletion (ii) the induction of anergy, an active non-responsiveness of T lymphocytes iii) immune-regulation through the effect of inhibitory co-receptors (immune checkpoints), regulatory T cells (T_{regs}) and the production of immunosuppressive cytokines such as TGF-β and IL-10 [63]. In the context of tumor immunology, primarily the process of peripheral tolerance is manipulated by the tumor to avoid an efficient immune attack.

Tumor progression from an immunological point of view

> "It is by no means inconceivable that small accumulations of tumour cells may develop and because of their possession of new antigenic potentialities provoke an effective immunological reaction with regression of the tumour and no clinical hint of its existence." – Macfarlane Burnet, Immunologist, 1957

The notion that the immune system is conducting a systematic surveillance to find and eradicate transformed cells is not new. However, tumor cells are self-cells, but with many mutations and damage associated structures that should be recognized as non-self, or danger, for the immune response. To avoid recognition and attack of our own body and in order to keep the tolerance to self, the immune response has developed certain strategies to regulate dangerous and overt immune responses. In a tumor context, the immune system therefore has been shown to play a paradoxical role, since it not only can protect the host from tumor cells but also can act in synergy with tumor cells and thus promote tumor development.

The hypothesis of immunoediting in cancer

The immune system has an inherent capacity of targeting and destroying spontaneously occurring tumor cells. This mechanism is termed immunosurveillance. Tumor immunology covers the transitioning from immunosurveillance into immune evasion. This transitioning is divided into three phases known as the "three Es" of cancer immunoediting; elimination, equilibrium and escape [64,65].

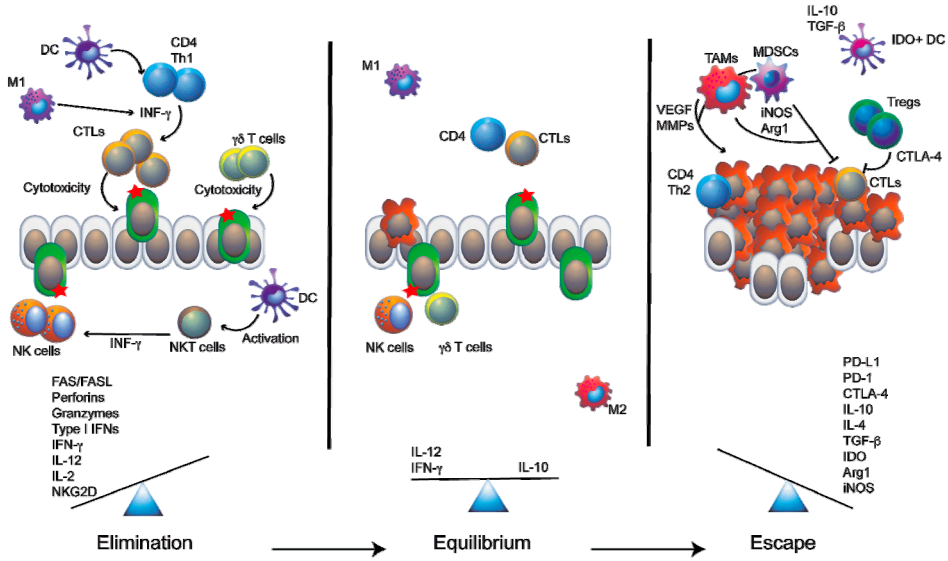

Figure 1. A brief overview of the three "Es" of cancer immunoediting.
In the elimination phase, transformed cells (green) are recognized and destroyed by the immune system. During the equilibrium phase, immunogenic tumor cells are removed, while less immunogenic tumor cells (red) are not. During the escape phase, the tumor cells that manage to avoid and escape immune destruction are expanding in an uncontrolled manner. Adapted from *Dunn, G.P., et al, 2002* and *Mittal, D., 2014* [64,66].

Elimination

The immunosurveillance or elimination of cancer cells is executed in three distinct ways; (i) the protection and suppression of virus infections that can induce cell transformations (ii) the ability of the immune system to suppress prolonged inflammations which have been shown to contribute to tumorigenesis [67] (iii) tumor antigens that are recognized by antigen-specific receptors of the adaptive immune system, and triggering of the innate immune response by DAMPs such as high mobility group box-1 (HMGB1) expressed by necrotic tumor cells [68].

Intense investigations have been conducted to solve the mechanisms of how the innate and adaptive immune system manages the elimination of transformed cells. Among the different possibilities are (i) tumor cells that are presenting tumor antigens in a MHC class I dependent manner will consequently trigger a cell mediated cytotoxic response involving CTLs [66] (ii) the release of endogenous "danger-signals", DAMPs. The release of DAMPs by dying tumor cells [69] or the expression of stress-associated molecules such as MICA/MICB on tumor cell surface can promote immune responses of NK, NTK and γδ T cells [70] (iii) the Type I IFNs, an extensively studied group of factors that has been associated with a tumoricidal immune response [68,69]. Type I IFNs are very important for NK cell effector functions [71]. Blocking of Type I IFNs in a mouse model enhances tumor

growth and progression [72] and it has been revealed that Type I IFNs drives the anti-tumor response through the activation of DCs and their cross-presentation capabilities that connects the $CD8^+$ CTL mediated elimination of tumor cells [66,73,74]. The initial signals that contribute to Type I IFN production are mostly through PRR stimulation caused by DAMPs that are released into the microenvironment by necrotic tumor cells [75], thus truly emphasizing how important the innate immune system is in tumor immunology.

Equilibrium

Equilibrium, is believed to extend over a period of several years, therefore making it the longest of the three phases [64,68]. During this phase, there is little, if any, growth of the primary tumor due to containment by the adaptive immune system. One study revealed that T_{h1} and CTLs of the adaptive immune system are highly involved in maintaining this equilibrium. Chemically induced sarcomas in wild-type immunocompetent mice showed that tumor outgrowth was initiated once the T_{h1} and CTLs, as well as IL-12 and IFN-γ were disabled by blocking antibodies [76]. They also revealed that depletion of NK cells or blocking NKG2D did not lead to tumor outgrowth, therefore concluding that only the adaptive immune system was responsible for maintaining tumor dormancy [76,77]. However, these experiments did not show that the induced tumors passed through the elimination phase first prior to the state of equilibrium. Immense efforts have been conducted to prove the state of equilibrium. It is even more challenging to show this in patients, due to tumors already being in the end-phase of the immunoediting process. It has, however, been suggested that the equilibrium state is dependent on the balance between IL-12 and IFN- γ (promoting tumor destruction) and IL-23 (promoting tumor persistence) [78]. Furthermore, the source of IL-23 has been found to originate from $CD11b^+$ macrophages in the tumor microenvironment [79]. Depletion of $CD11b^+$ macrophages (also known as tumor associated macrophages; TAMs) in tumors showed a reduction of tumor growth in mice models [80], indicating that they play an important role in maintaining the equilibrium state or even shift the balance towards tumor outgrowth and metastasis. Therefore, suggesting that there is a Darwinian selection of tumor cells that has acquired the appropriate mutations, allowing it to become less immunogenic, consequently, escaping the elimination process. This is termed immunological sculpting, and describes the co-evolution between tumor cells and the immune system [64].

Escape

During the escape phase, the tumors are growing aggressively and invasively, consequently leading to metastasis and death of the host if left untreated. The tumor cells have, through the process of immunological sculpting and a series of mutations and epigenetic changes, acquired a set of properties that allows them to evade,

inhibit and also to some extent employ the immune system in aiding disease progression.

There are several mechanisms that the tumor cells can use in order to escape the immune system.

(i) Decreased T cell function: A decreased expression of MHC class I has been observed in tumor cells [66,81]. Upregulation of inhibitory co-receptors (PD-L1:PD-1 or CTLA4) thus leading to T cell exhaustion or anergy [82]. Additional factors that can inhibit T cell activation or induce T cell anergy (indoleamine 2,3 dioxygenase [IDO] and arginase) are produced by immune cells with tolerogenic functions such as IDO^+ DCs, regulatory T cells (T_{regs}) and TAMs secreting arginase [66].

(ii) Immunological sculpting: Shedding of Killer activation receptors on innate or borderline innate cytotoxic lymphocytes (NK/NKT and γδ T cells). NKG2D-ligands MICA/MICB being released into the microenvironment that inhibits the effector function of the innate or borderline innate lymphocytes NK/NKT and γδ T cells [83,84].

(iii) Soluble regulatory factors: Another important aspect of the immune evasion or suppression is the microenvironment that is influenced by the factors produced by the tumor cells. A decrease in IFNs and IL-12 will abolish effector function of tumoricidal immune cells [66]. The production of TGF-β, IL-10, CXCL12 and GM-CSF in the tumor microenvironment promotes immune suppression and attraction of immune suppressive cells such as T_{regs}, TAMs and myeloid-derived suppressor cells (MDSCs) [85]. Therefore, skewing the immunological profile towards suppression of the tumoricidal immune response, and hence promoting disease progression.

Table 2.
Different strategies of escaping and inhibiting the immune response [83,85-87].

Different strategies	Mechanisms	Cells involved
Decreased T cell function	• Reducing the expression of MHC class I molecules on tumor cells • Expression of inhibitory co-receptors PD1/PD-L1, CTLA-4 • Immunosuppressive factors being released; eg. IDO, arginase	• T cells • APCs • Tregs • TAMs • IDO^+ DCs • MDSCs • Tumor cells
Immunological Sculpting	Shedding of Killer receptors; NKG2D-ligands, MICA/MICB	• NK • NKT • γδ T cells
Immunosuppressive tumor microenvironment	Production of TGF-β, IL-10, IL-13, IL-4, GM-CSF and CXCL12	• Tregs • TAMs • MDSCs • tolerogenic DCs

Inflammatory cells and mediators in the tumor microenvironment

"Tumors: Wounds that do not heal" – Dvorak, H.F., 1986

Lately, the promotion of disease progression in breast cancers has been linked to the function of non-malignant cells within the tumor microenvironment. Immune cells infiltrate the tumor microenvironment and through their inflammatory mediators, growth factors and remodeling of the ECM – they promote the malignancy of the disease.

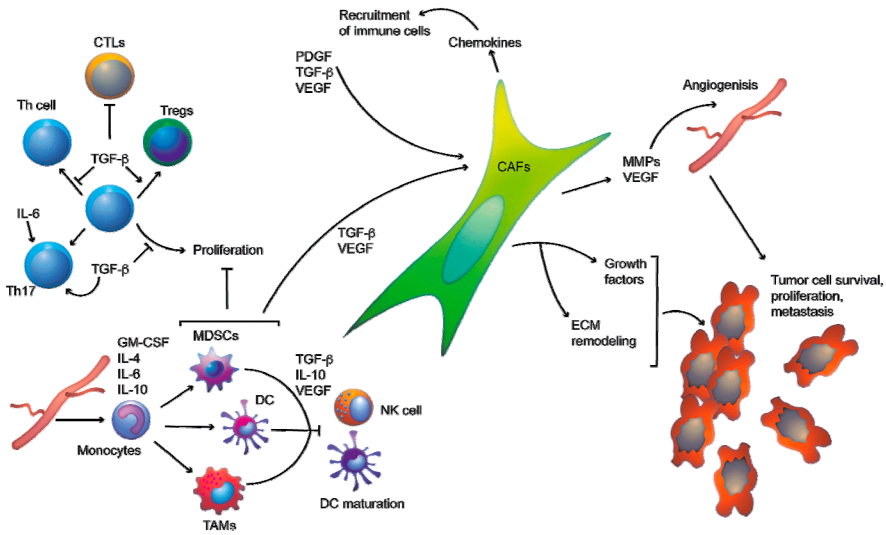

Figure 2. An overview of the tumor microenvironment.
The non-malignant cells included in the tumor microenvironment facilitate an active remodeling of ECM, promotion of angiogenesis, recruitment of immune cells, suppression of tumoricidal immune response and aiding tumor progression. Adapted from *Kalluri, R., 2016* [88].

Tumor infiltrating myeloid cells

Immune cells of the myeloid lineage have been associated with angiogenesis [89,90], suppressing tumoricidal immune response and aiding the tumor cells in migration, invasion and metastasis [91].

Monocytes

Monocytes are, as previously mentioned, a part of the innate immune system, constituting approximately 5-10% of all peripheral leukocytes in the blood of humans. The monocytes are a heterogeneous population that express high levels of CD14 [92]. Although, monocytes possess the ability of engulfing antigens and presenting antigens, they are not professional phagocytes like macrophages and DCs. Monocytes are plastic by nature and capable of giving rise to different cell types upon differentiation. Thus, when monocytes infiltrate tissues, they can become DCs or macrophages and maybe also myeloid-derived suppressor cells depending on the cytokine milieu [87,93].

In the context of a tumor, they have been described as proangiogenic [94]. Moreover, monocytes may be reprogrammed and skewed towards a more immunosuppressive phenotype by the tumor microenvironment [95,96]. In breast cancer, monocytes have been associated with increased invasiveness and metastatic progression [97]. Furthermore, it has been shown that tumor derived factors selectively recruit monocytes into the tumor tissue in early development of breast cancer [97,98]. It has also been noted that monocyte levels in the blood of breast cancer patients is elevated [98,99], especially monocytes with a monocytic myeloid-derived suppressor cell (Mo-MDSC) phenotype that are similar to the immunosuppressive monocytes observed in blood from septic patients [99]. In this thesis (Paper III), we show that TNBCs promote recruitment, survival, proliferation and differentiation of monocytes into myeloid cell populations with a tumor-aiding immunosuppressive phenotype, in situ in the tumor.

Macrophages

As previously mentioned, macrophages are highly specialized phagocytes that reside within tissue (resident) or originate from circulating monocytes (recruited). Generally, macrophages in tissue are identified through the pan-marker; CD68 [100]. Macrophages are furthermore highly plastic, capable of becoming either pro-inflammatory (M1 macrophages) or anti-inflammatory (M2 macrophages) depending on the environmental characteristics [101,102]. M1 and M2 macrophages

represents the two extremes of a continuum of various polarizations [101]. Also, M1 are prominent in expression of co-stimulatory molecules such as CD80 and CD86 as compared to M2 macrophages. This further indicates that M2 macrophages promote a more tolerogenic environment [101]. Table 3 summarizes the difference in characteristics and cytokine profile between the different macrophage phenotypes.

In a tumor context, the macrophages (TAMs) have been extensively studied and described as one of the main components among the tumor infiltrating leukocytes [85]. TAMs are mostly described as M2-like macrophages due to their immunosuppressive profile [85,103]. They inhibit anti-tumor immune responses through the expression of inhibitory co-stimulatory ligands and their immunosuppressive cytokine profile [104,105]. In addition, TAMs are highly pro-angiogenic and are believed to be one of the main drivers of the angiogenic switch in breast cancer [90,91]. TAMs are also involved in promoting invasiveness in breast cancer [106]. In clinical studies, infiltration of TAMs is generally associated with poorly differentiated tumors, higher tumor grade, hormone receptor negativity and poor prognostic outcome [89,107-109]. It was also found that $CD163^+$ myeloid cells present in luminal A ($ER^+PR^+HER2^-$) breast cancer were associated with poor survival outcome, indicating that these cells are powerful promoters of disease progression [108].

Table 3.
Summary of the different macrophage phenotypes [85,101,110,111].

Macrophage phenotypes	Cytokine profile	Molecular markers
M1, pro-inflammatory and tumoricidal	High IL-12 Low IL-10 Produces IL-6, IL-1β and TNF-α	CD80, CD86, CD16, iNOS, MHC class II^+
M2, anti-inflammatory	Low IL-12 High IL-10 Produces TGF-β, VEGF, MMPs and PGE2	CD163, Arginase, MHC class II^{low}
TAMs, anti-inflammatory and angiogenic	Low IL-12 High IL-10 Produces TGF-β, VEGF, COX2, PGE2 and MMPs	CD163, Arginase, MHC class II^{low}

Dendritic cells

DCs are the most efficient APCs. They are therefore the main players linking the innate with the adaptive immune response. In tissue, they are in an immature state that are mainly surveilling the surrounding tissue by internalizing antigens, presenting them in an MHC dependent manner without expressing co-stimulatory ligands such as CD80 or CD86, thus, no priming of any T cell response. Presenting self-antigens without any co-stimulatory ligands is a crucial regulation of

maintaining tolerance to self. However, upon PRR stimulation by either exogenous or endogenous "danger" signals the DC will start maturing as it migrates to nearby lymph nodes, hence, expressing co-stimulatory ligands as well as cytokine secretion to activate a T cell response [112,113]. There are different types of DCs, which can be characterized by specific cell surface markers, although the large variation between markers in mice compared to humans, as well as between different markers depending on which sites in our body the DCs are situated in, has made it difficult to categorize them in tumors (Table 4). DCs are also the main drivers of anti-tumor responses due to their ability of cross-priming CTLs. Upon PRR mediated response of the DCs, they will mature and initiate a T cell mediated anti-tumor response [114]. In addition, cancer therapies such as radiation or chemotherapy induces tumor cell death that will release DAMPs, therefore, triggering maturation of DCs [115]. However, in advanced tumors they are heavily suppressed by the tumor microenvironment – keeping the DCs in an immature state leading to the exploitation of checkpoint regulation and maintenance of central tolerance. Factors that are potent inhibitors of DC maturation includes TGF-β, VEGF and IL-10 [114,116,117].

Table 4.
Phenotypic description of the different DC subtypes [118].

Dendritic cell phenotype	Some Features
cDC1	Excellent cross-presenters and activators of CTLs T_{h1}/T_{h2} responses
cDC2	Excellent activators of $CD4^+$ T cells High expression of MHC class II molecules T_{h2}/T_{h17} responses
pDC	Express TLR7 and TLR9 that binds to viral RNA and DNA Anti-viral responses; Produces IFN-α
Mo-DC	Monocyte derived myeloid DCs Inflammatory responses

Myeloid-derived suppressor cells

More than a decade ago, *Gabrilovich et al* argued that the highly immunosuppressive myeloid cells found in association with acute inflammation, infections and tumors, should be termed myeloid-derived suppressor cells (MDSCs) [119]. MDSCs are believed to be generated as a systemic response to excessive inflammation [120]. They are considered highly immunosuppressive and their presence has been described in various tumors [121-123]. MDSCs have been reported to inhibit tumoricidal T cell activity, promote T_{reg} differentiation, inhibit DC maturation and promote M2-like macrophages. They are also known producers of IL-10, TGF-β, VEGF, IL-6 and GM-CSF all of which are associated with angiogenesis, ECM remodeling and tumor invasiveness, thus, contributing to disease progression [87,123-

[127]. Although MDSCs are a very heterogeneous population, they are divided into two main subsets: Monocytic MDSCs (Mo-MDSCs) and granulocytic MDSCs (G-MDSCs) [122,123]. Human Mo-MDSCs are defined as $CD11b^{+}CD14^{+}CD33^{+}HLA\text{-}DR^{-/low}Co\text{-}receptor^{-/low}$ and G-MDSCs as $CD11b^{+}CD15^{+}CD33^{+}Lin\text{-}HLA\text{-}DR^{-/low}$ [87]. In humans, it is difficult to study MDSCs in solid tumors due to their heterogeneity. However, there have been studies investigating circulating MDSCs in peripheral blood of cancer patients. In breast cancer patients, it was shown that the amount of circulating MDSCs in breast cancer patient blood was associated with metastasis and impaired clinical outcome [99,128,129].

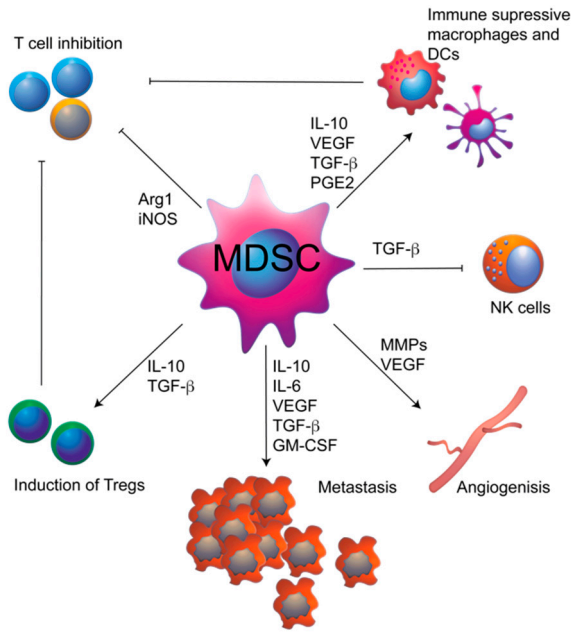

Figure 3. The role of MDSCs in immune suppression and tumor progression.
Through modulation of the cytokine milieu and thus by affecting other cell types, MDSCs induce immunosuppression and promote tumor progression. Adapted from *Millrud, C.R., 2017* [87].

Tumor infiltrating lymphocytes

Generally, tumor infiltrating lymphocytes (TILs) are associated with good clinical outcome in breast cancer patients [130-132]. However, the composition of the infiltrating lymphocyte subpopulations in the primary tumors is important due to the wide range of different functions exerted by the different TIL subpopulations. Determining the immunological profile of a solid tumor has become an important parameter when assessing how the patient will respond to different therapies.

$CD8^+$ cytotoxic T lymphocytes

Although, many tumor antigens can be recognized by CTLs, tumors still manage to avoid and escape the CTL immune response. Tumors manage to escape via a number of steps including down regulation of MHC class I molecules, expression of inhibitory immune checkpoint molecules (i.e. PD-L1) and secretion of TGF-β. Also, tumor microenvironment plays a crucial role in regulating CTL response such as recruited immunosuppressive leukocytes (T_{regs}, MDSCs, TAMs, IDO^+ DCs). Generally, high infiltration of $CD8^+$ T lymphocytes is associated with good clinical outcome; however, this is not always the case [86,133]. Lately, studies have shown that the clinical effect of infiltrating $CD8^+$ T lymphocytes depends on the overall immunological profile of the tumor. In breast cancer, one study could show that high infiltration of both $CD8^+$ and $CD4^+$ T lymphocytes, together with low numbers of macrophages, was associated with improved survival outcome [134]. This indicates that the clinical effect of CTLs is dependent on the polarization of the tumor microenvironment.

There have been several studies conducted on evaluating CTLs through the assessment of $CD8\alpha^+$ TILs in breast cancer. One study revealed that high infiltration of $CD8\alpha^+$ TILs was independently associated with good prognostic outcome in breast cancer patients [135]. On the contrary, another study with similar cohort size, could only show that high $CD8\alpha^+$ TILs were associated with improved prognosis in ER^- breast cancers [136]. It was later revealed that high infiltration of $CD8\alpha^+$ TILs was associated with a good prognosis in TNBCs and $HER2^+/ER^-$ breast cancer [132,137]. This indicates that a positive prognostic outcome of the $CD8\alpha^+$ TILs might be limited to certain subtypes of breast cancer. Another explanation could be that CD8α can be expressed by other immune cells than CTLs, which makes it difficult to determine which effect is carried out by the monitored $CD8\alpha^+$ TILs in the different breast cancer subtypes [133,138-140].

T_{h1} cells

Th1 cells is a subset of classical αβTCR $CD4^+$ T_h lymphocytes that mainly enhances the activation and infiltration of CTLs and their cytotoxic effector function. The master regulator of the T_{h1} polarization is T-bet, a transcription factor, that is upregulated when naïve $CD4^+$ T lymphocytes are exposed to IFN-γ and IL-12. T-bet further enhance the production of IFN-γ [61,141]. T_{h1} effector function can be negatively regulated via interaction of checkpoint molecules expressed on T_{h1} surface [142], but also secretion of immunosuppressive cytokines (i.e. IL-10 and IL-4) [141]. In a tumor context, T_{h1} cells plays an important part in mediating anti-tumor immune response via CTLs [143]. In breast cancer, clinical studies have shown that

tumors with a T_{h1} associated immune profile is associated with improved clinical outcome [144,145].

T_{h2} cells

T_{h2} cells are also classical αβTCR $CD4^+$ Th lymphocytes, but mainly involved in mediating host defense against foreign pathogens via activation and engagement of B-cells. The main inducer of T_{h2} cells is IL-4. The master regulator of T_{h2} is GATA-3 transcription factor that further enhances the production of IL-4 but also IL-13 and IL-10 [141]. Furthermore, IL-4 appears to inhibit the IL-12 signaling, thus, disrupting T_{h1} differentiation [61]. T_{h2} differentiation is inhibited by IFN-γ [141]. In a tumor context, typical T_{h2} cytokines including IL-4, IL-10 and IL-13 are important in tumor progression due to their ability to induce immunosuppressive macrophages and DCs similar to that of the wound-healing mechanism [146,147]. In breast cancer, T_{h2} cells seems to play a role in facilitating immunosuppression via IL-13 in early breast cancer [148,149].

T_{h17} cells

T_{h17} cells are commonly described as $CD4^+ROR\gamma^+$ T_h cells that express high amounts of the pro-inflammatory cytokine IL-17. IL-17 can promote production of IL-6 and TNF-α as well as facilitating recruitment of neutrophils to the site of inflammation. In addition, IL-17 has been associated with several autoimmune diseases [150]. The activation and differentiation of T_{h17} cells has been highly debated. In mice, the induction of T_{h17} cells requires a combination of TGF-β, IL-6, IL-23 and TCR stimulation [151]. However, induction of T_{h17} cells in humans remains a controversy. One study showed that differentiation of T_{h17} cells was independent of TGF-β [152]. Another study argued that TGF-β is crucial to effectively induce T_{h17} [153], whereas, another report claims that IL-1β is critical in order to drive the development of an inflammatory response that also includes induction of T_{h17} [154]. In a tumor context, T_{h17} cells have been associated with both good and bad clinical outcome [155]. The clinical impact of T_{h17} in breast cancer has been evaluated in smaller patient cohorts, however, with contradictory results [156-159]. One possible explanation is that T_{h17} cells are highly plastic, therefore, giving them the ability to adapt different cytokine profiles, thus making them highly contextual [151]. In addition, IL-17 is not exclusively produced by T_{h17} cells [160], hence, the difficulties in determining the exact clinical impact of T_{h17} cells. In Paper IV, we show that high infiltration of $IL\text{-}17^+$ T cells was associated with poor prognostic outcome in TNBCs specifically.

Regulatory T cells

T_{regs} are described as $CD4^+FoxP3^+CD25^+$ T lymphocytes that suppress conventional effector T cells. They exert their immune suppression through absorption of IL-2, the production of TGF-β and IL-10 or expression of inhibitory CTLA-4 [161], and play an important role in regulating anti-self immune response, thus maintaining central tolerance to self [162]. Induction of T_{regs} in the periphery requires TGF-β. In the context of a tumor, T_{regs} are strong suppressors of anti-tumor immune responses and associated with reduced clinical outcome in different malignancies [163]. Therefore, T_{regs} are an attractive therapeutic target in tumors [164]. However, the risk of autoimmunity is greatly increased when affecting T_{regs}, therefore, a strategy to specifically target tumor-associated T_{regs} is needed [163]. In ER^+ breast cancer, T_{regs} are commonly associated with a poor prognostic outcome [132,165-167]. However contradictory results have been obtained for ER^- breast cancers, where infiltrating T_{regs} have been associated with a good prognostic outcome instead [166,168].

γδ T cells

γδ T cells belong to the unconventional subgroup of T lymphocytes. They express γδTCR unlike the conventional αβTCR expressing lymphocytes. There are two main subtypes of human γδ T cells; Vδ1 and Vδ2. Vδ1 mainly resides in the tissue while Vδ2 circulates in the blood. They both recognize antigen presented on non-classical MHC molecules, and are both equipped with NKG2D (natural killer receptor; NKRs) that recognizes stress-related ligands such as MICA/MICB [169]. Vδ2 T cells uniquely recognize non-peptide antigens called phosphoantigens that are produced by bacteria but also in high quantities by tumor cells [170]. Vδ2 T cells are also equipped with FcγR (recognizes Fc-region of antibodies) that triggers ADCC mediated responses [171]. With regards to Vδ1 T cell, the antigens remain unclear. γδ T cells are attractive targets of immunotherapy. One approved drug, Zoledronate, indirectly causes accumulation of phosphorantigens in tumor cells, thus, triggering Vδ2 T cell mediated cytotoxicity [169]. The obstacle in such treatments is that γδ T cells become anergic when repeatedly exposed to phosphoantigens. Another interesting strategy is the triggering of ADCC mechanism via tumor-targeting antibodies [169].

In the context of tumors, γδ T cells have been reported to have dual-functions – with either tumoricidal or tumor promoting functions [171,172]. Although the role for γδ T cells in cancer has been thoroughly examined, their prognostic value has not been evaluated in detail. This is due to difficulties of detecting γδ T cells in paraffin embedded human tissue, and therefore few studies concerning the clinical relevance of γδ T cells in breast cancer have been done. A study with a small patient cohort

showed that γδ T cells were associated with HER2 subtype and poor prognosis [173]. However, recent studies revealed contrasting results reporting that elevated expression of genes associated with γδ T cells had a positive impact on clinical outcome of breast cancer patients [174,175].

NK and NKT cells

NK cells belong to the innate lymphoid cell compartment since they lack antigen binding receptors and their main function is instead to discriminate between cells that express MHC class I molecules on their surface or not. Whenever a cell downregulates MHC class I or over-express certain stress related molecules, NK cells will exert their effector function.

NKT cells are unconventional αβ T cells with a canonical recombination of the αβTCR. The most extensively studied NKT subtypes recognize antigens that are CD1d restricted [176]. It is believed that NKT cytotoxicity is dependent on the amount of CD1d molecules expressed by the target cell [177], however, the exact regulation of this mechanism is unclear [176]. Moreover, NKT cells can both be indirectly activated by APCs and directly activated by somatic cells. Also, NKT cells have the ability to further recruit and facilitate for the action of NK cells.

The mechanism of action for both NK and NKT cells is cytotoxicity via secretion of granzymes or mediated by FAS/FASL interaction [177]. NK and NKT cells are very efficient in killing tumor cells, thus including them in the immunosurveillance process. Furthermore, the NK cells are attractive therapeutic targets in immunotherapy since they express FcγR that can trigger an antibody dependent cellular cytotoxicity (ADCC). In breast cancer, this effect is observed when treating HER2 subtype with Herceptin (anti-HER2 antibody) [178].

TLRs, DAMPs and their role in tumors

Toll-like receptors (TLRs) are members of the PRR family that are mainly expressed by innate immune cells [179]. TLRs are capable of recognizing ligands that are both PAMPs and DAMPs. There are ten different TLRs described in humans; TLR1-10. They are either expressed on the cell surface (TLR1, TLR2, TLR4, TLR5 and TLR6) or intracellularly in vesicles (TLR3, TLR7, TLR8 and TLR9) [180-182]. TLRs can signal via myeloid differentiation factor 88 (MyD88)-dependent or independent ways. Myd88 is an intrinsic adaptor protein that connects TLR signaling with downstream signaling. TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 signal via MyD88, therefore, leading to NFκB activation and expression of pro-inflammatory mediators. In contrast, TLR3 and TLR4 signal via a MyD88-independent manner leading to activation of interferon regulatory factor-3 (IRF-3) and expression of type I IFNs [180,182]. The ligand for TLR10 is still unexplored [180]. However, it has recently been proposed that TLR10 negatively regulates both MyD88- dependent and independent TLR signaling [183]. TLRs are being addressed as the link between inflammation and tumor progression in different malignancies [179,184-186].

DAMPs represent a range of endogenous molecules that are released by injured or stressed cells, thus, triggering a sterile inflammation. Typical DAMPs are the HMGB1, S100 proteins, heat-shock proteins, adenosine triphosphate (ATP) and molecules that usually stay inside the cell in healthy conditions [115]. DAMPs trigger "danger-response", therefore, activating the innate immune system upon tissue injury [115]. In cancer, it appears that DAMPs are actively secreted by tumor cells [187]. In addition, one study showed that high presence of nuclear HMGB1 in tumor cells correlated with improved survival outcome in a large breast cancer cohort [188]. This indicates that secreted HMGB1 is not in favor of patient outcome. Another DAMP that is associated with tumor progression are the S100 proteins [189]. The S100 protein family constitutes of 21 members and is regulated by Ca^{+} binding [189]. In cancer, several S100 members are overexpressed [190]. In breast cancer, S100A9 and S100A8 are expressed in invasive, high-grade tumors that are of the basal-like subtype [191]. Moreover, S100A9 is secreted in tumors and may attract myeloid-derived suppressor cells (MDSCs), consequently, suppressing inflammatory macrophages,

the maturation of DCs, CTLs, recruitment of Tregs and promoting angiogenesis, invasiveness and metastasis [192]. Also, S100A9 and S100A8 can bind to PPRs, such as TLR4 and RAGE, and by consequence promote a NFκB mediated pro-inflammatory environment [193]. Therefore, a positive feedback loop is created where S100A9/A8 or S100A9/A9 may play a role in maintaining an inflammatory environment and hence recruit additional MDSCs to suppress anti-tumor immunity cells and promote disease progression.

Table 5.
Different TLRs in human, their typical ligand and the source of the ligand [115,180,187,193,194].

TLRs	Ligands (PAMPs and DAMPs)	Source of the ligand
TLR1	Triacyl lipopeptides	Bacteria
TLR2	Peptidoglycan Lipoproteins HSP	Gram-positive bacteria Various bacterias Host
TLR3	Double-stranded RNA	Viruses
TLR4	LPS HMGB1 S100A9 HSP	Gram-negative bacteria Host Host Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptide Lipotheicoic acid	Mycoplasma Gram-positive bacteria
TLR7	Single-stranded RNA	Viruses
TLR8	Single-straded RNA	Viruses
TLR9	CpG DNA HMGB1	Bacteria Host
TLR10	Unknown	

Immunotherapy – recent advances

> "The battlefield is a scene of constant chaos. The winner will be the one who controls that chaos, both his own and the enemies." – Napoleon Bonaparte

In recent time, a novel therapeutic strategy has emerged in oncoimmunology. The common name for this therapy is immune checkpoint inhibitors (ICIs). These therapies aim to activate or re-activate the adaptive anti-tumor immune response. As previously mentioned, immune checkpoints are crucial for peripheral central tolerance in order to induce T cell anergy or T cell exhaustion. This is mediated by inhibitory ligand: receptor pairs such as B7:CTLA-4 and PD-L1:PD-1. The tumor cells and the tumor microenvironment have adapted these regulatory mechanisms. The ICIs focuses on disrupting the inhibitory signals using monoclonal antibodies, thus re-activating anti-tumor immune responses [195]. Today, CTLA-4, PD-1 and PD-L1 inhibitors have been approved to treat advanced melanoma, non-small cell lung cancer, bladder cancer, renal cancer and Hodgkin lymphoma [195,196]. Further investigations have suggested that the response of ICIs depends on how immunogenic the tumor is. Therefore, the combination with adjuvant therapies or irradiation that may drive tumors into becoming more immunogenic is being intensely studied [197]. Other strategies that have been suggested are combining ICIs with drugs to act on multiple targets depending on the immunological profile of the tumor, such as IDO inhibitors, DC based vaccines or anti-inflammatory myeloid cell (M2 macrophages and MDSCs) inhibitors [198]. In addition, targeting TLRs to trigger anti-tumor immune response is an attractive therapeutic strategy [199]. Furthermore, targeting S100A9 to disrupt TLR4 mediated pro-inflammatory feedback loop has been revealed to inhibit tumor growth [200].

Current investigation and aims

Aims

The common aims of this thesis were to investigate the presence and function of both inflammatory mediators and the non-malignant cells (immunosuppressive myeloid cells, TILs and CAFs) in the tumor microenvironment of breast cancer and how they may affect the clinical outcome.

The specific aims of each project were:

I. To study the expression and function of TLRs in breast cancer, their role in the production of inflammatory mediators and their clinical relevance.

II. To investigate the expression and localization pattern of S100A9 in breast cancer, what role they play in mediating an inflammatory microenvironment and how this affects clinical outcome.

III. To study the role of myeloid cells in different breast cancer microenvironments, and their effect on stroma formation.

IV. To study the prognostic impact of alternative T cell populations as compared to conventional CTLs and T cells in general, in a larger retrospective consecutive breast cancer TMA cohort.

Paper I - Expression of functional toll like receptor 4 in estrogen receptor/progesterone receptor–negative breast cancer.

Results and Discussion

TLRs (TLR1-10) are a part of the PRR family that are mainly expressed by the innate immune cells. TLRs can bind both DAMPs and PAMPs, leading to activation of NFκB and production of pro-inflammatory mediators [201]. Previous studies have revealed that TLR4 is expressed in breast cancer and that it is associated with resistance to paclitaxel [202,203]. We decided to investigate the TLR expression patterns across several human breast cancer cell lines representing different breast cancer

subtypes. In addition, we evaluated the function of TLR4 in detail in breast cancer cells and the clinical relevance of TLR4 in a breast cancer cohort.

First, we wanted to investigate the TLR expression across different breast cancer cell lines using three ER^+PR^+ cell lines (MCF-7, T47D and CAMA-1) and four with TN phenotype (MDA-MB-231, MDA-MB-468, SUM149 and SUM159). Gene expression analysis revealed that TLR2 and TLR4 were highly expressed in all TN cell lines except for MDA-MB-468. TLR3 was expressed in all TN cell lines while TLR9 was generally expressed in all cell lines. Furthermore, CD14 and MD2 (necessary components for a functional TLR4) were also highly expressed in all TN cell lines, except in MDA-MD-468 where MD2 was totally absent. This indicates that three out of four TN cell lines might have a functional TLR4. TNBCs are known producers of pro-inflammatory cytokines such as IL-6 and IL-8, which are partially mediated by a constitutively active NFκB [204]. Therefore, we set out to investigate if the TLRs could affect or be partially responsible for the expression of pro-inflammatory cytokines through the activation of NFκB.

We investigated whether TLR4 was functional by adding LPS, the ligand for TLR4 [205], to our cell lines. Then we performed a cytokine bead array (CBA) analysis to detect soluble, secreted cytokines. IL-6, IL-8 and TNF-α were increased in supernatants harvested from MDA-MB-231, SUM149 and SUM159 upon stimulation with LPS. MDA-MB-468 did not show any effect upon stimulation of LPS. These findings were then supported by gene expression analysis of IL-8 and IL-6 that were in line with the CBA results. This experiment demonstrated that LPS induced an increase of pro-inflammatory cytokines specifically in TN cell lines, which thus is likely to be induced via a functional TLR4/TLR2 pathway. To confirm this, we silenced TLR4/TLR2 in MDA-MB-231 and observed a decreased secretion of IL-6 and IL-8. In addition, we transfected MDA-MB-231 with an NFκB luciferase reporter plasmid and observed an elevated activity of NFκB upon LPS stimulation. Although LPS is a well-established ligand of TLR4/TLR2 it remains a pathogen associated molecule. Therefore, we investigated a cancer-related DAMP known to be a TLR4 ligand, S100A9 [206]. It was revealed to increase secretion of IL-6 and IL-8 in MDA-MB-231, SUM149 and SUM159.

We next chose to evaluate the clinical relevance of TLR4 expression in a breast cancer cohort of 144 cases. High TLR4 staining intensity was positively associated with ER^-PR^- breast cancer and the basal-like status marker CK5. Furthermore, TLR4 expression significantly decreased recurrence-free survival, which is in line with results from previous studies that demonstrate how TLR4 expression is associated with metastasis in breast cancer models [202,203].

These findings show that TLR4 in ER^-PR^- breast cancers is biologically functional and is likely to enhance the secretion of pro-inflammatory cytokines into the tumor microenvironment upon stimulation with either PAMPs or DAMPs. Furthermore,

we did not see any decreased ERα expression when MCF-7 was transfected with a functional TLR4 plasmid, thus, indicating that TLR4 may not induce ER^-PR^- breast cancer subtypes. Therefore, we suggest that TLR4 in ER^-PR^- breast cancer should be considered as a potential therapeutic target.

Paper II - S100A9 expressed in ER(⁻)PgR(⁻) breast cancers induces inflammatory cytokines and is associated with an impaired overall survival.

Results and Discussion

In this paper, we set out to investigated the expression of S100A9 in breast cancer cells, what role it plays in a tumor context and if it is of any clinical relevance. S100A9 signals as a DAMP via TLR4/NFκB pathway [193,207,208]. The expression of S100A9 in breast cancer cohorts have already been investigated [191]. In addition, recruitment of MDSCs requires the expression of S100A9 [189]. In a previous study, we show that $CD163^+$ immunosuppressive myeloid cells were preferentially residing within the stromal compartment of TNBCs and were associated with worse prognostic outcome [108]. Therefore, we decided to investigate the expression and localization patterns of S100A9 in breast cancer.

We started with exploring the expression patterns of S100A9 in the following breast cancer cell lines: MCF-7, T47D, CAMA (ER^+ cell lines), MDA-MB-231, MDA-MB-468 (TN cell lines) and SKBR3 (HER2 amplified cell line). It was revealed that both MDA-MB-468 and SKBR3 cells expressed S100A9 on both mRNA and protein levels. Then we stimulated the previously mentioned cell lines with rS100A9 and saw that pro-inflammatory cytokines were only upregulated in MDA-MB-231 cells, most likely due to their expression of TLR4 as previously shown in Paper I. In line with the results of Paper I, we saw an up-regulation of NFκB activity upon rS100A9 stimulation in MDA-MB-231 but not in MDA-MB-468 or SKBR3 cells. Given that MDA-MB-468 and SKBR3 cells expressed high levels of EGFR, we added recombinant-EGF (rEGF) to the cell culture and measured gene expression levels of S100A9. Upon stimulation of rEGF, S100A9 was induced in SKBR3, but not in MDA-MB-468 cells. This indicates that production of S100A9 is not only dependent on EGFR stimulation.

In a breast cancer cohort of 144 cases we could see that S100A9 stained positive both in the cytoplasm and the nucleus of malignant cells as well as in the stromal compartment. S100A9 staining (irrespective of location) was positively associated with ER^-PR^- breast cancers. In addition, cytoplasmic S100A9 was positively associated with Ki67. Stromal expression of S100A9 was positively associated with $CD163^+$ myeloid cells, Ki67, tumor size, $HER2^+$ tumors and with a borderline significant association with EGFR expressing tumors. Furthermore, all of the

HER2$^+$ and EGFR positive tumors were positive for cytoplasmic expression of S100A9. The strong association of S100A9 with HER2$^+$ and EGFR expressing tumors were in line with our findings on gene expression and with what has been previously shown [191]. Finally, the expression of S100A9 in both malignant cells and in the stromal compartment was associated with a reduced overall survival.

In conclusion, S100A9 is expressed by both malignant tumor cells that are mainly ER$^-$PR$^-$, and by myeloid cells residing in the stroma. Given that S100A9 is both a chemo-attractant of MDSCs and a DAMP that triggers TLR4 signaling, this may indicate that tumors can exploit potential DAMP:TLR4 molecular mechanisms to create an environment that furthers disease progression.

Paper III - Cancer associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers.

Results and Discussion

This paper was initiated based on two previous findings; (i) that CD163$^+$ myeloid cells residing in the stromal compartment of breast cancers correlated with granuline (GRN) expression, TNBCs/basal phenotype and poor prognosis [108] and (ii) that bone marrow cells could promote a reactive stroma in TNBCs via secreted GRN resulting in activation of fibroblasts [209]. Therefore, we decided to investigate if CD163$^+$ myeloid cells had any contribution to the stroma formation in TNBCs.

The first step into finding out the relevance of myeloid cells in tumor progression and stroma formation was to co-transplant luminal A (MCF-7 or T47D) or TN (MDA-MB-231 or SUM159) breast cancer cell lines with or without primary human monocytes (proposed precursors CD163$^+$ myeloid cells) from healthy donors. These cells were co-injected subcutaneously into highly immunodeficient NSG-mice. NSG-mice are deficient in T- B- and NK cells. Most importantly, functional mouse DCs and macrophages are impaired in these mice, but engrafted human monocytes would be fully functional. Tumors were excised, embedded in paraffin and then analyzed with IHC. The common myeloid surface marker; CD11b was present in all co-transplants, however, they were more abundant in TNBC co-transplanted xenografts. Furthermore, the expression of CD163 and CD68 was expressed at a significantly higher density in the TNBC compared with luminal A co-transplanted xenografts. The myeloid cells in the TNBC co-transplanted xenografts did not only express CD163 but also expressed the MDSC marker S100A9. In this setting, monocytes are co-injected with cancer cell lines from the start, thus addressing how monocytes are recruited to the tumor site is not possible using this model.

We next investigated why the myeloid cells were numerous in the TNBC co-transplanted xenografts and therefore analyzed proliferation and survival advantage in vivo and in vitro. The Ki67 staining revealed that the primary human myeloid cells were proliferating. Moreover, in our in vitro experiments we confirmed that the primary human monocytes survived and proliferated more in TNBC conditioned media. As shown in Paper I, both monocytes and M2 macrophages migrated more towards conditioned media produced by TNBC cell lines. Thus, concluding that monocytes are attracted to the TNBC microenvironment where they survive, proliferate and differentiate.

Staining with mouse specific β2-microglobulin revealed a high infiltration of mouse specific cells in the TNBC co-transplants. Therefore, we speculated whether these cells were fibroblasts. Using antibodies specific for PDGFRβ, we could identify the infiltrated cells as fibroblasts. Moreover, the fibroblasts were also active in large numbers specifically in our TNBC co-transplants indicated by the IHC staining of αSMA. Luminal A co-transplants showed only modest αSMA staining. To investigate why they were numerous in TNBC co-transplants specifically, we set up a range of in vitro experiments using primary fibroblasts from nude mice. The mouse primary fibroblasts survived and proliferated more in conditioned media from TNBC/monocyte co-cultures. In addition, they expressed the fibroblast activation protein (FAP) indicating activated fibroblasts. This indicates that monocytes in combination with TNBC tumor cells activates and promotes both survival and proliferation of fibroblasts.

Given that the fibroblasts were active in our TNBC co-transplants, we decided to investigate how this affected the ECM composition in the tumors. The ECM is comprised of different components including collagens and it is already known that the tumor microenvironment promotes remodeling of collagens, thus, promoting disease progression [210]. Examining the collagen deposition in the xenografts using Sirius Red stain (detects classical collagens type I, III and IV) revealed that it was increased in all co-transplants of any breast cancer subtype. The expression of the classical collagens was however, significantly less in TNBC as compared to the luminal A co-transplants, despite the numerous activated fibroblast in the TNBC co-transplants. This might be explained by the high amount of MMPs produced in these tumors, that can degrade and remodel collagen depositions [211]. Nonetheless, the expression of the non-classical collagen (type VI) was expressed in TNBC co-transplants. A set of in vitro experiments indicated that the production of collagen type VI is most likely caused by the monocytes. Collagen VI is a beaded filament that is associated with tumor progression, drug resistance and being an anti-apoptotic factor for fibroblasts [212].

Lastly, we found that primary fibroblasts from human TNBCs expressed the chemokine CXCL16 in the supernatant. CXCL16 was shown to attract both

monocytes and M2 macrophages in vitro, which might offer an explanation as to why $CD163^+$ myeloid cells were preferentially located in the stroma of TNBCs [108]. CXCL16 gene expression was induced in primary fibroblasts from nude mice cultured in conditioned media produced by TNBC MDA-MB-231/monocyte, MDA-MB-468/monocyte, SUM159/monocyte and SUM159 alone. This data was supported by gene expression analysis of both TNBC PDX grafts and TNBC syngeneic mouse models.

In conclusion, our findings show that myeloid cells are recruited to TN breast tumors where they become $CD163^+S100A9^+$ immunosuppressive cells, activate fibroblasts to produce CXCL16 that in turn recruit more myeloid cells.

Paper IV - Infiltration of γδ T cells, IL-17^+ T cells and $FoxP3^+$ T cells in human breast cancer.

Results and Discussion

Most clinical studies agree that high infiltration of TILs in breast cancers correlates with a good clinical outcome. Studies that investigate the clinical impact of TILs are mostly based on the evaluation of the pan-T cell marker CD3, the CTL marker CD8α or the T_{reg} marker $FoxP3^+$ [213]. Since the function of different T cell subsets vary considerably, we set out to investigate the clinical relevance of less conventional TILs; γδ T cells, IL-17^+ T cells, $FoxP3^+$ T cells, as compared to the common TIL markers CD3 and CD8α, in a retrospective consecutive breast cancer cohort of 498 cases.

The breast cancer cohort was setup in a TMA, thus allowing us to perform a series of IHC staining. γTCR and CD3 were co-stained to ensure the specificity of anti-γTCR and annotated manually. Foxp3 and IL-17 were scored using an algorithm based image analysis to quantify the amount of positive staining with lymphocytic morphology.

Our data shows that high infiltration of $CD8\alpha^+$ T cells was positively associated with TNBCs and inversely associated with ER^+ breast cancer. CD3 and γδ T cells were positively associated with TNBCs and HER2 subtype but inversely associated with both luminal A as well as ER^+ breast cancers. T_{regs} were positively associated with TNBCs and HER2-subtype but inversely associated with both luminal A and ER^+ breast cancers. Lastly, IL-17^+ T cells were inversely associated with TNBCs only.

Survival analysis indicated that generally high infiltration of $CD3^+$ T cells as well as γδ T cells was associated with favorable clinical outcome. Infiltration of T_{regs} was associated with worse survival outcome. Furthermore, multivariate Cox regression

analysis showed that both CD8α$^+$ and CD3$^+$ TILs were independently associated with improved survival, thus confirming previous reports [130,132,135].

When stratifying according to different breast cancer subtypes, we show that both CD3 and γδ T cells correlated with improved clinical outcome in luminal A and ER$^+$ breast cancer. In addition, γδ T cells also correlated with improved clinical outcome in luminal B and HER2-subtype but the amount of cases in these groups were too small to draw any conclusions. CD8α$^+$ T cells only indicated improved survival outcome in the HER2-subtype. T_{regs} were associated with worse survival outcome, specifically in ER$^+$ breast cancer, which is in line with previous studies [132]. Intriguingly, IL-17$^+$ T cells indicated worse survival outcome in TNBCs. It is known that TNBCs produces a highly inflammatory microenvironment, thus it is likely that it attracts T_{h17} cells or that they even play a role in regulating microenvironment [155]. Lastly, CD8α$^+$ and γδ T cells were the only subtypes independently associated with improved survival in patients treated with endocrine therapy.

The molecular markers CD8α, IL-17 or FoxP3 have been found to be expressed by several different cell types. We can therefore not draw any conclusions on the exact T cell subtype responsible for the different outcomes in this study. Hence, we cannot exclude that CD8α, FoxP3 or IL-17 may indeed be expressed by γδ T cells and not on conventional αβ T cells [214-216]. This would have to be performed using double staining in the future. This study, not only highlights the difficulties of evaluating T cells in a clinical setting, but also emphasizes the importance of characterizing the immunological profile in tumors as complement to molecular subtyping and prediction of therapeutic response.

Conclusions

I. ER^-PR^- breast cancer subtype express functional TLR4 to further enhance pro-inflammatory tumor microenvironment. Therapeutic inhibition of TLR4 could help decrease the pro-inflammatory microenvironment of ER^-PR^- breast cancer, thus, arresting the disease progression.

II. ER^-PR^- breast cancer subtypes express S100A9 that might act as both chemo-attractant of MDSCs and a DAMP - triggering TLR4 mediated inflammatory response. Therefore, S100A9 inhibition might serve as therapeutic target – preventing tumor progression.

III. These findings demonstrate that in the TNBC environment, monocytes survive, proliferate and become anti-inflammatory $CD163^+S100A9^+$ myeloid cells, that are capable of activating fibroblasts that further recruit additional monocytes.

IV. General infiltration of $CD3^+$ T cells and γδ T cells was associated with good prognosis in breast cancer patients, while $Foxp3^+$ T cells was associated with worse prognosis. High infiltration of γδ T cells and $CD8\alpha^+$ T cells has a favorable outcome in endocrine therapy treated patients. This further demonstrates the importance of evaluating specific subsets of T cells in clinical setting.

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Paper I

Mehmeti et al. Breast Cancer Research (2015) 17:130
DOI 10.1186/s13058-015-0640-x

Breast Cancer RESEARCH

RESEARCH ARTICLE Open Access

Expression of functional toll like receptor 4 in estrogen receptor/progesterone receptor-negative breast cancer

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Abstract

Introduction: Toll-like receptors (TLRs) are a family of pattern recognition receptors that are expressed on cells of the innate immune system. The ligands can be pathogen derived (pathogen associated molecular patterns; PAMPs) or endogenous (damage associated molecular patters; DAMPs) that when bound induces activation of nuclear factor kappa B (NF-κB) and transcription of pro-inflammatory genes. TLRs have also been discovered in various malignant cell types, but with unknown function.

Methods: In this study we performed a detailed analysis of TLR and co-receptor expression pattern and function in breast cancer. Expression patterns were examined using real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry (IHC) on three estrogen receptor-positive (ER^+) and four estrogen receptor/progesterone receptor-negative (ER^-/PR^-; ER/PR-negative) breast cancer cell lines, and a breast cancer cohort consisting of 144 primary breast cancer samples. The function was investigated using *in vitro* assays comprising PAMP/DAMP-stimulation, downstream signaling and TLR-silencing experiments.

Results: We found that TLR4 was expressed in a biologically active form and responded to both PAMPs and DAMPs primarily in ER/PR-negative breast cancers. Stimulation of TLR2/4 *in vitro* induced expression of pro-inflammatory genes and a gene expression analysis of primary breast cancers showed a strong correlation between TLR4 expression and expression of pro-inflammatory mediators. In line with this, TLR4 protein expression correlated with a decreased survival.

Conclusions: These findings suggest that TLR4 is expressed in a functional form in ER/PR-negative breast cancers. Studies regarding TLR4-antagonist therapies should be focusing on ER/PR-negative breast cancer particularly.

Introduction

Breast cancer is the most common form of cancer among women today [1]. The prognosis of breast cancer patients varies depending on the breast cancer subtype. Clinical breast cancer classification is based on expression of various immunohistochemical markers, with the hormone receptors being the most important. One of the worst prognosis subtypes is the triple-negative (TN) breast cancer subtype, where the malignant cells lack expression of the hormone receptors, estrogen receptor (ER) and progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2) ($ER^-PR^-Her2^-$). The treatment options are few for patients with TN breast cancer [2–4].

Toll-like receptors (TLRs) are a family of receptors that are expressed on innate immune cells [5]. They are part of the pattern recognition receptor (PRR) family and recognize molecular patterns from pathogens (pathogen-associated molecular patterns; PAMPs) or from endogenous stress-induced proteins (damage-associated molecular patterns; DAMPs) [6–9]. Signaling via TLRs leads to activation of nuclear factor kappa B (NFκB) and a subsequent expression of pro-inflammatory genes [10]. There are 10 different TLRs (TLR1-10) in humans, and these are divided into two subgroups depending on cellular localization; on the surface of the cell (TLR1, TLR2, TLR4, TLR5 and TLR6), or in vesicles such as endoplasmic reticulum, endosomes or lysosomes (TLR3, TLR7,

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TLR8 and TLR9). Lately, expression of different TLRs has been described in various malignancies, although their function is as yet unclear [5, 11, 12].

TLR2 and TLR4 respond to the typical PAMP from Gram-negative bacteria, lipopolysaccharide (LPS). Different variants of LPS (from *Escherichia coli* and *Salmonella typhimurium*) induce different TLR-intracellular signals [13]. DAMPs can also bind to and activate TLR2 or TLR4, and two endogenous ligands that are well-described are HMGB1 and S100A9 [14–19]. To signal via TLR2 or TLR4, different ligands may also require the co-receptors CD14 or MD2 [20–23]. All TLR ligands initiate activation of NFκB, but also mitogen-activated protein kinase (MAPK) pathways that affect protein translation and processing rather than transcription can be activated [24]. TLR4 has previously been shown to be expressed in breast cancer [25, 26].

The transcriptional factors ERα and NFκB are synergistically interrelated, although their exact interactions are unknown [10, 27–31]. NFκB is a transcriptional factor that induces a wide array of pro-inflammatory mediators and is also related to several oncogenic processes [32]. Both ER and NFκB have previously been shown to attenuate each other in different ways. In line with this observation, ER^- breast cancers have a stronger pro-inflammatory phenotype and microenvironment. NFκB has even been shown to downregulate ERα expression in breast cancer cells [29], but there is no direct proof that constitutive NFκB would generate ER^- breast cancers in general. On the other hand, a recent positive synergy between ER and NFκB was published, where TNFα and estrogen were shown to remodulate the ERα-promoter landscape in an NFκB and FoxA1 dependent manner resulting in an altered gene expression pattern [33].

In this study we performed an analysis of TLR expression patterns and function in breast cancer. Using a carefully validated TLR4-specific antibody for immunohistochemistry (IHC), we found that TLR4 protein expression was primarily present in breast cancers of ER/PR-negative phenotype. Using three cell lines of ER^+ phenotype and four cell lines of the TN phenotype, we further showed that the expressed TLR4 was biologically active and hence responding to both PAMPs and DAMPs, primarily in the TN breast cancer cell lines. Finally, TLR4 protein expression correlated with a decreased survival in a cohort of 144 primary breast cancer patients. We propose that novel therapies targeting TLR4 may be of value, in particular in ER/PR-negative breast cancers.

Methods

Cell culture

The human breast cancer cell lines MCF-7, T47D, MDA-MB-231 and MDA-MB-468 were purchased from ATCC and were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) (Biosera, Boussens, France), 1 % sodium pyruvate, 1 % HEPES and penicillin/streptomycin (100 U/ml and 100 μg/ml respectively); CAMA-1 (also purchased from ATCC) was cultured in MEM/EBSS supplemented with 10 % FBS and penicillin/streptomycin, and SUM-149 and SUM-159 were cultured in F-12 HAM'S medium supplemented with 5 % FBS, 1 mM L-Glutamine, 1 μg/ml hydrocortisone (BD BioScience, San Diego, CA, USA) and 5 μg/ml insulin (Novo Nordisk A/S, Måløv, Denmark). The SUM-149 and SUM-159 cell lines were produced by Professor S Ethier. Media and supplements were purchased from Thermo Scientific HyClone (South Logan, UT, USA) unless otherwise stated.

Compounds and cytokine analysis

LPS was purchased from Sigma Aldrich (St Louis, MO, USA) and originated from *S. Typhimurium* (LPS1) and *E. Coli* (LPS2), respectively. All stimulations were performed for a total of 6 h except for rhS100A9 (20 h). IL-1β and HMGB1 was from R&D Systems. Recombinant human S100A9 (rhS100A9) was a gift from Active Biotech AB and a detailed description on endotoxin-free S100A9 generation and purification has been published previously [15] and was used in the presence of calcium and zinc (Ca^{2+} ≥200 μM; 10 μM $ZnCl_2$ [34, 35]). Supernatants from stimulated or siRNA transfected cells were harvested and analyzed using human inflammatory cytokine cytometric bead array (CBA; BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions or using IL-6 and IL-8 Quantikine ELISA (R&D Systems, Minneapolis, MN, USA). Annexin V-allophycocyanin (APC) and propium iodide (PI) staining was performed according to the manufacturer's instructions (BD Biosciences). The cycloheximide (CHX) experiments (Sigma Aldrich) where performed by adding 10 μg/ml CHX, with or without 100 ng/ml LPS for 6 h.

Preparation of necrotic cell supernatant (NCS)

Confluent monolayers of MDA-MB-231 cells were harvested by trypsinization and 3.2×10^6 cells were resuspended in 2 ml serum-free RPMI-1640 medium. Necrosis was induced by performing three freeze-thaw cycles and NCS was separated from the necrotic cell pellet by centrifugation.

Tissue microarray (TMA) and immunohistochemistry

The breast cancer cohort analyzed in this study consists of 144 patients diagnosed with invasive breast cancer at Skåne University Hospital, Malmö, Sweden, between 2001 and 2002. The cohort and TMA have previously been described in detail [36–38] and [39]. TMA sections of 4 μm thickness were mounted onto glass slides and deparaffinized followed by antigen retrieval using the PT-link system (DAKO, Glostrup, Denmark) and stained

in an Autostainer Plus (DAKO) with the EnVisionFlex High pH-kit (DAKO). Antibody used for TLR4 IHC was anti-TLR4 NB100-56566 at 1:250 (Novus Biologicals, Littleton, CO, USA). TLR4 expression in TMA tumor samples was estimated as cytoplasmic staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong intensity and 4 = very strong intensity).

Ethical considerations

Ethical permit was obtained from the regional ethical committee at Lund University (Dnr 447/07), waiving the requirement for signed informed consent. Patients were offered to opt out of research. Ethical permission for using blood from healthy blood donors was obtained from the regional ethical committee at Lund University (Dnr 2012/689).

Gene expression profile array

The publicly available database R2: microarray analysis and visualization platform [40]; Tumor breast EXPO-351 was used for gene expression profile analysis.

Quantitative real-time PCR (RT-qPCR)

RNeasy Plus kit was used to extract total RNA according to the manufacturer's instructions (Qiagen, Hilden, MD, USA). Random hexamers and the M-MuLV reverse transcriptase enzyme (Thermo Scientific) was used and quantitative real-time PCR (RT-qPCR) were performed in triplicates for the genes analyzed using Maxima SYBR Green/Rox (Thermo Scientific) according to the manufacturer's instructions. RT-qPCR analysis was performed on the Mx3005P QPCR system (Agilent Technologies, Santa Clara, CA, USA) and the relative mRNA expression was normalized to *YWHAZ*, *UBC* and *SDHA* and calculated using the comparative cycle threshold (Ct) method [41]. For primers see Additional file 1: Table S1.

Transient transfections

siRNA transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA): 2 μM of the following silencer select siRNA oligonucleotides from Ambion (Carlsbad, CA, USA) were used; Silencer Select Negative Control #2: 4390846, siTLR2 #1: s168, siTLR2 #2: s170, siTLR4 #1: s14194, siTLR4 #2: s14195. Analyses were performed 48 h and 72 h post transfection. For luciferase assays, breast cancer cells were co-transfected using Lipofectamine 2000 with a total of 0.6 μg pNFκB-luciferase (BD Biosciences) and 0.06 μg TK-renilla-luciferase (Promega, Madison, WI, USA) plasmids and was subsequently analyzed using Dual-Luciferase Reporter System (Promega). For TLR4 transfections breast cancer cells were transfected using Lipofectamine 2000 with a total of 1.0 μg pDUO-MD2/hTLR4 or pUNOI-hTLR4-GFP (Invivogen, San Diego, CA, USA) per 24 wells for 72 h or 48 h, respectively, and was subsequently analyzed using immunofluorescence (×40 magnification) or ELISA as described in the figure legends.

Statistical analyses

Graph Pad Prism software was used to perform analysis of variance (ANOVA) or Students *t* test for the *in vitro* experiments as indicated. Spearman's Rho and the chi-square (χ^2) test was used for correlation analysis and Kaplan-Meier analysis with the log-rank test was used to illustrate differences in survival. All statistical tests were two sided and $P \leq 0.05$ was considered significant. Calculations were performed with IBM SPSS Statistics version 19.0 (SPSS Inc).

Results

TLR and co-receptor mRNA expression pattern in breast cancer cell lines

Most studies of TLRs in breast cancer have been performed using the ER^+ cell line MCF-7 and the TN cell line MDA-MB-231 [5]. To our knowledge, a detailed comparison between ER^+ and TN cell lines or cancers has not been published. We initially performed a broad analysis on TLR and TLR2/4 co-receptor (CD14 and MD2) mRNA expression patterns in various breast cancer cell lines. We used three cell lines with an ER^+PR^+ phenotype (MCF-7, T47D and CAMA-1) and four with an $ER^-PR^-Her2^-$ (TN) phenotype (MDA-MB-231; MDA-MB-468, SUM-149 and SUM-159). As shown in Fig. 1a-c, *TLR2*, *TLR3* and *TLR4* were preferentially expressed in the TN cell lines while *TLR9* was more generally expressed (Fig. 1d). Only MDA-MB-468 had low/absent mRNA expression levels of *TLR2* and *TLR4* of the TN cell lines. Similarly, the TLR4 co-receptors *CD14* and *MD2* were expressed primarily in the TN cells lines (Fig. 1e, f). Again, the TN cell line MDA-MB-468 stood out with high *CD14* mRNA expression levels, but low *MD2* levels (Fig. 1e, f). This means that three out of the four TN breast cancer cell lines had the necessary proteins for a functional TLR4 signal to occur.

The TLRs are functional and activation promotes expression of pro-inflammatory genes

To investigate whether the expressed TLRs were functional in the breast cancer cells following LPS stimulation, we analyzed the expression levels of some pro-inflammatory genes that are known targets for NFκB. The pro-inflammatory cytokines IL-6 and IL-8 were expressed at both protein (Fig. 2a and b) and mRNA (Fig. 2c and d) levels and only in the TN breast cancer cells but not the ER^+ breast cancer cells. The TLR2/4-ligand LPS induces different TLR downstream signaling pathways when originating from different bacterial strains [13]. When the breast cancer cells were stimulated with

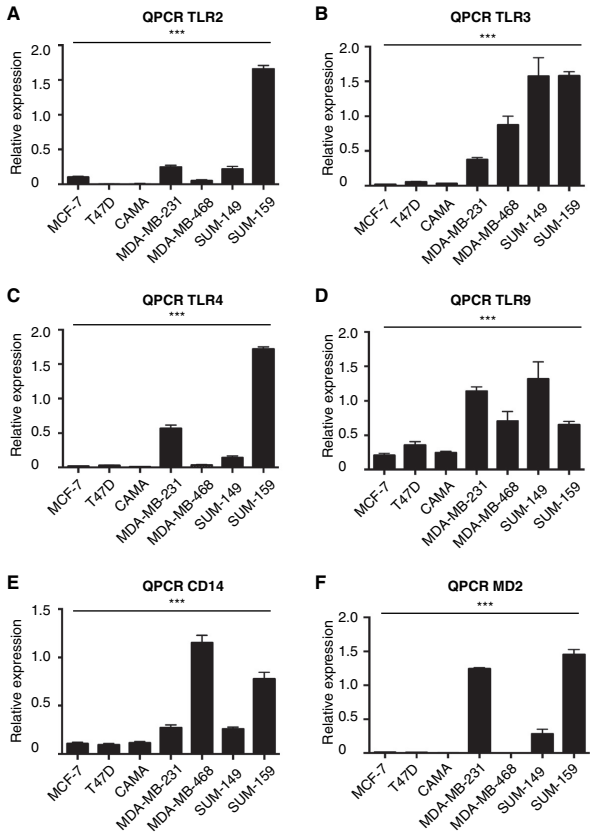

Fig. 1 Breast cancer cell line mRNA expression levels of Toll-like receptor 2 (*TLR2*), *TLR3, TLR4, TLR9* and co-receptors *CD14* and *MD2*. **a**-**f** The relative expression of indicated mRNA using quantitative real-time PCR (*QPCR*) on mRNA from the cell lines indicated. *Error bars* standard error of the mean: n = 6 – 9; ***P <0.001 (analysis of variance)

LPS for 6 h (LPS1 from *S. Typhimurium* and LPS2 from *E. Coli*), we could see that IL-6, IL-8 and TNFα were induced by both LPS1 and LPS2 in the MDA-MB-231, SUM-149 and SUM-159 cell lines, but not the MDA-MB-468 cells with inherent low expression of TLR4 (Fig. 2b). A slight effect of LPS2 was seen in the TLR2/4-negative cell line, CAMA-1, which might represent unspecific binding to other receptors. This was supported by the finding that the mRNA levels of *IL-6* and *IL-8* increased in a similar manner in all TN cell lines except MDA-MB-468, and not in the CAMA cell line (Fig. 3a and data not shown). Interestingly, TLR signaling affected not only the transcription of *IL-6* and *IL-8*, but also the protein translation as judged by cycloheximide (CHX) experiments showing a decreased release of both IL-6 and IL-8 after LPS1 stimulation upon simultaneous treatment with LPS and CHX (Fig. 3b).

A TLR4-specific DAMP induces pro-inflammatory cytokines in breast cancer cells

We further investigated whether DAMPs could induce TLR2/4-signaling in TN cell lines (MDA-MB-231, SUM-149 and SUM-159) and found that LPS (LPS1 and LPS2), but not the endogenous DAMP HMGB1, significantly induced IL-6 and IL-8 release in MDA-MB-231 cells and SUM-159 cells (Fig. 3c and e), whereas in SUM-149 cells LPS2 induced IL-6 and IL-8 release primarily (Fig. 3d). This finding might reflect that TLR2/4-induced transcription v/s translation might be differentially regulated in breast cancer cells. IL-1β was used

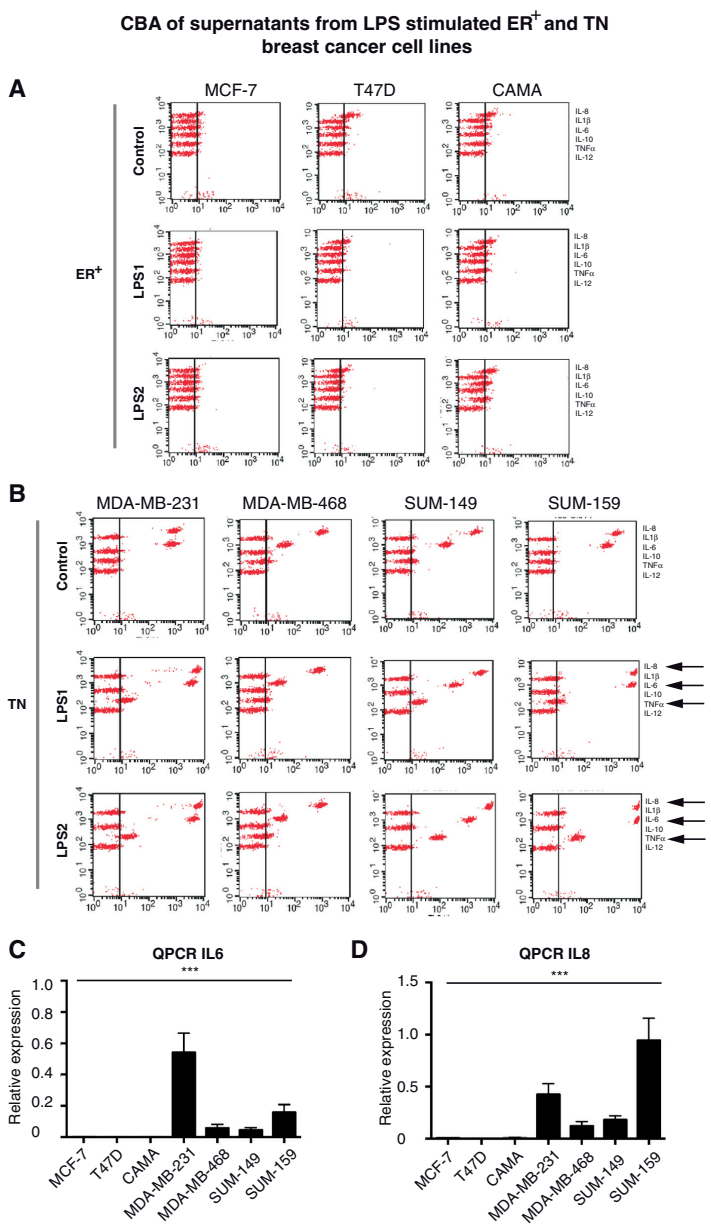

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Lipolysaccharide (*LPS*) induced cytokine release in human breast cancer cells in vitro. Release of cytokines by breast cancer cells of estrogen receptor-positive (ER^+) origin (**a**) and triple-negative (*TN*) origin (**b**) was analyzed using cytokine bead array (*CBA*). Unstimulated breast cancer cells of TN origin (**b**) produce IL-6 and IL-8 at high levels. LPS1 (from *S. Typhimurium*) and LPS2 (from *E. Coli*) stimulation for 6 h induced release of IL-8, IL-6 and TNFα from indicated breast cancer cell lines. At least three experiments were performed for each cell line. The relative mRNA expression of *IL-6* (**c**) and *IL-8* (**d**) in unstimulated cells was measured using quantitative real-time PCR (*QPCR*). At least five experiments were performed for each cell line. *Error bars* standard error of the mean; ***P <0.001 (analysis of variance)

as a positive control. Using dual luciferase assays and an NFκB reporter, we confirmed that LPS stimulation of MDA-MB-231 cells induced activation of NFκB but HMGB1 did not (Fig. 3f). We continued with another cancer-related DAMP reported to be a TLR4 ligand, the S100A9 protein [35]. Indeed, stimulating MDA-MB-231, SUM-149 and SUM-159 cells with rS100A9 for 20 h induced a significant increase in both IL-6 and IL-8 release (Fig. 3g). We also tested whether stimulating with HMGB1 for 20 h would induce cytokine release but with negative results (data not shown). Finally, by introducing the MD2/TLR4 complex (pDUO-MD2/TLR4) in otherwise negative MCF-7 cells, we could see a significant expression of both IL-6 and IL-8 as compared to control MCF-7 cells (Fig. 3h). pDUO-MD2/TLR4 is an expression vector that is designed to co-express the *MD2* and *TLR4* genes needed to interact with each other for functional signaling to occur upon ligand binding [42].

Constitutive expression of IL-6 and IL-8 is inhibited by silencing of TLR4

The impact of TLR4 signaling (possibly by endogenous DAMPs) on the constitutive expression of IL-6 and IL-8 seen in the MDA-MB-231 cells was analyzed. To this end we used negative control (nc) siRNA or siRNA specific for *TLR2* (siTLR2#1 and #2) and *TLR4* (siTLR4#1 and #2) (Fig. 4a), and analyzed the IL-6 and IL-8 levels 72 h post transfection. Both siTLR2 and siTLR4 slightly decreased the endogenous levels of IL-6 and IL-8 (Fig. 4b).

TLR2/4 expression affects migration and invasion

The TLR2/4-induced pro-inflammatory cytokines can be chemoattractants for myeloid cells. We therefore next investigated whether primary human $CD11b^+$ myeloid cells would migrate toward supernatants collected from breast cancer cells with a TN phenotype as compared to ER^+ breast cancer cells. Indeed, primary human myeloid cells migrated significantly more to supernatants collected from MDA-MB-231 cells as compared to from ER^+ MCF-7 or T47D cells, but as expected also to the TLR4-negative, but pro-inflammatory cytokine-secreting, MDA-MB-468 cells (Fig. 4c).

Other parameters that might be affected by TLR4 expression in breast cancer cells were also investigated; invasion, apoptosis and proliferation (Fig. 4d and Additional file 2: Figure S1A-B). In summary, invasion into matrigel invasion chambers by MDA-MB-231 breast cancer cells was increased when the TLR2/4 ligand LPS was added to the invading cells (Fig. 4d). Apoptosis in MDA-MB-231 breast cancer cells where TLR2 or TLR4 was silenced gave either conflicting results (TLR2) or was not affected (TLR4) (Additional file 2: Figure S1A), and finally proliferation using ^{3}H-incorporation assays of MDA-MB-231 breast cancer cells where TLR4 was silenced was not affected as compared to control (Additional file 2: Figure S1B).

To finally evaluate if other relevant TLRs were functional in the TN breast cancer cells we also performed stimulations of MDA-MB-231 cells with necrotic cell supernatant (NCS; TLR3 ligands [43]). Release of IL-8 but not IL-6 was affected by addition of NCS in a concentration-dependent manner (see Additional file 2: Figure S1C).

TLR4 is expressed in ER/PR-negative breast cancers and correlates with poor survival

TLR proteins are difficult to analyze because the antibody specificity is generally poor. We carefully evaluated several antibodies and found one to be highly specific. This antibody was confirmed first by using human tonsil tissue as positive control, showing the typical pattern of TLR4-expressing cells surrounding the follicles (Fig. 5a). Having optimized IHC, we subsequently stained formalin-fixed and paraffin-embedded cell pellets of the cell lines used in this study. All ER^+ cell lines were negative for TLR4, whereas three out of four TN cell lines displayed marked cytoplasmic positivity, corroborating our mRNA results (Fig. 5b). The cytoplasmic localization of TLR4 in breast cancer cells was supported by transfection of breast cancer cells using a green fluorescent protein (GFP)-tagged TLR4 plasmid (pUNOI-hTLR4-GFP) (Fig. 5c). We found that in cells expressing both the TLR4 co-receptors MD2 and CD14 (MDA-MB-231 cells; Fig. 5c left) TLR4-GFP was expressed in a vesicular pattern in the cytosol, whereas in breast cancer cells lacking both MD2 and CD14 (MCF-7 cells; Fig. 5c right), TLR4-GFP was expressed evenly in the cytoplasm.

A TMA of 144 breast cancer patients was subsequently stained and analyzed for correlation with other histological and clinical parameters. The staining was judged as cytoplasmic staining of intensities 0, 1, 2, 3 and 4 (see "Methods") (Fig. 6a). When the analysis was

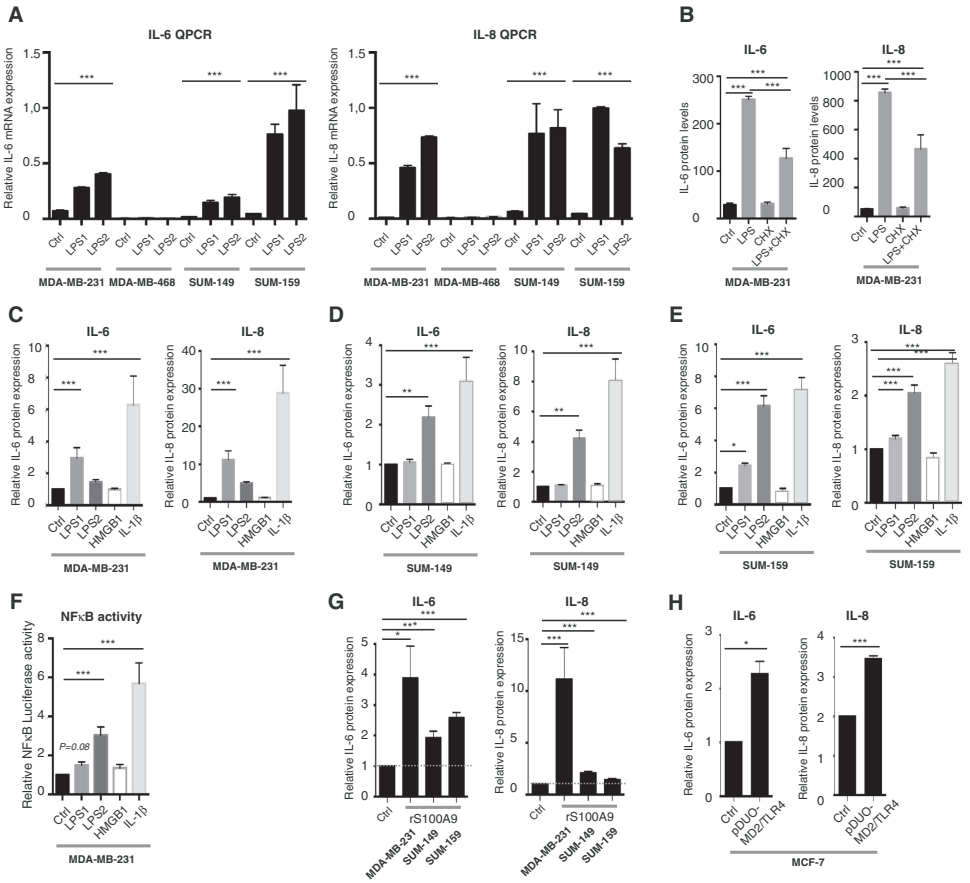

Fig. 3 Pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern (DAMPs) induce cytokine expression in human breast cancer cells in vitro. **a** Relative expression of *IL-6* and *IL-8* mRNAs after 6 h stimulation with lipopolysaccharide 1 (*LPS1*) and LPS2, using quantitative real-time PCR (*QPCR*) on mRNA from the cell lines indicated; n = 6. *Error bars* standard error of the mean (SEM); ***P <0.001 (analysis of variance (ANOVA)). **b** Release of IL-6 and IL-8 after stimulation with LPS1 and cycloheximide for 6 h, using ELISA on supernatants from stimulated MDA-MB-231 cells; n = 3. **c-e** Relative release of IL-6 and IL-8 after stimulation with LPS1, LPS2, the DAMP HMGB1 or IL-1β for 6 h, using ELISA on supernatants from stimulated MDA-MB-231 cells (**c**), SUM-149 cells (**d**) and SUM-159 cells (**e**); n = 10. *Error bars* SEM; *P <0.05, ***P <0.001 (ANOVA). **f** Dual luciferase reporter assays of MDA-MB-231 cells transfected with an NFκB reporter. TK-Renilla was co-transfected as control (*Ctrl*). LPS1, LPS2, HMGB1 or IL-1β was added to stimulate NFκB activity as described in "Methods"; n = 14. *Error bars* SEM; ***P <0.001 (ANOVA). **g** The relative release of IL-6 and IL-8 after stimulation with the DAMP S100A9 for 20 h, using ELISA on supernatants from stimulated MDA-MB-231, SUM-149 and SUM-159 cells; n = 6. *Error bars* SEM. *P <0.05, ***P <0.001 (ANOVA). **h** Transient transfection of pDUO-MD2/hTLR4 for 72 h in MCF-7 cells induces release of both IL-6 and IL-8 measured by ELISA; n = 6. *Error bars* SEM; *P <0.05, ***P <0.001 (Students *t* test)

performed these cytosolic scoring parameters were grouped into 0–2 (0) and 3–4 (1) critical cutoffs. Table 1 shows the clinical parameters and correlations found with the TLR4-specific antibody. The intensity groups 3–4 (1) correlated significantly with the ER/PR-negative patient group and the basal-like status marker CK5. It did not correlate to Her2 expression (or lack of expression), however (Tables 1, 2 and 3). Tables 2 and 3 specifically show the correlation between Her2 and TLR4. Survival curves using the two cutoff groups indicate that as expected, the group with high TLR4 expression had significantly worse recurrence-free survival (P <0.029)

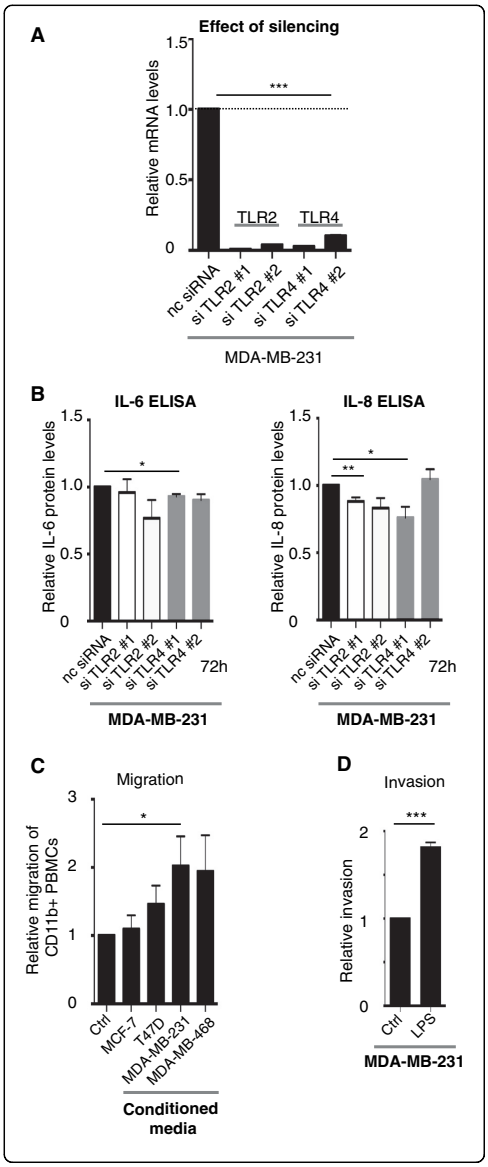

Fig. 4 Toll-like receptor 4 (*TLR4*) silencing decreases endogenous levels of pro-inflammatory cytokines. **a** Effect of TLR2/4 silencing in breast cancer cells transfected with negative control (*nc*) siRNA, or siRNA directed against *TLR2* mRNA (*si#1* and *si#2*) or *TLR4* mRNA (si#1 and si#2) was analyzed using quantitative real-time PCR; ***P <0.001 (analysis of variance (ANOVA)). **b** IL-6 (*left*) and IL-8 (*right*) ELISA on supernatants from MDA-MB-231 breast cancer cells transfected with nc siRNA, or siRNA directed against *TLR2* mRNA (si#1 and si#2) or *TLR4* mRNA (si#1 and si#2); n = 4. *Error bars* standard error of the mean (SEM); *P <0.05, **P <0.01, ***P <0.001 (ANOVA). **c** Boyden chamber migration assays. Migration of primary human myeloid cells towards supernatants from different cell lines indicated. Human primary peripheral blood mononuclear cells were isolated as previously described [48] and allowed to migrate through a Costar Transwell® Permeable Support 8.0-μm 24-well plate (Corning) to the supernatants of breast cancer supernatants cultured under serum-free conditions. Percentage of migrated $CD11b^+$ cells was analyzed using a flow cytometer and CD11b-APC antibodies (BD Sciences); n = 4. *Error bars* SEM; *P <0.05, **P <0.01, ***P <0.001 (ANOVA). **d** Matrigel invasion assays. Invasion of lipopolysaccharide (LPS)-stimulated/un-stimulated MDA-MB-231 cells into matrigel invasion chambers (BD Sciences) as indicated: 25×10^3 MDA-MB-231 cells were stimulated or not with LPS and allowed to invade from 72 h. Amount of invaded cells was analyzed using crystal violet staining and manual counting in four separate experiments; n = 4. *Error bars* SEM; *P <0.05, **P <0.01, ***P <0.001 (Student's t test)

(Fig. 6b). Membranous staining was also scored (0, 1) but there was no strong correlation (data not shown).

Finally, using a publicly available data site (R2: microarray analysis and visualization platform [40]; Tumor breast EXPO-351) with gene expression profiles of 351 primary breast cancers, we found positive correlation between expression of *TLR4* mRNA and *IL-6* (r value 0.231, P = 1.3e-05) (Fig. 6c).

Discussion

Breast cancers with an ER-negative phenotype have previously been shown to promote a strong pro-inflammatory microenvironment [44]. Furthermore, historically there is a negative relationship between ERα and NFκB that has previously been described in depth [10, 27–30]. Despite the fact that ER signaling can inhibit NFκB activity and vice versa, there is no evidence that the development of ER-negative breast tumors are caused by constitutive NFκB activity. Rather, it may be a result of the typical molecular gene landscapes found in luminal A compared to basal breast cancers, respectively. A link between PRR, e.g., TLR-induced activation of NFκB in breast cancer and its relation to expression of ER, has not been described. Both IL-6 and IL-8 can be highly expressed in TN breast cancers and this has partly been attributed to constitutively active NFκB [44]. In order to investigate whether TLRs, which are known to induce strong activation of NFκB, are expressed primarily in TN breast cancers and if this might affect the expression

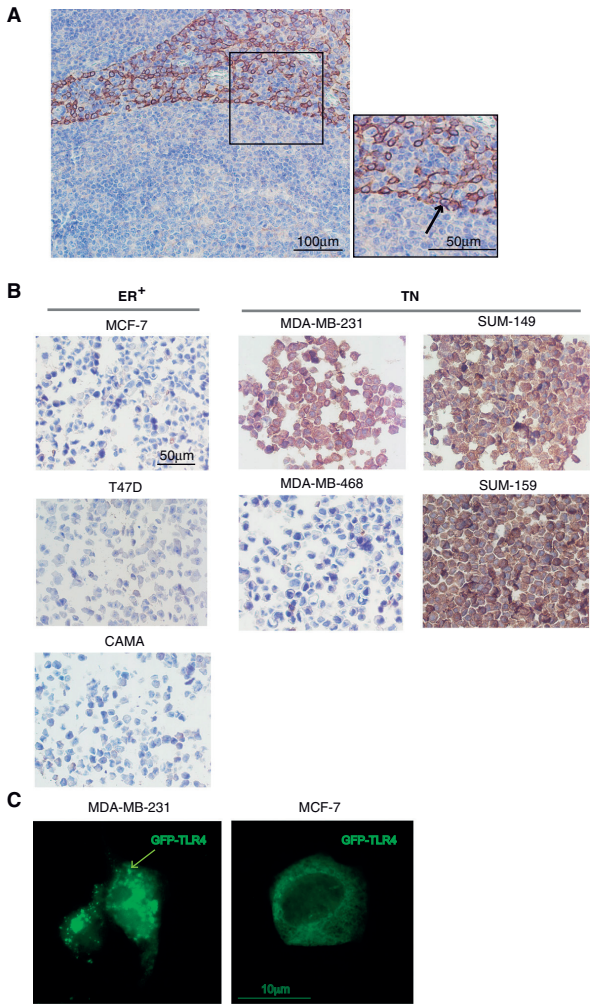

Fig. 5 Analysis of Toll-like receptor 4 (*TLR4*) protein expression in tonsil and breast cancer cells lines. Immunohistochemical analysis using an anti-human TLR4-specific antibody on paraffin-embedded tonsil (**a**) or cell pellets from the cell lines indicated (**b**). *Arrow* indicates the membranous staining. **c** MDA-MB-231 cells expressing the TLR co-receptors MD2 and CD14 but not ERα (*left*), and MCF-7 cells expressing ERα but not MD2 or CD14 (*right*) were transfected with *GFP*-tagged hTLR4 (pUNOI-hTLR4-GFP; Invivogen) for 48 h. Localization was investigated using immunofluorescence microscopy. The green fluorescent protein (GFP)-tagged hTLR4 was expressed in a vesicular pattern in the cytoplasm of MDA-MB-231 cells and evenly in the cytoplasm of MCF-7 cells. *Arrow* indicates the vesicular pattern. *ER+* estrogen receptor-positive, *TN* triple-negative

of pro-inflammatory genes in the same, we investigated the functional role of TLRs and co-receptors in breast cancer.

In immune cells, TLR expression is generally inhibited by prolonged activation of NFκB [45]. In contrast, our findings show that TLRs (TLR2, TLR 3, TLR 4) are preferentially expressed in TN breast cancer cell lines with constitutive NFκB activity, suggesting that the TLRs may be responsible for the NFκB activation pathway rather than induced by the same. Although introduction of a functional MD2/TLR4 complex in an ER^+ cell line has been shown to induce expression of pro-inflammatory

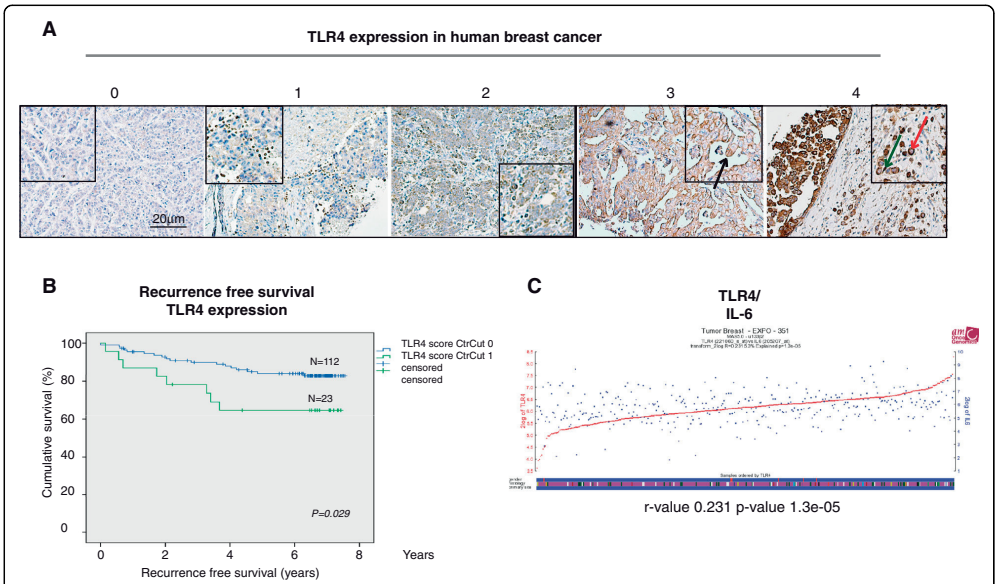

Fig. 6 Toll-like receptor 4 (*TLR4*) expression in breast tumors and survival curves. **a** Immunohistochemical TLR4 expression sample images from breast cancer tissue microarray cores, from *left* to *right*: negative (0), weak (1), moderate (2), strong (3) and very strong (4). Boxes with magnification and arrows to indicate localization (*black arrow* membranous, *green arrow* leukocyte, *red arrow* cytoplasmic staining. **b** Kaplan-Meier curves illustrating differences in recurrence-free survival according to TLR4 cytoplasmic expression in breast tumors. Cytosolic scoring parameters were grouped into 0–2 (0) and 3–4 (1) critical cutoffs. **c** Gene expression profile analysis of *TLR4* mRNA in relation to *IL-6* in (R2: microarray analysis and visualization platform [40]; Tumor breast EXPO-351)

cytokines, silencing of TLR4 in TN cells only caused a slight decrease in pro-inflammatory mediator release, indicating that the constitutive NFκB activation seen in TN cells in general is caused by another mechanism [44]. Apart from MDA-MB-468, the TN breast cancer cells were also demonstrated to express the co-receptors CD14 and MD2 meaning that they harbor the necessary proteins for a functional TLR4 signal to occur [20–22]. The exception, MDA-MB-468, only expressed CD14 and in line with this showed no biological TLR function. In the patient cohort we found correlation between TLR4 expression and ER/PR-negative tumors, but not TN tumors. This strengthens the interrelationship between TLR4, ER and NFκB activity, as expression of HER2 was not correlated in the TLR4-expressing primary tumors. We did not perform our in vitro analyses on any $Her2^+$ breast cancer cell line. Interestingly, the typical membrane staining seen in immune cells was not as obvious in the malignant cells, indicating that different regulation of TLR expression and signaling could be possible in cancers. This was previously described in neuroblastoma cells

Table 1 Correlation between TLR4 expression and clinicopathologic features in primary breast cancer (n = 144 patients)

Clinicopathologic features	Toll-like receptor 4		
	Correlation coefficient	*P* value (two-tailed)	Number
Age	−0,042	0,629	135
Nodal stage	0,042	0,646	122
Tumor size	−0,006	0,944	135
Ki67	0,003	0,971	117
nhg	0,094	0,276	135
Her2 subtype	0,158	0,072	131
ER status	−0,170	0,049*	135
PR status	−0,206	0,016*	135
CK5	0,184	0,037*	129

*P <0.05 using SPSS and Spearman's Rho test. *Her2* human epidermal growth factor receptor 2, *ER* estrogen receptor, *PR* progesterone receptor, *nhg* Nottingham histological grade

Table 2 Crosstab over Toll-like receptor 4 (TLR4) (0, 1) and Her2 (0, 1)

		TLR4 (0)	TLR4 (1)	Total
Her2	(0)	83	33	116
	(1)	6	6	12

Chi square test value 2.366, $P = 0.124$. *Her2* human epidermal growth factor receptor 2

Table 3 Crosstab over Toll-like receptor 4 (TLR4) (0,1) and ER/PR-negative/positive Her2+ tumors

	TLR4 (0)	TLR4 (1)	Total
ER/PR-negative Her2+	1	3	4
ER/PR-positive Her2+	5	3	8

Chi square test value 1.375, $P = 0.241$. *Her2* human epidermal growth factor receptor 2, *ER* estrogen receptor, *PR* progesterone receptor

[46] and is also supported by our finding that a GFP-tagged hTLR4 primarily showed a vesicular cytoplasmic localization in breast cancer cells. Furthermore and supporting this observation, it was recently reported that the TLR4-specific DAMP, S100A9, needs to be internalized to be able to signal via TLR4 [15]. Indeed, scoring of membrane TLR4 expression in breast cancer lesions did not reveal as much as that of cytoplasmic staining, and both TLR2 and TLR4 have been reported to be expressed intracellularly as well [47].

The DAMP, HMGB1, has previously been shown to signal via TLR4 in myeloid cells [6, 19]. Although we have also previously shown this in primary myeloid cells [48], we did not see an effect of HMGB1 on breast cancer cells in vitro. This could be due to different culture conditions, or to receptor expression patterns in myeloid as compared to cancer cells which might also reflect the fact that different sources of LPS generate different signals in the different cell lines in this study. Instead, we could show that the DAMP, S100A9, also induced pro-inflammatory proteins in breast cancer cells expressing TLR4.

It has previously been shown that NFκB [29] and targets (IL-6) [49] can downregulate ERα. We also investigated whether overexpression of TLR4 would affect ERα expression per se in ER^+ MCF-7 cells. We did see a slight although non-significant decrease of ERα after 72 h (data not shown), a finding that is probably explained by the significantly increased levels of IL-6 we observed in these experiments (Fig. 3h). In spite of this, we suggest that the ER/PR-negative breast cancer subtype probably is not caused by expression of TLRs and their downstream mediators, but rather further affected by them. Perhaps the expression of TLRs is even affected by the ERα/FoxA1/GATA3 network [50]. We show that both PAMPs and DAMPs induced release of pro-inflammatory mediators in ER/PR-negative breast cancer cells in vitro, a process that was regulated both at the transcriptional and post-transcriptional level. This means that although ER-negative breast cancer cells express high endogenous levels of pro-inflammatory mediators, a functional TLR4 is still likely to enhance their phenotype and surrounding inflammatory microenvironment, and this is also reflected by the decreased recurrence-free survival seen in the patients with tumors expressing TLR4 at high levels. In support of this, previous studies have shown that TLR4 expression promotes metastasis in a breast cancer model, an effect that was even enhanced by Paclitaxel [25, 26].

Conclusion

The findings presented in this study suggest that TLR4 is expressed in a functional form in ER/PR-negative breast cancers primarily. We suggest that TLR4 should be viewed as a possible therapeutic target in ER/PR-negative breast cancers to decrease the pro-inflammatory environment and hence the metastatic spread.

Additional files

Additional file 1: Table S1. Primer sequences. (PDF 94 kb)

Additional file 2: Figure S1. A Annexin V staining of MDA-MB-231 cells using flow cytometry to investigate apoptosis of MDA-MB-231 cells transfected with negative control (*nc*) siRNA, or siRNA directed against *TLR2* mRNA (*si#1* and *si#2*) or *TLR4* mRNA (*si#1* and *si#2*). TLR2 (si#1 and #2) gave contradicting results while TLR4 si#1 and #2 gave no effect (n = 3). Error bars indicate standard error of the mean (SEM); **P* <0.05, ***P* <0.01, ****P* <0.001 (analysis of variance (ANOVA)). **B** 3H-incorporation assay using previously published methods [48] to investigate proliferation of MDA-MB-231 cells transfected with negative control (*nc*) siRNA, or siRNA directed against *TLR2* mRNA (*si#1* and *si#2*) or *TLR4* mRNA (*si#1* and *si#2*) (n = 6). Error bars indicate SEM; **P* <0.05 ** *P* < 0.01, ****P* <0.001 (ANOVA). **C** IL-6 (*left*) and IL-8 (*right*) ELISA performed on supernatants from MDA-MB-231 breast cancer cells stimulated with increasing amounts of necrotic cell supernatants (*NCS*): 100 μl = 1:1, 50 μl = 1:4, 25 μl = 1:8 (n = 4). Error bars indicate SEM; **P* <0.05, ***P* <0.01, ****P* <0.001 (ANOVA). (PDF 176 kb)

Abbreviations

ANOVA: analysis of variance; CBA: cytokine bead array; CHX: cycloheximide; DAMP: damage-associated molecular pattern; ELISA: enzyme-linked immunosorbent assay; FBS: fetal bovine serum; ER: estrogen receptor; GFP: green fluorescent protein; Her2: human epidermal growth factor receptor 2; HMGB1: High mobility group protein B1; IL: interleukin; LPS: lipopolysaccharide; MAPK: mitogen-activated protein kinase; NCS: necrotic cell supernatant; NFκB: nuclear factor kappa B; PAMP: pathogen associated molecular pattern; PR: progesterone receptor; PRR: pattern recognition receptor; RT-qPCR: quantitative real-time PCR; TLR: toll-like receptor; TMA: tissue microarray; TN: triple-negative ($ER^-PR^-Her2^-$); TNF: tumor necrosis factor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MM performed the majority of experiments and analyzed data. RA and CB performed experiments and analyzed data. LHS was involved in revising the manuscript critically for important intellectual content, contributed to analysis and interpretation of data and also was responsible for linguistic correction. SPE produced and provided the TN breast cancer cell lines SUM149 and SUM159, contributed to analysis and interpretation of data and was involved in revising the manuscript critically for important intellectual content. As a clinical pathologist MEJ verified the IHC staining, and helped and mentored RA in scoring of the IHC. KJ was responsible for the breast cancer clinical samples and verified the IHC staining and TMA scoring. KL designed the experiments, wrote the manuscript and interpreted and analyzed the data. All authors read and approved the manuscript and were involved in revising the manuscript critically for important intellectual content.

Acknowledgements
The authors thank Ms Elise Nilsson for professional technical skills in preparation of TMAs and IHC. This work was supported by grants from the Swedish Research Council, The Swedish Cancer Society, Kocks Foundation, Österlunds Foundation, Gunnar Nilsson Cancer Foundation, MAS Cancer Foundation, and Åke Wibergs Foundation.

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Received: 8 May 2015 Accepted: 9 September 2015
Published online: 22 September 2015

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Keywords: breast cancer; human; S100A9; myeloid cell; MDSC; TLR4

S100A9 expressed in ER$^-$PgR$^-$ breast cancers induces inflammatory cytokines and is associated with an impaired overall survival

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Background: Breast cancer is the most common cancer form among women today. Depending on hormone receptor status, breast cancers are divided into different subtypes with vastly varying prognosis. S100A9 is a calcium-binding protein that is associated with inflammation and expressed not only in myeloid cells but also in some tumours. The role for S100A9 in the malignant cells is not well characterised; however, previous studies have shown that the protein could have important immune-modulating properties.

Methods: Using a human breast cancer cohort consisting of 144 tumour samples and *in vitro* analysis of human breast cancer cell lines, we investigated the expression and function of S100A9 in human breast cancer.

Results: We show that S100A9 expression in breast cancer correlated with the ER$^-$PgR$^-$ breast tumour subtype ($P<0.001$) and with Ki67 ($P=0.024$) and was expressed both in the malignant cells and in the tumour-infiltrating anti-inflammatory CD163$^+$ myeloid cells ($P<0.001$). Stromal expression of S100A9 also correlated to nodal stage, tumour size and Her2 positivity. Within the ER$^-$PgR$^-$ subgroup, all Her2$^+$ and EGFR$^+$ tumours expressed S100A9 in the cytoplasm. Both cytoplasmic staining in the malignant cells as well as stromal S100A9 expression in myeloid cells correlated with a decreased overall survival in breast cancer patients. Furthermore, rS100A9 homodimers induced expression of pro-inflammatory cytokines (IL-6, IL-8 and IL-1β) in a TLR4- and EGFR-dependent manner in human breast cancer cells *in vitro*.

Conclusion: We suggest that S100A9 could be viewed as a novel therapeutic target for patients with ER$^-$PgR$^-$ breast cancers.

Breast cancer is the most common cancer form among women (Kamangar *et al*, 2006). Clinical breast cancer classification is partly based on hormone receptor status of oestrogen receptor (ER), progesterone receptor (PgR) and Her2 receptor (Sorlie *et al*, 2001; Allred *et al*, 2004; Schnitt, 2010). Depending on hormone receptor status, breast cancers are divided into different subtypes with vastly varying prognosis and treatment strategies. ER$^+$ breast cancer patients have the best prognosis. In contrast, hormone receptor-negative breast cancers, that is, ER$^-$, PgR$^-$ and Her2$^-$ (also denoted triple-negative (TN) subtype) have the worst prognosis (Schnitt, 2010). Although there are numerous treatment options for hormone receptor-positive patients, there are inadequate options for TN breast cancer patients (Schnitt, 2010). Therefore, novel treatment options are urgently needed for this breast cancer subgroup particularly.

Cancer cells are dependent on an uncontrolled and indefinite cell division. This can be mediated by different mutations and mechanisms supporting the cancer cell proliferation. Two typical

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Received 24 June 2015; revised 3 August 2015; accepted 4 September 2015; published online 8 October 2015

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growth factor receptors that are commonly upregulated in breast cancers are the epidermal growth factor receptor (EGFR/ERBB1) and human epidermal growth factor 2 receptor (Her2/ERBB2) (Slamon *et al*, 1987; Hanahan and Weinberg, 2000). These are also often mutated to generate a constitutive activation and thus enhanced cell proliferation. The expression of EGFR and Her2 in breast cancers is generally not beneficial for survival (Hanahan and Weinberg, 2000), although patients with $Her2^+$ tumours have a slightly better prognosis owing to specialised treatment options (Schnitt, 2010).

S100A9 is a calcium-binding protein involved in inflammatory processes (Lagasse and Clerc, 1988; Zwadlo *et al*, 1988; Edgeworth *et al*, 1991; Hessian *et al*, 1993; Heizmann *et al*, 2002; Marenholz *et al*, 2004) and the protein can form homodimers or heterodimers with S100A8. S100A9 is mainly expressed in neutrophils and myeloid cells where it induces inflammatory cascades. Neutrophils primarily express S100A9/S100A8 heterodimers, thought to be involved in inflammatory diseases. In myeloid cells, it is believed that S100A9 signals as a Ca^{2+}-and Zn^{2+}-dependent damage-associated molecular pattern, via Toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE) whereby activation of NFκB is induced (Roth *et al*, 2003; Sunahori *et al*, 2006; Benedyk *et al*, 2007; Sinha *et al*, 2008; Riva *et al*, 2012). S100A9 has a strong immunosuppressive function in tumour-infiltrating myeloid-derived suppressor cells (MDSCs) (Ostrand-Rosenberg and Sinha, 2009). This is in sharp contrast to the role for S100A9 in tumour-infiltrating neutrophils (Yui *et al*, 2003). Furthermore, S100A9 has been shown to act as a chemo-attractant for immunosuppressive cells such as MDSCs or anti-inflammatory myeloid cells in tumours (Srikrishna *et al*, 2001; Ostrand-Rosenberg and Sinha, 2009). The protein expression of S100A9 in breast cancer has previously been studied in smaller cohorts (Arai *et al*, 2004; Cross *et al*, 2005; Arai *et al*, 2008) and in a large protein profiling study performed on breast cancers (Goncalves *et al*, 2008) where only cytoplasmic S100A9 expression in the malignant cells was scored. The functional role of the protein in malignant breast epithelial cells is controversial (Arai *et al*, 2004, 2008; Moon *et al*, 2008; Yin *et al*, 2013; Cormier *et al*, 2014; Gumireddy *et al*, 2014; Bresnick *et al*, 2015).

We have previously shown that anti-inflammatory myeloid cells are preferentially expressed in the tumour stroma of TN breast tumours (Medrek *et al*, 2012). The reason to this preferential location is unknown and one factor could be expression of chemo-attractants, such as S100A9. In this study, we investigated the expression and localisation pattern of S100A9 in 144 breast tumours and found a strong correlation not only to ER^-PgR^- tumours (irrespective of S100A9 localisation) and to the proliferation marker Ki67 (cytoplasmic and stromal localisation) but also to the presence of anti-inflammatory myeloid cells. Stromal S100A9 localisation further correlated to parameters such as larger tumour size, Her2 positivity and nodal stage. Within the ER^-PgR^- subgroup, all $Her2^+$ and $EGFR^+$ tumours expressed S100A9. This was further supported by gene expression profile analyses where ER, PgR, Her2 or EGFR mRNA expression strongly correlated with S100A9 expression, respectively. Also, stimulation with rEGF induced S100A9 mRNA expression. When breast cancer cell lines were stimulated with homodimers of rS100A9, induction of pro-inflammatory cytokines (IL-6, IL-8 and IL-1β) was observed in a TLR4-dependent manner. This was not observed when stimulating with S100A9/A8 heterodimers. Importantly, cytoplasmic staining of S100A9 in the malignant cells, as well as in stromal myeloid cells, correlated with an impaired overall survival in breast cancer patients. We suggest that S100A9 should be viewed as a potential therapeutic target for patients with ER^-PgR^- breast cancers.

MATERIAL AND METHODS

Cell culture. The human breast cancer cell lines MCF-7, T47-D, MDA-MB-231 and MDA-MB-468 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Biosera, Boussens, France), 1% sodium pyruvate, 1% HEPES and penicillin/streptomycin (100 U ml^{-1} and 100 μg ml^{-1}, respectively), CAMA-1 was cultured in MEM/EBSS supplemented with 10% FBS and penicillin/streptomycin, and SKBR3 was cultured in McCoys 5 A medium (without phenol red) supplemented with 10% FBS and penicillin/streptomycin. All cell lines were from ATCC (Wesel, Germany). Media and supplements were purchased from Thermo Scientific HyClone (South Logan, UT, USA) unless otherwise stated.

All stimulations were performed for a total of 20 h. Recombinant human S100A9 (rS100A9) and S100A9/S100A8 was a gift from Active Biotech AB (Lund, Sweden), and a detailed description on endotoxin-free S100A9 and S100A8/A9 generation, purification, bioactivity and binding has been published previously (Bjork *et al*, 2009; Riva *et al*, 2012; Bjork *et al*, 2013; Riva *et al*, 2013). Both S100A9 and S100A8/A9 were used at a concentration of 10 μg ml^{-1}. As proper S100A9 homodimer activity and binding of S100A9 to its receptors requires the presence of calcium and zinc (Bjork *et al*, 2009; Markowitz and Carson, 2013), cells were pretreated with 10 μM $ZnCl_2$ $\geqslant$15 min prior stimulation with rS100A9 at the indicated concentrations. All purchased media contained Ca^{2+} ($\geqslant$200 μM).

Compounds and cytokine analysis. Antibodies used for S100A9 western blotting (WB), immunoprecipitation (IP) and IHC were Calgranulin B (clone MRP 1H9; Santa Cruz, Dallas, TX, USA), Lamin B (clone C-20; Santa Cruz) and Actin (clone C-4; MP Biomedicals, Solon, OH, USA). Supernatants from rS100A9-stimulated or siRNA-transfected cells were harvested and analysed using human inflammatory cytokine cytometric bead array (BD Biosciences, San Diego, CA, USA) or quantikine human CXCL8/IL-8 immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturers' instructions. IL-10 and IL-12 were largely undetectable and hence excluded in this study.

Tissue microarray. The large breast cancer cohort analysed in this study consists of 144 patients diagnosed with invasive breast cancer at Skåne University Hospital, Malmö, Sweden between 2001 and 2002. The cohort and TMA has previously been described in detail (Elkabets *et al*, 2011; Gronberg *et al*, 2011; Svensson *et al*, 2011) and (Medrek *et al*, 2012).

Ethical considerations. Ethical permit was obtained from the local ethical committee at Lund University (Dnr 447/07). Signed informed consent was not collected but the patients were offered to opt out.

Immunohistochemistry. Four-μm-thick TMA sections were mounted onto glass slides and deparaffinised followed by antigen retrieval using the PT-link system (DAKO, Glostrup, Denmark) and stained in a Autostainer Plus (DAKO) with the EnVisionFlex High pH-kit (DAKO). Antibodies used were: anti-Calgranulin B (S100A9; clone MRP 1H9 dilution 1 : 200; Santa Cruz), anti-CD163 (10D6 dilution 1 : 250; Novocastra, GmbH, Nuβloch, Germany), and anti-CD68 (dilution 1 : 1500; DAKO).

Gene expression profile array. Correlations between S100A9 and ER, PgR, EGFR or Her2 in a separate data set (Tumour breast EXPO-351) was performed using the publicly available database R2: microarray analysis and visualisation platform (http://r2.amc.nl).

Quantitative real-time PCR (qRT–PCR). Total RNA was extracted using the RNeasy Plus Kit according to the manufacturer's instructions (Qiagen, Hilden, MD, USA). Equal amounts of

RNA were used for cDNA synthesis using random hexamers and the M-MuLV reverse transcriptase enzyme (Thermo Scientific). qRT–PCR was performed in triplicates using Maxima SYBR Green/Rox (Thermo Scientific) according to the manufacturer's instructions. qRT–PCR analysis was performed on the Mx3005P QPCR system (Agilent Technologies, Santa Clara, CA, USA) and the relative mRNA expression was normalised to *YWHAZ*, *UBC* and *SDHA* and calculated using the comparative Ct method (Vandesompele *et al*, 2002). For primer sequences, see Supplementary Table S1.

Transient transfections and luciferase assays. Transient siRNA transfections were performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). In all, 10 nM of the following silencer select siRNA oligonucleotides were used: Silencer Select Negative Control #2, siS100A9 #1 (s12425), siS100A9 #2 (s12426), siTLR4 #1 (s14194) and siTLR4 #2 (s14195), all purchased from Ambion (Carlsbad, CA, USA). For proliferation and viability assays, the cells were re-seeded into 96-well plates after 24 h. All analyses were performed 72 h posttransfection.

For transient transfection and luciferase assays, breast cancer cells were co-transfected with a total of 0.5 μg pNFκB-luciferase (BD Biosciences) and 0.05 μg TK-renilla-luciferase (Promega, Madison, WI, USA) plasmids, w/wo 1 μg pDUO-MD2-hTLR4 (Invivogen, San Diego, CA, USA) plasmid or 10 nM of the respective siRNA, using Lipofectamine 2000. rS100A9 stimulation was performed 24 h posttransfection as described above. The NFκB activity was analysed using the Dual-Luciferase Reporter Assay System (Promega).

Cell proliferation and viability assays. The effects of rS100A9 or S100A9 knockdown on cell proliferation and viability was assessed using thymidine incorporation, WST-1 assay and Annexin V-staining. In all, 1 μ Ci [methyl-^{3}H] thymidine was added 18 h prior to analysis, and thymidine incorporation was determined in a Microbeta Counter (PerkinElmer, Waltham, MA, USA). The WST-1 assay was performed according to the manufacturer's recommendations (Roche Applied Science, Indianapolis, IN, USA). For Annexin V staining, cells were detached using EDTA-free trypsin (Thermo Scientific), washed twice in PBS prior to Annexin V-APC and PI staining according to the manufacturer's instructions (BD Biosciences). The amount of viable cells was analysed using a FACSVerse (BD).

Statistics and annotation. ANOVA or Student's *t*-test were used for *in vitro* experiments as indicated, using the Graph Pad Prism software (La Jolla, CA, USA).

Immunohistochemical S100A9 expression in TMA breast cancer tumours was estimated in fraction (percent) and intensity (0–3) separately annotated for nuclear, cytoplasmic, membrane and stromal compartments. A multiplier of percent and intensity for each core was constructed, yielding a score of 0–3, and a mean value of the two cores was used in the analyses. Spearman's Rho and χ^2 test was used for comparison of CD163, CD68 and S100A9 expression. Classification and regression tree (CRT) was used for cutoff purposes, and Kaplan–Meier analysis with log rank tests were used to illustrate differences in survival. All statistical tests were two sided and $P \leqslant 0.05$ were considered significant. Calculations were performed with IBM SPSS Statistics version 19.0 (IBM Corp, Armonk, NY, USA).

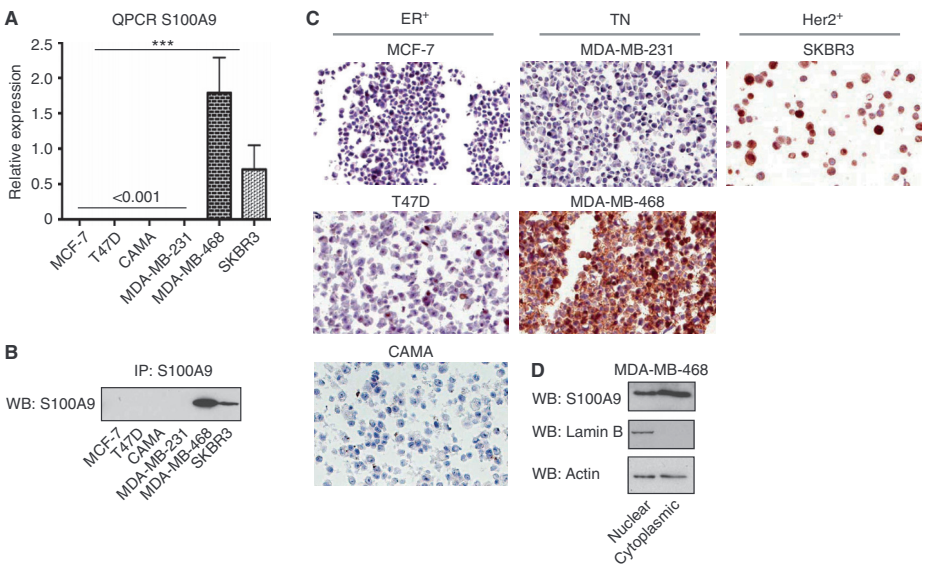

Figure 1. Analysis of S100A9 protein and mRNA expression in breast cancer cells lines. (**A**) The relative expression of S100A9 mRNA using Q-PCR on mRNA from the cell lines indicated. Error bars indicate s.e.m. ***$P<0.001$ using ANOVA test. (**B**) WB of immunoprecipitated (IP) cell lysates from the indicated cell lines. (**C**) IHC using an anti-human S100A9-specific antibody on paraffin-embedded cell pellets from the cell lines indicated. (**D**) WB of nuclear and cytoplasmic fractions. Nuclear extracts was controlled with the Lamin B-specific antibody. Actin shows the loading control for each lane. IHC, IP and WB antibody was the same in panels (**A**–**C**).

RESULTS

S100A9 is expressed in ER$^-$PgR$^-$ breast cancer cell lines expressing EGFR. To investigate the expression pattern of S100A9 in human breast cancers, we started by verifying a human specific anti-calgranulin B (S100A9) antibody (clone MRP 1H9). To this end, we first characterised the S100A9 expression pattern in six different breast cancer cell lines (MCF-7, T47D, CAMA-1 (all three ER$^+$PgR$^+$) and MDA-MB-231 (TN), MDA-MB-468 (TN EGFR^{++}), SKBR3 (ER$^-$PgR$^-$Her2$^+$)), by using Q-PCR, WB and IHC of paraffin-embedded cell pellets (Figure 1). The endogenous mRNA levels of S100A9 were compared using Q-PCR of the six cell lines (Figure 1A). Very low levels (<0.001 relative to control) of S100A9 were observed not only in all the ER$^+$ cell lines but also in the TN MDA-MB-231 cell line. In contrast, a high level of S100A9 mRNA was found in MDA-MB-468 and SKBR3 cells (Figure 1A). A similar expression pattern could be seen at the protein level using IP with subsequent WB of cell lysates prepared from the cell lines (Figure 1B). IP was used as a weak background band and was present in all cell lysates at the size of S100A9 (14 kDa; data not shown). We then proceeded with an IHC staining of S100A9 on paraffin-embedded cell pellets from the cell lines used (Figure 1C). The cytoplasmic IHC staining was intense in the two cell lines expressing S100A9 (SKBR3 and MDA-MB-468; Figure 1C, right and center). A membranous as well as a

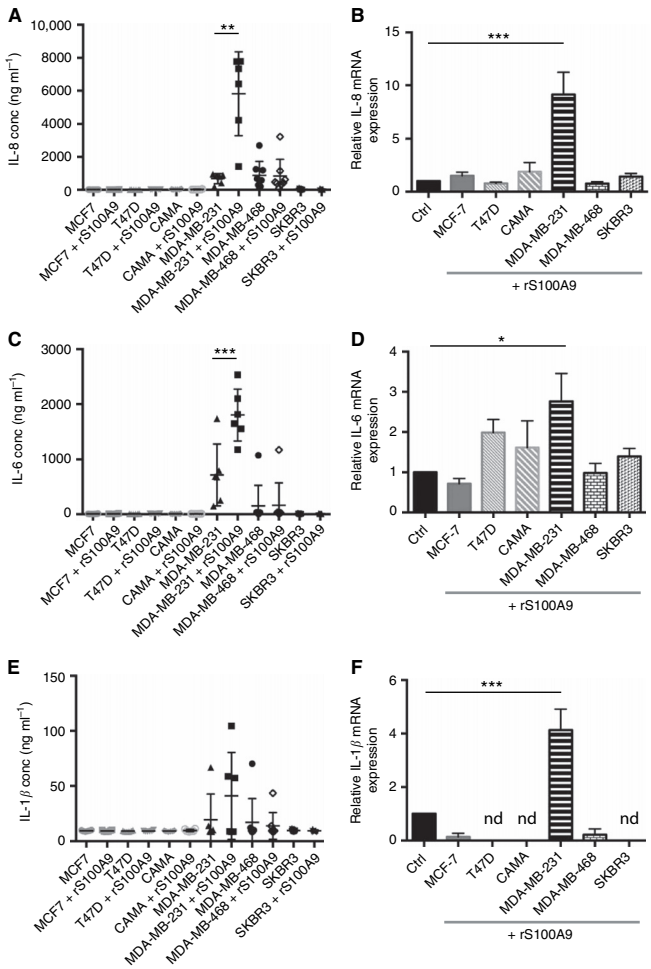

Figure 2. S100A9 induced cytokine expression in human breast cancer cells *in vitro*. rS100A9 (10 μg ml^{-1}) induced (**A**) IL-8, (**C**) IL-6 and (**E**) IL-1β levels were measured using cytometric bead array (CBA) in supernatants from stimulated breast cancer cell lines as indicated. The relative expression of corresponding mRNA was measured using Q-PCR (**B**) IL-8, (**D**) IL-6 and (**F**) IL-1β. Error bars indicate s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using ANOVA test.

nuclear staining was seen in these S100A9-expressing cell lines (Figure 1C). We next silenced S100A9 in MDA-MB-468 cells using siRNA and verified the knockdown of S100A9 and the specificity of the IHC stainings using Q-PCR (Supplementary Figure S1A) and IHC of paraffin-embedded cells (Supplementary Figure S1B), respectively. To confirm the nuclear staining in the S100A9-expressing cells, we also performed nuclear lysates from MDA-MB-468 cells showing that S100A9 was present in both the cytosol and nucleus (Figure 1D).

Cytokine release is induced upon rS100A9 stimulation. The functional role for S100A9 in human breast epithelial cells is not clear (Arai *et al*, 2004, 2008; Moon *et al*, 2008; Yin *et al*, 2013; Cormier *et al*, 2014; Gumireddy *et al*, 2014). As S100A9 is known to induce inflammatory cascades in certain cell types, we wanted to investigate whether rS100A9 could do this in malignant epithelial breast cancer cells as well. Using rS100A9 in Ca^{2+}/Zn^{+} physiological conditions has previously been shown to trigger a conformational change of S100A9 (Bjork *et al*, 2009; Markowitz and Carson, 2013). We therefore added rS100A9 in Ca^{2+}/Zn^{2+}-high conditions and analysed whether inflammatory cytokines were released upon S100A9 stimulation of breast cancer cell lines. Indeed, in one of the cell lines we found a significantly enhanced expression of the cytokines IL-8, IL-6 and IL-1β at both the protein level (Figure 2A, C and E) and mRNA level (Figure 2B, D and F) upon stimulation with rS100A9 homodimers. This was preferentially seen in MDA-MB-231 cells that lack endogenous expression of S100A9 (TN cell line) and to some extent in the MDA-MB-468 cells (TN $EGFR^{+\,+}$). Stimulation with S100A9/A8 heterodimers did not induce this cytokine release (Supplementary Figure S1C). Interestingly, only very modest or no decrease in cytokine expression was seen when S100A9 was silenced in the S100A9-expressing cells (Supplementary Figure S1D).

S100A9 affects NFκB activity in breast cancer cells. To investigate whether the S100A9-induced cytokine release was caused by an S100A9-induced activation of NFκB activity, we performed NFκB Dual-Luciferase reporter assays. As shown in Figure 3A and B, addition of rS100A9 induced a significant NFκB activity in the MDA-MB-231 cell line but not the MDA-MB-468 or SKBR3 cell lines. Addition of S100A8/A9 heterodimers induced a modest but significant NFκB activity in the MDA-MB-231 cell line (Figure 3B) but as described above absolutely no increase in cytokine expression (Supplementary Figure S1C). The most logical explanation to this is that S100A8/A9-induced NFκB activity is too low for pro-inflammatory cytokine induction. We next performed

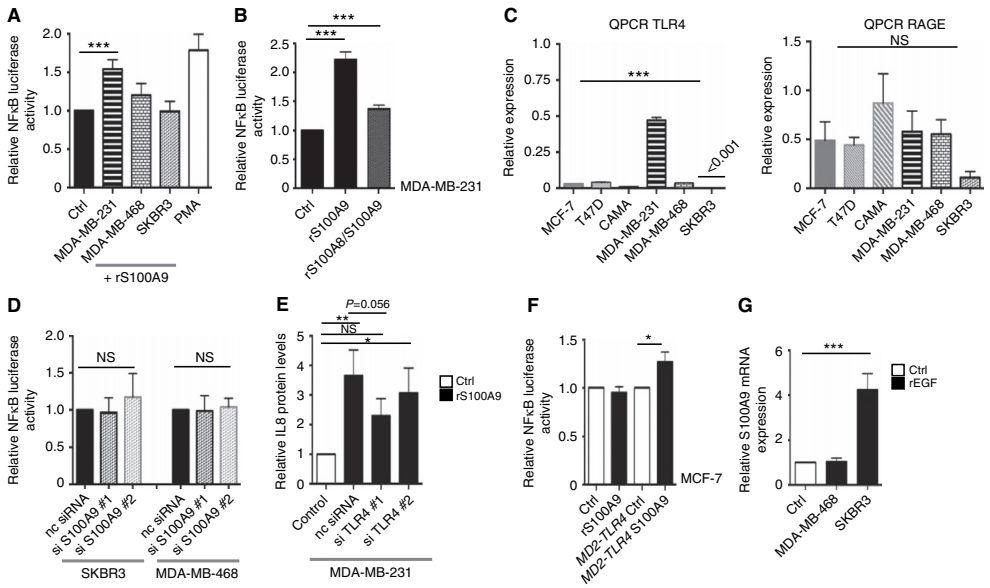

Figure 3. S100A9 induces NFκB in breast cancer cells *in vitro* in a TLR4-dependent manner. (**A**) Dual luciferase reporter assays of breast cancer cells transfected with a NFκB reporter. TK-Renilla was co-transfected as control. rS100A9 was added to stimulate NFκB activity as described in Material and Methods. Error bars indicate s.e.m. *$P<0.05$ using Student's *t*-test. (**B**) Dual luciferase reporter assays of MDA-MB-231 breast cancer cells transfected with a NFκB reporter. TK-Renilla was co-transfected as control. rS100A9 (10 $\mu g\,ml^{-1}$) or rS100A8/A9 (10 $\mu g\,ml^{-1}$) was added to stimulate NFκB activity in MDA-MB-231 cells as described in Material and Methods. Error bars indicate s.e.m. *$P<0.05$ using Student's *t*-test. (**C**) TLR4 and RAGE mRNA expression levels in human breast cancer cell lines as measured by Q-PCR. Error bars indicate s.e.m. ***$P<0.001$ using ANOVA test. (**D**) Dual luciferase reporter assays of breast cancer cells transfected with siRNA directed against S100A9 mRNA together with a NFκB reporter and TK-Renilla as control. Error bars indicate s.e.m. **$P<0.01$ using ANOVA test. (**E**) IL-8 protein levels in supernatants from MDA-MB-231 breast cancer cells transfected with negative control (nc) siRNA or siRNA directed against TLR4 mRNA (si#1 and si#2) and subsequently stimulated or not with rS100A9 homodimers. Cytometric bead array (CBA) was used to measure IL-8 levels. Error bars indicate s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using ANOVA test. (**F**) Dual luciferase reporter assays of MCF-7 breast cancer cells transfected with the MD2-hTLR4 complex (pDUO-MD2-hTLR4) together with a NFκB reporter and TK-Renilla as control. Error bars indicate s.e.m. **$P<0.01$ using ANOVA test. (**G**) EGF induced S100A9 expression in human breast cancer cells *in vitro*. rEGF stimulation of indicated breast cancer cell lines induced S100A9 mRNA levels as measured using Q-PCR. Error bars indicate s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using ANOVA test.

a Q-PCR analysis of the potential receptors for Ca^{2+}-dependent S100A9 signalling: TLR4 and RAGE (Markowitz and Carson, 2013), and could show that only MDA-MB-231 cells expressed high levels of TLR4 (Figure 3C, left). RAGE was expressed at relatively similar levels (Figure 3C, right). As the S100A9-expressing cell lines SKBR3 and MDA-MB-468 cells did not express TLR4 (Figure 3C, left) but expressed RAGE at varying levels (Figure 3C, right), we next investigated the NFkB activity upon S100A9 silencing in SKBR3 and MDA-MB-468 cells (Figure 3D). Silencing of S100A9 did not promote a decreased NFkB activity in SKBR3 or MDA-MB-468 cells (Figure 3D). In line with this and as mentioned above, only very modest or no decrease in cytokine expression was seen when S100A9 was silenced in the S100A9-expressing cells that lack TLR4 or RAGE (Supplementary Figure S1D). We therefore performed a TLR4-silencing experiment and could show that the rS100A9-induced IL-8 release was decreased when TLR4 was silenced in MDA-MB-231 cells and subsequently treated with rS100A9 (Figure 3E). Also, introduction of the MD2-hTLR4 complex (pDUO-MD2-hTLR4) in otherwise MD2- and TLR4-negative MCF7 cells promoted a significant NFκB activity upon S100A9 stimulation (Figure 3F).

Because of the inherent expression of EGFR in MDA-MB-468 (amplified; $EGFR^{++}$) and SKBR3 ($EGFR^{+}$) cells, we also wanted to analyse whether EGF could induce S100A9 *per se*. Indeed, we found that rEGF induced S100A9 at the mRNA level in SKBR3 but not in the EGFR-amplified MDA-MB-468 cells (Figure 3G). In an attempt to investigate further functions of S100A9, we performed an array of *in vitro* experiments, including cell cycle, proliferation, apoptosis, actin polymerisation, epithelial-to-mesenchymal transition (EMT) and migration analyses. Using the mentioned breast cancer cell lines rS100A9 homodimers or siRNA towards S100A9,

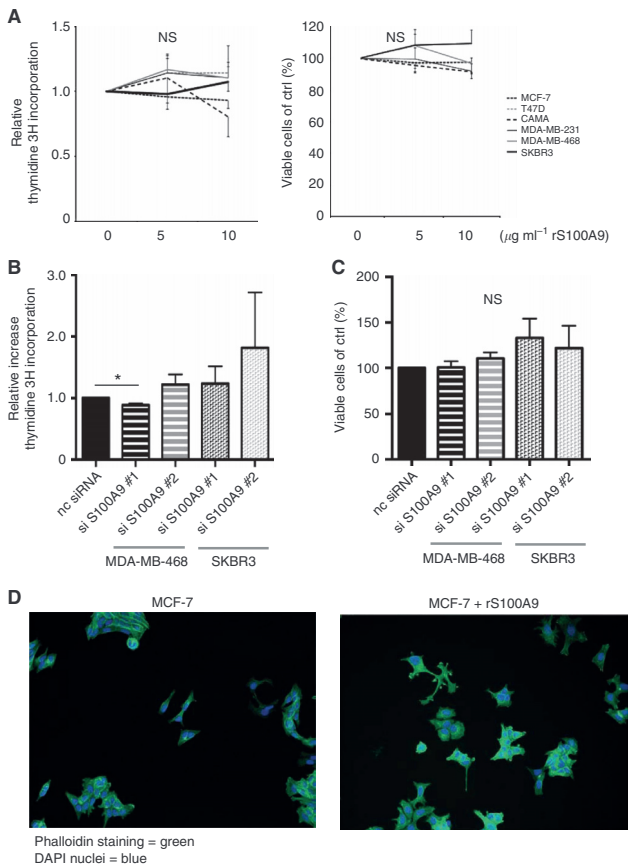

Figure 4. Effects of S100A9 on breast cancer cell proliferation, survival and F-actin polymerisation *in vitro*. (**A**) Effects of rS100A9 on breast cancer cell proliferation using thymidine ^3H incorporation (left) or WST-1 assay (right) on the different breast cancer cell lines indicated. (**B**) Breast cancer cell proliferation using thymidine ^3H incorporation on cell lines transfected with either negative control (nc) or silencing (si) S100A9 siRNAs as indicated. Error bars indicate s.e.m. *$P<0.05$ using ANOVA test. (**C**) Breast cancer cell proliferation/viability using WST-1 assay (right) on cell lines transfected with either negative control (nc) or silencing (si) S100A9 siRNAs as indicated. Error bars indicate s.e.m. NS = not significant using ANOVA test. (**D**) Effects of S100A9 on F-actin polymerisation in MCF-7 cells using phalloidin IF (green) staining. Blue staining = DAPI.

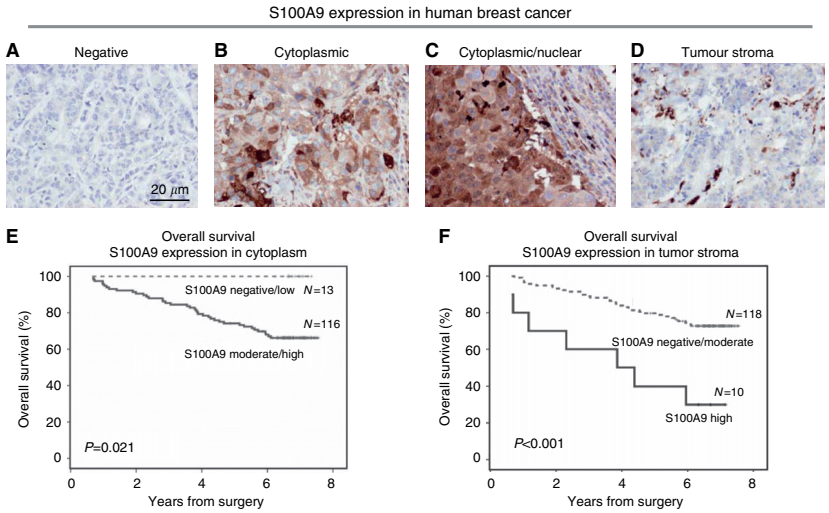

Figure 5. S100A9 expression in breast tumours and survival curves. (**A–D**) Immunohistochemical S100A9 expression sample images from breast cancer TMA cores, from left to right: negative (**A**), cytoplasmic expression (**B**), cytoplasmic/nuclear (**C**), and stromal expression (**D**). (**E**) Kaplan–Meier curves illustrating differences in overall survival according to S100A9 cytoplasmic expression in breast cancer tumours. (**F**) Kaplan–Meier curves illustrating differences in overall survival according to S100A9 stromal expression in breast cancer tumours.

Table 1a. Correlations between S100A9 expression and clinicopathological features in primary breast cancer (*N* = 144)

	S100A9 cytoplasmic	S100A9 nuclear	S100A9 membrane	S100A9 stroma
Clinicopathological features	**Correlation coefficient, *P* value, *N* (two-tailed)**	**Correlation coefficient, *P* value, *N* (two-tailed)**	**Correlation coefficient, *P* value, *N* (two-tailed)**	**Correlation coefficient, *P* value, *N* (two-tailed)**
Age	0.040, 0.656, 129	−0.028, 0.752, 129	0.029, 0.47, 129	−0.026, 0.770, 128
Nodal stage	0.030, 0.747, 117	−0.048, 0.610, 117	−0.021, 0.822, 117	0.205, 0.028*, 116
Tumour size	0.117, 0.188, 129	0.037, 0.678, 129	0.097, 0.276, 129	0.257, 0.003**, 128
Ki67	0.213, 0.024*, 112	0.141, 0.138, 112	0.153, 0.108, 112	0.318, 0.001**, 111
Her2	0.078, 0.384, 126	0.064, 0.478, 126	0.117, 0.194, 126	0.211, 0.018*, 125
ER status	−0.306, 0.000**, 129	−0.296, 0.001**, 129	−0.348, 0.000**, 129	−0.346, 0.000**, 128
PR status	−0.231, 0.009**, 129	−0.215, 0.014*, 129	−0.336, 0.000**, 129	−0.283, 0.001**, 128
EGFR	0.171, 0.064, 118	0.014, 0.884, 118	0.140, 0.132, 118	0.099, 0.286, 117
Basal	0.316, 0.000**, 129	0.262, 0.003**, 124	0.291, 0.001**, 124	0.245, 0.006**, 123
Luminal A	−0.300, 0.001**, 122	−0.238, 0.008**, 122	−0.300, 0.001**, 122	−0.402, 0.000**, 121
Luminal B	0.003, 0.977, 123	−0.050, 0.584, 123	−0.024, 0.791, 123	0.178, 0.050*, 122

Abbreviations: EGFR = epidermal growth factor receptor; ER = oestrogen receptor; Her2 = human epidermal growth factor 2 receptor; PR = progesterone receptor. Shaded boxes are significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using Spearmans Rho test.

we found no significant effects on cell cycle, apoptosis, EMT or migration (data not shown). We did, however, see a small but significant effect on breast cancer cell proliferation in siS100A9-transfected MDA-MB-468 cells (Figure 4A–C) and also on actin polymerisation in rS100A9-stimulated MCF-7 cells (Figure 4D) as previously shown (Yin *et al*, 2013; Cormier *et al*, 2014).

S100A9 expression correlates with ER$^-$, PgR$^-$, Her2$^+$ and EGFR$^+$ expression in human breast tumours. To investigate the relation between S100A9 expression and ER, PgR, Her2 (ERBB2) and EGFR expression in primary breast cancers in more detail, we next used a publicly available data site (R2: microarray analysis and visualisation platform http://r2.amc.nl; Tumour breast EXPO-351) with gene expression profiles of 351 primary breast cancers. We found a very strong negative correlation between expression of S100A9 mRNA with either ER (*r*-value −0.518; $P = 1.7e\text{-}25$) and PgR (*r*-value −0.468; $P = 1.8e\text{-}20$), and a positive correlation between expression of S100A9 mRNA with either ERBB2 (*r*-value 0.238; $P = 6.8e\text{-}06$) or EGFR (*r*-value 0.319; $P = 9.6e\text{-}10$) (Supplementary Figures S2A–D).

Following IHC treatment of our TMA comprising 144 breast cancers, cytoplasmic and nuclear S100A9 staining was successfully annotated in 129 (89.6%) tumours and 128 tumours (88.9%) for stromal compartment. In line with both the IHC data from breast cancer cell lines, as well as the gene expression profiling data, S100A9 expression (cytoplasmic, nuclear, membrane as well as

stromal; Figure 5A–D) correlated to ER^-PgR^- hormone receptor status of the breast tumours ($P<0.001$; Tables 1a–c). Cytoplasmic S100A9 also correlated to expression of the Ki67 proliferation marker (Table 1a). Tumours presenting Her2 or EGFR positivity were relatively few ($n=12$ and $n=11$, respectively). Despite this, stromal S100A9 expression correlated to Her2 positivity ($P=0.018$; Table 1a) and presented a borderline correlation to EGFR positivity ($P=0.064$; Table 1a). All of the $Her2^+$ ($n=12$) as well as all $EGFR^+$ ($n=11$) showed cytoplasmic S100A9 expression (Table 1b).

Stromal S100A9 expression correlates to the presence of anti-inflammatory $CD163^+$ myeloid cells. Tumour stromal S100A9 expression has previously been described in different tumours (e.g., prostate cancer) (Tidehag *et al*, 2014). In this study, we found that stromal S100A9 expression (predominantly in cells with a leukocyte morphology) correlated not only with ER^-PgR^- tumours as mentioned above but also to parameters such as larger tumour size and Ki67 positivity and nodal stage (Table 1a; S100A9 stroma; right). We have previously shown that anti-inflammatory $CD163^+$ myeloid cells are located preferentially in the tumour stroma of TN breast cancer and also that this correlated to higher grade, larger tumour size and Ki67 positivity (Medrek *et al*, 2012). As shown in Table 1c, breast cancer stromal S100A9 correlated with anti-inflammatory $CD163^+$ myeloid cells but interestingly not to the macrophage marker CD68. This suggests that the myeloid cells expressing S100A9 are of immature myeloid origin (e.g., MDSCs). Supporting this notion, also anti-inflammatory $CD163^+$ myeloid cells in the tumour nest actually expressed S100A9, but $CD68^+$ macrophages did not (Table 1c).

S100A9 expression in breast cancer correlates with a poor survival. S100A9 expression in cytoplasm and in stroma was found to be significantly associated with an impaired survival (Figure 5). The same trend but non-significant was seen for membrane and nuclear expression (data not shown). For cytoplasmic expression, CRT analysis revealed a cutoff at 0.149/3.00 (Supplementary Figure S3A) where patients having positive (any) S100A9 expression denoted as moderate–high had significantly impaired survival ($n=116/129$, $P=0.021$, Figure 5E). For stromal expression, CRT analysis that revealed an optimal cutoff at 1.95/3.00 (Supplementary Figure S3B) also had significantly impaired survival ($P<0.001$, Figure 5F). This is in line with what we previously showed concerning correlation between impaired overall survival and the presence of macrophages and anti-inflammatory myeloid cells in the tumour stroma, with a preferential presence of anti-inflammatory myeloid cells in the tumour stroma of TN breast cancers particularly (Medrek *et al*, 2012).

Table 1b. S100A9 expression in Her2-cutoff group (upper) and EGFR-cutoff group (lower): all $Her2^+$ and $EGFR^+$ cases also express S100A9

	S100A9 cytoplasmic		
	0	1	Total
Her2 cutoff			
0	4	106	110
1	0	12	12
Total	4	118	122
EGFR cutoff			
0	3	104	107
1	0	11	11
Total	3	115	118

Abbreviations: EGFR = epidermal growth factor receptor; Her2 = human epidermal growth factor 2 receptor.

DISCUSSION

S100A9 expression in breast cancer has previously been explored *in vitro* and in small cohorts of patients (Arai *et al*, 2004; Cross *et al*, 2005; Arai *et al*, 2008; Moon *et al*, 2008; Markowitz and Carson, 2013; Yin *et al*, 2013; Cormier *et al*, 2014; Gumireddy *et al*, 2014). Also, a large protein profiling study was performed on breast cancers (Goncalves *et al*, 2008) where cytoplasmic S100A9 expression in the malignant breast epithelial cells was investigated. Studies concerning the role for S100A9 in breast cancer cells *in vitro* are indefinite (Markowitz and Carson, 2013) and one reason for this is the limited supply of reagents. As S100A9 acts as a chemo-attractant for certain immunosuppressive cells and is expressed in myeloid suppressor cells that are important in diseases such as cancer (Ostrand-Rosenberg and Sinha, 2009), we decided to perform a deeper analysis concerning the expression and localisation pattern of S100A9 and its correlation to the presence of different tumour-infiltrating myeloid cells. We stained TMA sections containing samples of 144 breast cancers using a carefully validated antibody towards human S100A9 and using a publicly available gene expression data set consisting of 351 primary breast tumours. The findings were endorsed by analysis regarding the functional role of S100A9 specifically using stimulation with rS100A9 or silencing of S100A9 *in vitro*.

Although S100A9 is expressed in some epithelial cell types and also in certain cancers, its functional role in these cell types is still obscure (Markowitz and Carson, 2013). Our *in vitro* experiments regarding cell cycle analysis, apoptosis, migration and EMT markers, using stimulation with rS100A9 or silencing of S100A9, did not yield consistent results. However, as published previously, both cell proliferation and f-actin polymerisation was affected slightly by S100A9 in some cell lines and should therefore be warranted more studies in the future (Yin *et al*, 2013; Cormier *et al*, 2014). Instead we found that inflammatory cytokines were released upon S100A9 stimulation of breast cancer cell lines but only in cells with a high expression of TLR4 (MDA-MB-231), similar to what has previously been published regarding myeloid cells (Riva *et al*, 2012). In line with this, only very modest or no decrease in cytokine expression was seen when S100A9 was

Table 1c. Correlations between S100A9 expression and myeloid cell markers (CD68 and CD163) in primary breast cancer ($N=144$)

	S100A9 cytoplasmic	S100A9 nuclear	S100A9 membrane	S100A9 stroma
Presence of myeloid cells	**Correlation coefficient, *P* value, *N* (two-tailed)**	**Correlation coefficient, *P* value, *N* (two-tailed)**	**Correlation coefficient, *P* value, *N* (two-tailed)**	**Correlation coefficient, *P* value, *N* (two-tailed)**
CD68 tumour nest	0.176, 0.079, 101	0.055, 0.588, 101	0.008, 0.939, 101	0.161, 0.109, 100
CD68 tumour stroma	−0.070, 0.484, 101	0.022, 0.827, 101	0.050, 0.620, 101	0.059, 0.563, 100
CD163 tumour nest	0.130, 0.183, 107	0.133, 0.173, 107	0.162, 0.095, 107	0.209, 0.032*, 106
CD163 tumour stroma	0.074, 0.439, 112	−0.068, 0.471, 112	0.080, 0.403, 112	0.197, 0.038*, 111

Shaded boxes are significant. *$P<0.05$, **$P<0.01$, ***$P<0.001$ using Spearmans Rho test.

silenced in the S100A9-expressing cells that lack TLR4 or RAGE. Only, S100A9 signalling, but not S100A9/S100A8, induced significant expression of inflammatory proteins. Indeed, S100A9 homodimers but not S100A9/S100A8 heterodimers have been proposed to signal via TLR4 in a Ca^{2+}-dependent manner (Markowitz and Carson, 2013). As both membranous and vesicular TLR4 has been reported in cancer cells, the cytoplasmic S100A9 expression pattern could represent intracellular as well as extracellular signalling pathways (Hassan *et al*, 2006).

Breast tumours of the TN and ER^-PgR^- subtypes most often express pro-inflammatory proteins that activate STAT3 (Sasser *et al*, 2007). STAT3 has previously been shown to induce S100A9 (Li *et al*, 2004; Lee *et al*, 2012). This does, however, not reflect the expression pattern of S100A9 in breast cancer cell lines *in vitro*, as a high S100A9 expression is seen only in two out of three ER^-PgR^- breast cancer cell lines tested. Hence other explanations as to why S100A9 is expressed in malignant epithelial breast cells are likely to be found in the future. A link between S100A9 expression and EGFR has been suggested for other cancer types before (Kim *et al*, 2011, 2014). The fact that the two cell lines expressing S100A9 both expressed EGFR (MDA-MB-468, $EGFR^{++}$ amplified; SKBR3, $EGFR^+$) led us to the finding that also rEGF could induce S100A9 expression but only in EGFR-non-amplified SKBR3 cells. It would be interesting to investigate whether the tumour microenvironment as such could effect S100A9 expression *in vivo*.

After careful validation, we considered the S100A9 antibody used in this study to be specific using IHC on paraffin-embedded samples. We found that S100A9 was expressed primarily in tumours with an ER^-PgR^- phenotype. This correlation was found irrespective of the localisation of S100A9 (cytoplasmic, nuclear, membrane) in the malignant cells and was supported by a strong inverse correlation of the genes in a gene expression profile analysis made of 351 breast tumours (R2: microarray analysis and visualisation platform (http://r2.amc.nl; Tumour breast EXPO-351). Even stromal expression of S100A9 correlated not only with the ER^-PgR^- breast cancer phenotype but also to parameters such as larger tumour size, Her2 positivity and nodal stage. Stromal expression of S100A9 was mostly present in cells with a leukocyte morphology and correlated with the presence of anti-inflammatory myeloid cells ($CD163^+$), but not with the macrophage marker CD68, in the same tumours. This support previous findings that S100A9 is expressed by MDSCs (Ostrand-Rosenberg and Sinha, 2009). In this context, it is interesting to note that we previously have shown that anti-inflammatory myeloid cells ($CD163^+$) are preferentially enriched in the stromal areas of breast tumours of the TN subtype. Cytoplasmic S100A9 expression did not, however, correlate to Her2 in our breast cancer cohort. Instead we found that, out of the 11 $Her2^+$ tumours, all (100%) expressed cytosolic S100A9 in the malignant cells. The same was true for $EGFR^+$ tumours ($n=12$). The fact that no correlation can be found between S100A9 expression and Her2 or EGFR positivity is most likely due to the small amount of tumours with this particular subtype. This is supported by the gene expression profile analysis that showed a strong positive correlation between S100A9 and either ERBB2 (Her2) or EGFR at the gene expression level, as well as our *in vitro* analysis of breast cancer cell lines with this phenotype. A link between S100A9 expression and EGFR has previously been published regarding bladder cancer (Kim *et al*, 2011, 2014). Our findings that S100A9 expression correlates to ER^-PgR^- and $Her2^+$ or $EGFR^+$ breast tumours is also supported by a large protein profiling study performed on breast cancers (Goncalves *et al*, 2008).

The effect of S100A9 expression on primary breast cancer patient survival has not been studied in depth before, but the expression of S100A9 in metastatic lesions has been shown to correlate with a worse prognosis (Acharyya *et al*, 2012) and cytoplasmic S100A9 expression in malignant breast epithelial cells was correlated to worse overall survival, although it was concluded that this might be due to its correlation with other parameters such as grade, ER or ERBB2 status (Goncalves *et al*, 2008). In our study, patients having positive (any) cytoplasmic S100A9 expression were found to have a significant reduction in overall survival, where the main discrepancy was seen for patients having a minor increase in expression, and this was also seen for ER^+PgR^+ tumours. Even more accentuated, a similar trend was seen for stromal S100A9 expression, where a higher expression was progressively associated with a reduced overall survival. This result fits very well to what was found when we analysed the presence of stromal $CD163^+$ anti-inflammatory myeloid cells previously, again indicating that stromal S100A9 expression is caused by myeloid cells primarily (Medrek *et al*, 2012). In summary, this suggests that S100A9 can be expressed both in malignant cells and in myeloid cells in breast tumours. It is most likely so that S100A9 might have different roles for these cell types, but according to this study also similar functions are expected. This would primarily concern induction of an inflammatory environment. When the chemo-attractive effects on MDSCs are added to these processes, it is not surprising that both a stromal as well as malignant S100A9 expression pattern will affect breast cancer patient's overall survival. With these data, we propose that S100A9 would be a good future candidate drug target for patients with ER^-PgR^- breast cancer, especially in patients with tumours that express Her2 or EGFR.

ACKNOWLEDGEMENTS

We thank Ms. Elise Nilsson for professional technical skills in preparation of TMAs and IHC. This work was supported by grants from the Swedish Research Council, The Swedish Cancer Foundation, Kocks Stiftelser, Österlunds Stiftelse, Gunnar Nilsson Cancer Foundation, MAS Cancer Foundation and Åke Winbergs Foundation.

CONFLICT OF INTEREST

TL is a part-time employee at Active Biotech that develops S100A9 inhibitors for the treatment of autoimmune diseases and cancer.

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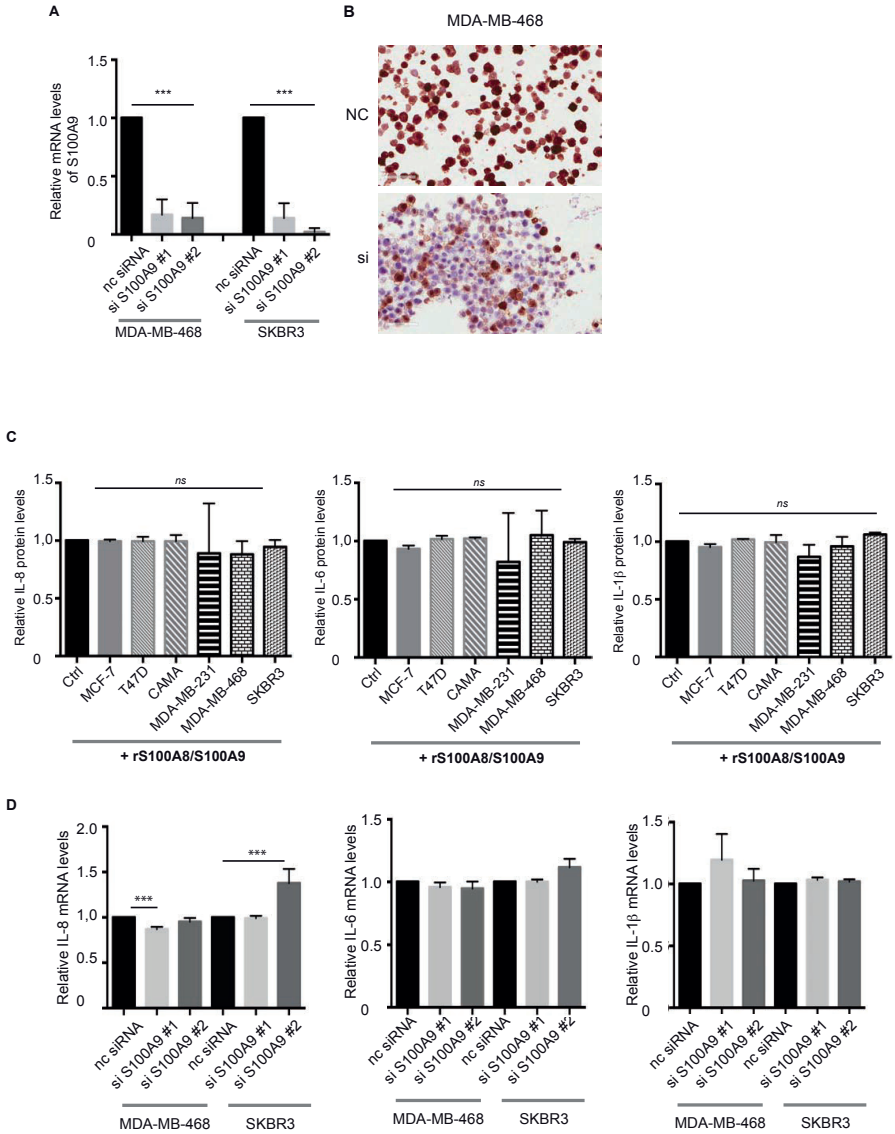
Supplemental Figure 1

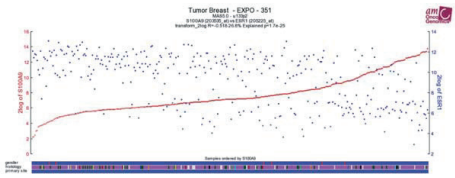


Supplemental Figure 2

Gene expression profile correlation analysis between S100A9 expression and EGFR or Her2 in human breast cancers

A

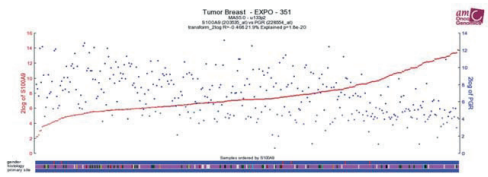
ESR1/ S100A9



r-value -0.518 p-value 1.7e-25

B

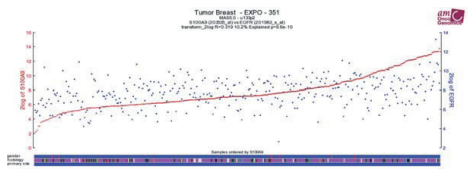
PGR/ S100A9



r-value -0.468 p-value 1.8e-20

C

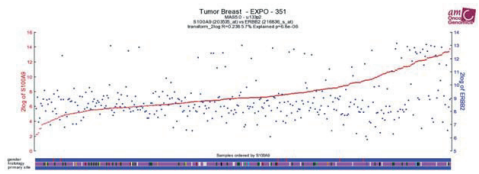
EGFR/ S100A9



r-value 0.319 p-value 9.6e-10

D

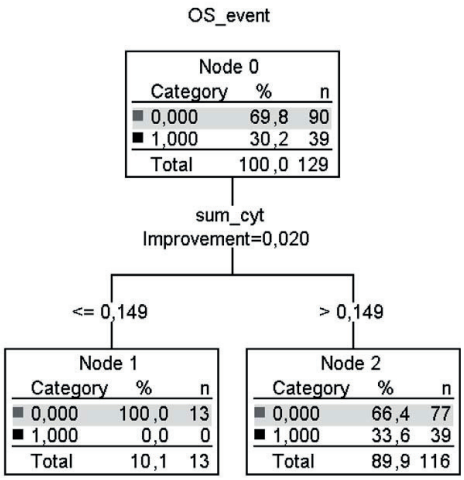
ERBB2/ S100A9



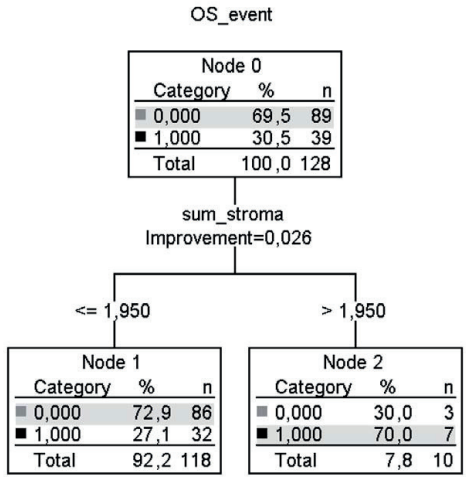
r-value 0.238 p-value 6.8e-06

Supplemental Figure 3

A



B



Paper III

nature COMMUNICATIONS

ARTICLE

Received 29 Jun 2016 | Accepted 22 Aug 2016 | Published 11 Oct 2016

DOI: 10.1038/ncomms13050 OPEN

Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers

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Triple-negative (TN) breast cancers ($ER^-PR^-HER2^-$) are highly metastatic and associated with poor prognosis. Within this subtype, invasive, stroma-rich tumours with infiltration of inflammatory cells are even more aggressive. The effect of myeloid cells on reactive stroma formation in TN breast cancer is largely unknown. Here, we show that primary human monocytes have a survival advantage, proliferate *in vivo* and develop into immunosuppressive myeloid cells expressing the myeloid-derived suppressor cell marker S100A9 only in a TN breast cancer environment. This results in activation of cancer-associated fibroblasts and expression of CXCL16, which we show to be a monocyte chemoattractant. We propose that this migratory feedback loop amplifies the formation of a reactive stroma, contributing to the aggressive phenotype of TN breast tumours. These insights could help select more suitable therapies targeting the stromal component of these tumours, and could aid prediction of drug resistance.

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Breast cancer is the most common cancer among women today and the prognosis is dependent not only on the stage of disease at detection, but also on the type of breast cancer. Breast cancers can be divided into several subtypes, mainly based on expression of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Using global gene expression profiling, breast cancers can be further categorized into molecular subtypes including the basal-like and luminal subtypes[1]. Triple-negative breast cancers ($ER^-PR^-HER2^-$; TNBC) constitute a heterogeneous group of breast cancers that largely coincide with the basal-like subtype. TNBCs are highly metastatic tumours with a poor prognosis and there are few treatment options for patients with these cancers[2]. Infiltration of inflammatory cells or the presence of a stroma with reactive, invasive properties, have been associated with poor prognosis in patients with TNBC[3–5]. Furthering our understanding of the role of the tumour stroma and inflammatory cells in TNBC will help elucidate how the tumour microenvironment may contribute to disease progression, drug resistance or may enable treatments to be tailored to patients more effectively.

The tumour microenvironment is composed of extracellular matrix (ECM) and non-malignant stromal cells including fibroblasts, pericytes, immune cells and endothelial cells. The cells of the tumour microenvironment communicate via soluble mediators or intercellular receptor-ligand interactions. Cancer-associated fibroblasts (CAFs), pericytes and innate immune cells, especially tumour-associated macrophages (TAMs), are the main cell types constituting the tumour stroma. It is generally thought that CAFs are recruited from resident fibroblasts or bone marrow-derived progenitor cells (BMDCs), or trans-differentiated from mesenchymal or tumour-derived cells[6]. These cells are then activated by factors in the tumour microenvironment, such as TGF-β, to become myofibroblasts (αSMA^+/$vimentin^+$) that promote invasion and metastasis. How CAFs are recruited and activated is still under intense investigation[7–9].

Monocytes are immune cells of the myeloid lineage that are plastic by nature and can give rise to macrophages, dendritic cells and probably also monocytic-myeloid-derived suppressor cells (MDSCs)[10,11]. Tumour-infiltrating myeloid cells, particularly TAMs and MDSCs negatively affect survival in breast cancer patients[12–16]. This negative effect has been ascribed to their immunosuppressive roles and their effects on tumour cell invasion and angiogenesis[7,17]. Both monocytes and BMDCs can promote metastasis to distant sites[18,19]. We have previously shown that a subpopulation of anti-inflammatory myeloid cells ($CD163^+$) is present in the tumour stroma of TN breast tumours and is associated with unfavourable clinicopathologic features[4]. However, the effects of myeloid cells on stroma formation in TN breast tumours have not been investigated in detail.

Stroma interactions and the effects on tumour development and progression are complex, and it is therefore important to understand the intricate networks within specific tumour types and the cells of their particular tumour microenvironment[20]. In 2011, Elkabets *et al.* showed that mouse BMDCs could promote stroma formation in TNBCs, specifically by recruitment or activation of non-bone marrow-derived fibroblasts via secreted granulin (GRN)[19]. Using the same human breast cancer cohort as Elkabets *et al.*, we have shown that the presence of myeloid $CD163^+$ cells in the stromal areas of human TN breast tumours specifically correlates with these GRN expressing cells[4].

In this study, we investigated whether myeloid cells could affect stroma formation in breast cancer. We show that primary human monocytes, co-transplanted with either luminal A or TNBC cells in highly immunodeficient NSG-mice, differentiated into $CD163^+$ myeloid cells and promoted an increased stroma formation in both tumour types. However, only the TNBC/monocyte co-transplants developed immunosuppressive $CD163^+$ myeloid cells, and were able to activate fibroblasts. The findings were validated in patient-derived xenografts (PDXs) as well as syngeneic breast cancer models of luminal and TNBC. Interestingly, we also found that the monocyte chemoattractant CXCL16 was induced in primary fibroblasts cultured under TNBC/monocyte conditions *in vitro*. In line with these data, primary CAFs isolated from TN tumours specifically expressed CXCL16, a finding that was supported by analysis of a human breast cancer RNAseq data set and a human breast cancer tissue microarray. Our data indicate that in a TNBC environment, myeloid cells can activate the stromal fibroblasts to express CXCL16 that, in turn, recruits more myeloid cells and fibroblasts. On the basis of these findings, we propose that drugs targeted at immunosuppressive myeloid cells, or the circuits mediated by them, such as immune checkpoint inhibitors or immunomodulatory drugs, should be investigated to treat TN breast tumours. Our findings will be important for predicting drug resistance and outcome in patients treated with drugs targeting tumour-infiltrating myeloid cells in the future.

Results

Immunosuppressive myeloid cells in TNBC xenografts. To investigate whether myeloid cells can promote stroma formation in TN tumours, or if the stroma of TN tumours secretes factors that attract $CD163^+$ myeloid cells, we first generated breast cancer xenograft models. To that end, we co-xenotransplanted primary human monocytes (Mo; the proposed precursors of $CD163^+$ myeloid cells) from healthy blood donors together with human breast cancer cells of the luminal A (MCF-7 or T47D cells) or TN (MDA-MB-231 or SUM-159 cells) subtypes in highly immunodeficient NSG-mice. These cell lines were chosen since they lack endogenous expression of the myeloid-derived suppressor cell marker, S100A9 (ref. 21), and the NSG-mice were chosen since they are deficient in T-, B- and NK-cells and also have defective macrophages and dendritic cells, but allow engraftment of functional human myeloid cells[22].

Tumours that formed were excised, and their biological characteristics, their myeloid cell content and stroma formation were analysed by immunohistochemistry (IHC) (Supplementary Tables 1 and 2, Figs 1 and 2 and Supplementary Figs 1 and 2). Grafted monocytes (expression of human myeloid markers CD11b and CD163 and very weak expression of human macrophage marker CD68; Fig. 1, Supplementary Tables 1 and 2, Supplementary Figs 1 and 2A) were present in the xenografts at the time of dissection (day 21 for MDA-MB-231, T47D and SUM-159 grafts; day 21 and day 90 for MCF-7 grafts). The presence of $CD163^+$ or $CD68^+$ cells was significantly higher in TN as compared with luminal A xenografts (Supplementary Tables 1 and 2). The luminal T47D/monocyte grafts showed the lowest density of $CD11b^+$ cells and lacked $CD163^+$ cells, whereas the TN SUM-159/monocyte grafts had the highest density of $CD163^+$ cells (Supplementary Fig. 1). In addition, mouse macrophages (F4/80 staining Supplementary Fig. 2B), human myeloid dendritic cells (CD208) and human fibroblasts (CD90) were all absent (Supplementary Fig. 2B). Gr-1, a mouse myeloid cell marker, was present only at low levels in some xenograft sections (Supplementary Fig. 2C). Of importance, the myeloid-derived suppressor cell marker S100A9 was expressed solely in the myeloid cells that had been co-transplanted with TNBC cells (Supplementary Tables 1 and 2), indicating that these transplanted myeloid cells had acquired an immunosuppressive character[16] (Figs 1, 2a, Supplementary Figs 1 and 2A).

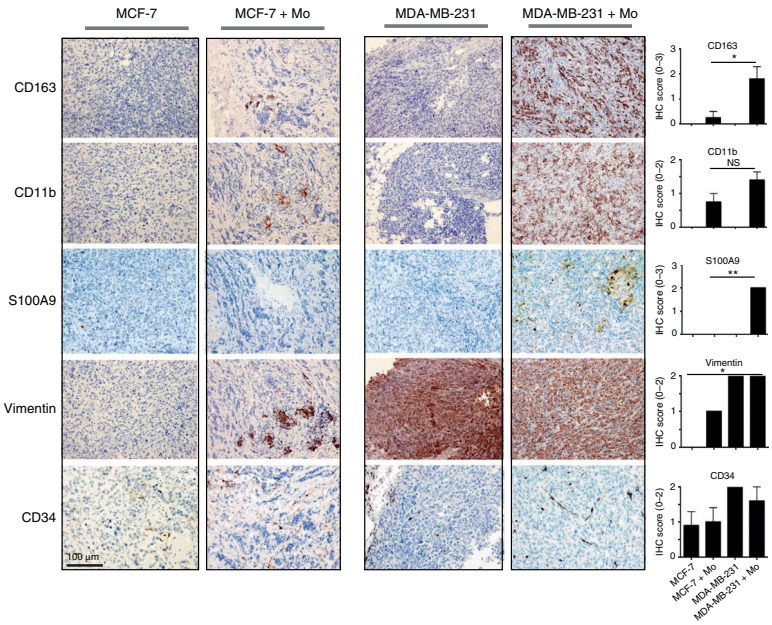

Figure 1 | IHC of xenografts. Tumour xenografts consisting of TN MDA-MB-231 breast cancer cells co-transplanted with primary human monocytes, express more myeloid-related and immunosuppressive markers than luminal A MCF-7/monocyte xenografts. The xenografts were grown in highly immunodeficient NSG-mice (see 'Methods' section), and sections from the tumours were stained with myeloid (CD163, CD11b, S100A9) tumour (vimentin) and endothelial markers (CD34). The two cell lines chosen are negative for S100A9 (ref. 21). IHC was performed using the indicated antibodies. All histological sections were counterstained with HE. $N=5$ mice were analysed for each group; MCF-7 grafts were analysed on day 21 and 90 post-graft with similar results—day 90 is shown here; TN MDA-MB-231 grafts were grown to day 21 only. The histograms show the mean value for each IHC score with statistical analyses. IHC scores are shown in Supplementary Table 1. $^{*}=P<0.05$, $^{**}=P<0.01$ ANOVA non-parametric Kruskal–Wallis test. $N=5$. Error bars indicate s.e.m.

Vimentin is normally expressed by MDA-MB-231 breast cancer cells, but not by MCF-7 cells. In the MCF-7 co-transplants, the vimentin staining is predominantly in the co-transplanted myeloid cells (Fig. 1), as supported by immunofluorescence (Supplementary Fig. 3A), IHC (Supplementary Fig. 3B) and western blotting (Supplementary Fig. 3C). Both MCF-7 and MDA-MB-231 monocyte co-transplants had slightly increased levels of the mouse endothelial marker CD34, suggesting that monocytes promote angiogenesis equally well in luminal A and TN breast xenografts (Fig. 1). Taken together, these data suggest that grafted monocytes survive transplantation in both luminal A and TN breast xenografts. However, myeloid cells were numerous in the TN xenotransplants and expressed the myeloid immunosuppressive marker, S100A9, only in the TN xenotransplants.

Monocytes survive and proliferate in a TNBC environment. We next investigated why the proportion of $CD11b^{+}CD163^{+}$ myeloid cells was high in the TN co-transplant tumours. In general, the monocyte co-transplanted tumours were smaller than the corresponding tumour cell-only transplants, and in one case even failed to grow (see Supplementary Tables 1 and 2) with the exception of the TN SUM-159 co-transplants that increased significantly in size in the presence of monocytes (Supplementary Table 2 and Fig. 3a,b). We used the Ki67 proliferation marker to show that the monocytes in the TN, but not the luminal tumours, were actively proliferating (Fig. 3c). This observation was supported by the fact that primary human monocytes proliferated when cultured in conditioned medium from MDA-MB-231 or SUM-159 cells, but did not proliferate in MCF-7- or T47D-conditioned medium as compared with control medium (Fig. 3d). Conditioned medium from a third TN cell line (MDA-MB-468 cells) did not induce monocyte proliferation (Fig. 3d). To evaluate monocyte survival in different tumour microenvironments, we cultured primary human monocytes in conditioned medium from five different breast cancer cell lines; three TN (MDA-MB-231, MDA-MB-468 and SUM-159) and two luminal A (MCF-7 and T47D). Indeed, survival of monocytes was significantly increased in the TNBC supernatant cultures, as measured by staining with the apoptosis markers annexin V and 7AAD, compared with in the luminal A breast cancer conditioned medium cultures (Fig. 3e and Supplementary Fig. 4A). As we have shown previously[23], both primary human monocytes and M2 macrophages migrated significantly more towards conditioned medium from TN cells than from luminal A cells (Fig. 3f and Supplementary Fig. 4B). Hence, our data indicate that the TN tumour microenvironment promotes increased survival and proliferation of co-transplanted myeloid cells.

Soluble mediators in monocyte and TNBC cell co-cultures. To search for soluble factors that might be critical for the increased

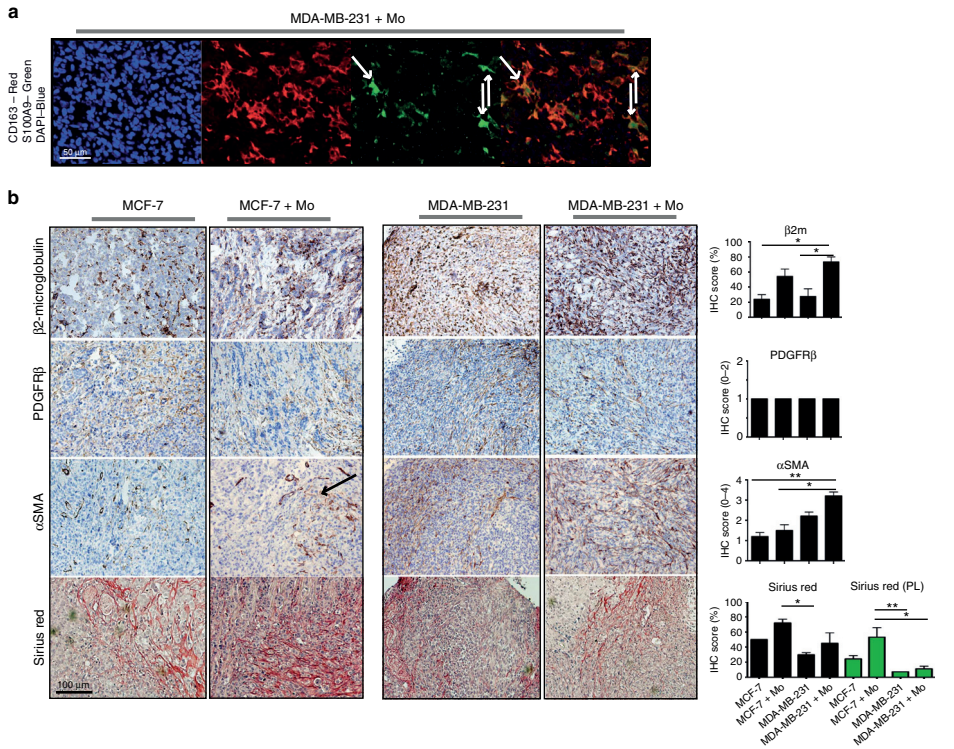

Figure 2 | IHC of xenografts. Tumour xenografts consisting of TN MDA-MB-231 breast cancer cells co-transplanted with primary human monocytes, express more activated stromal markers than luminal A MCF-7/monocyte xenografts. Xenografts were grown in highly immunodeficient NSG-mice (see 'Methods' section). (**a**) Immunofluorescence staining of CD163 (TRITC, red) and S100A9 (FITC, green) in sections from MDA-MB-231/monocytes xenograft tumours (day 21). DAPI (blue) shows nuclear staining. Overlay of colours is shown in lower right panel. S100A9 expression is located in the $CD163^+$ human myeloid co-transplanted cells. White arrows indicate three cells with co-expression of $CD163^+$ and S100A9. The cell lines are negative for S100A9 (ref. 21). (**b**) IHC was performed using the indicated antibodies and histological stains. All histological sections were counterstained with HE. $N=5$ mice were analyzed for each group; The black arrows indicate activated stroma. MCF-7 grafts were analysed on day 21 and 90 post-graft with similar results—day 90 is shown here; TN MDA-MB-231 grafts were grown to day 21 only. The histograms show the mean value for each IHC score with statistical analyses. IHC scores are shown in Supplementary Table 1. * = $P<0.05$, ** = $P<0.01$ ANOVA non-parametric Kruskal–Wallis test. $N=5$. Error bars indicate s.e.m.

monocyte survival and proliferation in the TN grafts, we compared proteome arrays conducted on supernatants collected before and after co-culture with monocytes, using either TN (MDA-MB-231, MDA-MB-468 and SUM-159) or luminal A (MCF-7 and T47D) breast cancer cells (Supplementary Fig. 4C–E). The TNBC cell line supernatants collected before monocyte co-cultures showed a typical expression pattern of chemokines, angiogenesis and invasion related proteins (Supplementary Fig. 4C; purple box), whereas the luminal A breast cancer cells (blue box) expressed fewer factors. The proteins that were more upregulated in TNBC cell lines/monocyte co-cultures than in luminal A/monocyte co-cultures were GM-CSF, MMP9, endothelin-1 and CXCL4 (platelet factor 4), and as shown previously, levels of IL-8 and CCL2 increased when monocytes were added to the TN breast cell lines and T47D cells[24] (Supplementary Fig. 4D,E; green and pink box). Thus, the TN tumour cell environment, alone or together with monocytes, harbour important myeloid cell survival, proliferation and differentiation factors.

Monocytes induce stroma formation. To examine whether co-transplanted monocytes would affect stroma formation or activation, we next analysed the stromal component of the tumour xenografts. Recruited cells of mouse origin were present in all xenografts (mouse β2-microglobulin staining; Fig. 2), but the TN co-transplants had the highest number of recruited mouse cells (Supplementary Table 1). The recruited cells were of fibroblast origin since they expressed the fibroblast marker PDGFRβ (Fig. 2 and Supplementary Table 1).

As activated fibroblasts are central players in the tumour microenvironment we also analysed whether the stromal fibroblasts were activated in the tumours using the marker αSMA (Supplementary Tables 1 and 2, Fig. 2 and Supplementary Fig. 1).

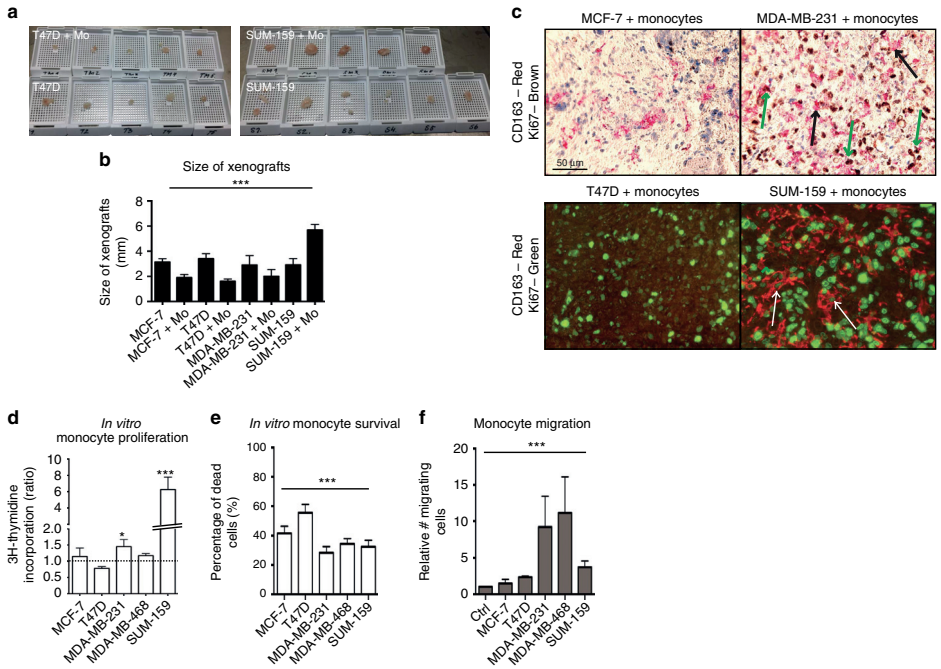

Figure 3 | Characterization of primary monocytes in TNBC cultures. Primary human monocytes show increased proliferation, survival and migration in a TNBC/monocyte environment than in a Luminal A/monocyte context. (**a**,**b**) The size of the xenografts differed between groups. Co-transplantation of monocytes generally decreased the tumour size slightly (**b**) except in the TN SUM-159/monocyte group, co-transplantation of monocytes (SUM-159 + Mo) increased tumour growth significantly (**a**,**b**). *** = $P<0.001$ ANOVA. $N=5$. (**c**) Myeloid cells proliferate *in vivo*. Upper panel: Double staining IHC of CD163 and Ki67 in xenografts from MCF-7/monocytes co-transplant (left) or MDA-MB-231/monocyte co-transplants (right) tumours as indicated. Black arrows show single staining with only CD163 and green arrows show double staining. Lower panel: Double staining immunofluorescence of CD163 and Ki67 in xenografts from T47D/monocytes co-transplant (left) or SUM-159/monocyte co-transplants (right) tumours as indicated. White arrows show $CD163^+$ cells with a clear nuclear Ki67 staining. (**d**) Proliferation of primary human monocyte cultured in control medium or in conditioned medium from different cell lines, was assessed using the thymidine incorporation assay. * = $P<0.05$ ANOVA. $N=14$. (**e**) Survival of isolated human primary monocytes in breast cancer cell conditioned medium, grown for 7 days, was assessed. Annexin V staining was performed to analyse the total content of apoptotic/dead cells. *** = $P<0.001$. ANOVA. $N=5$. Error bars indicate s.e.m. (**f**) Boyden chamber migration assay of primary human monocytes migrating towards control medium or MCF-7, T47D, MDA-MB-231, MDA-MB-468 or SUM-159 breast cancer cell conditioned medium. *** = $P<0.001$ ANOVA. $N=5$. Error bars indicate s.e.m.

MCF-7 and T47D tumours that had been co-transplanted with monocytes showed a very modest, if any increase in fibroblast αSMA expression (Fig. 2, Supplementary Fig. 1 and Supplementary Tables 1 and 2). By contrast, the MDA-MB-231 and SUM-159 tumours that had been co-transplanted with monocytes showed a major increase in fibroblast αSMA expression (Fig. 2, Supplementary Fig. 1 and Supplementary Tables 1 and 2). These fibroblasts also showed the typical spindle shaped morphology in the TN reactive stroma (Fig. 2). Since the αSMA antibody recognizes both human and mouse αSMA, we co-stained with the human myeloid cell markers CD163 as well (Supplementary Fig. 5A). Most αSMA^+ cells were CD163 negative and therefore of mouse origin (black arrows), although a few of the αSMA^+ cells were of human origin and stained positive for CD163 (green arrows; Supplementary Fig. 5A). The αSMA^+ cells did not stain for the mouse myeloid marker Gr-1 (Supplementary Fig. 2C). Altogether, this indicates that the majority of αSMA^+ cells are activated mouse fibroblasts, and that they are significantly increased in the TN xenografts co-transplanted with monocytes.

The tumour stroma in TN PDX and syngeneic models. To verify that our findings were applicable in other preclinical models, we next generated PDXs using tumour tissue from one luminal (HCI-011) and four TN (HCI-001, 002, 004 and 010) breast cancers (Fig. 4). We also generated syngeneic mouse tumours from one luminal (67NR) and one TN (4T1.13) breast cancer cell line (Supplementary Fig. 5B,C). As indicated in Fig. 4, there were significantly more myeloid cells of mouse origin (Ly6C; Fig. 4a,b), significantly more S100A9-expressing cells of mouse origin (Fig. 4a,b) and significantly more activated fibroblasts as indicated by the increased αSMA staining (Fig. 4a,b) in the TN PDX grafts as compared with the luminal PDX graft.

Similarly, Ly6C, mouse S100A9 and αSMA expression were increased in the TN as compared with the luminal grafts in the

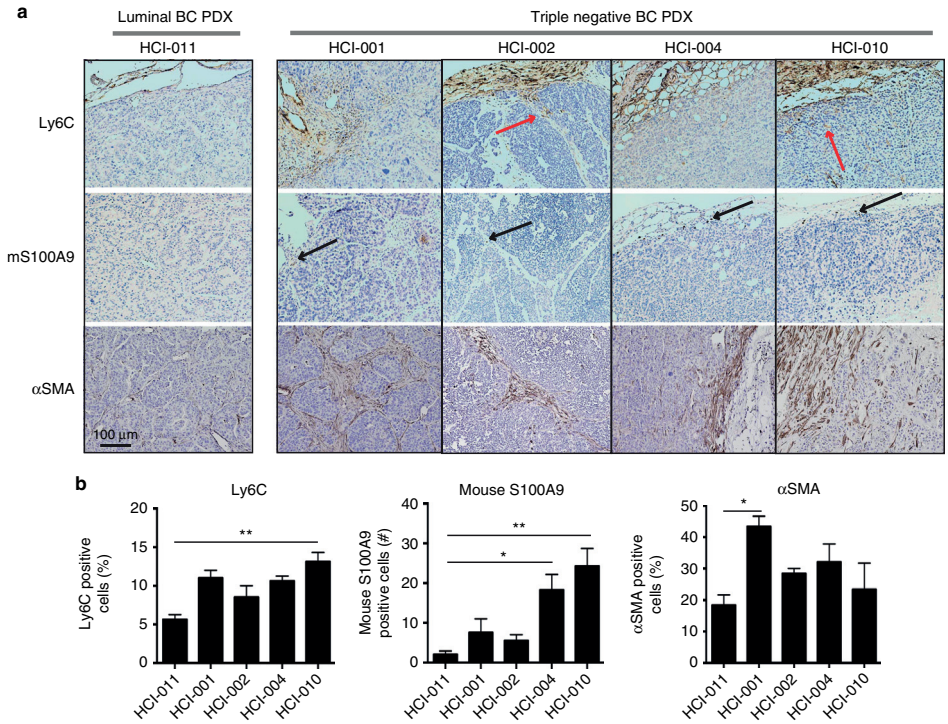

Figure 4 | IHC of PDXs. PDXs from TN breast tumours show increased myeloid cell infiltration, expression of S100A9 and activated fibroblasts. PDXs from one luminal (HCI-011) and four TN (HCI-001, HCI-002, HCI-004 and HCI-010) breast cancers grown in non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice were analysed for presence of monocytes (Ly6C mouse specific), expression of mouse S100A9 (mouse specific) and activated fibroblast (αSMA; recognizes human and mouse). (**a**) Ly6C positive cells were present in the tumour borders, with some cells infiltrating the stromal areas in particular (red arrows). Mouse S100A9 positive cells were present in the tumour borders and areas where myeloid cells were also present, and importantly mainly in the TN PDX grafts (black arrows). αSMA was expressed in the stromal areas of the PDX grafts, representing activated fibroblasts. (**b**) Quantitation of the immunohistochemical stains as presented by the histograms showing the mean value for each protein. For each PDX graft, 2–4 sections were stained and scored ($N=3$–6). For αSMA and Ly6C the percentage (%) of positive cells was scored and for mouse S100A9 the numbers (n) of infiltrating cells expressing S100A9 was scored. A significantly higher level of Ly6C positive cells (left), of mouse S100A9 positive cells (center) and of αSMA (right) was seen in the TN PDX grafts as compared with the luminal PDX graft. $^{*}=P<0.05$ $^{***}=P<0.001$. ANOVA non-parametric Kruskal–Wallis test. $N=(3$–$6)$. Error bars indicate s.e.m.

syngeneic model systems (Supplementary Fig. 5B,C). The increased expression of S100A9 in the TN grafts has previously been reported to associate with an increased metastatic property in the TN 4T1.2 as compared with luminal 67NR grafts[25]. Indeed, when mRNA expression profiles of the syngeneic grafts were analysed, also *ACTA2* (αSMA) was significantly increased in whole tumour all exon array data of 4T1.2 versus 67NR tumours (Supplementary Table 3)[25]. S100A9 mRNA was not upregulated *in vitro*, a finding that might indicate *in vivo* requirements for S100A9 expression or be explained by its post-transcriptional regulation[26].

Less classical collagen depositions in TN xenografts. Collagen is the major component of the ECM and the tumour micro-environment actively promotes degradation and re-deposition of collagen to promote tumour progression[27]. Collagens can be divided into fibrillar (for example, Type I, II, III, V) and non-fibrillar collagens (for example, Type IV and VI). Collagen IV is a basement membrane collagen and collagen VI is a beaded filament-forming non-classical collagen that is associated with ECM[28]. The main collagens present in tumours are Type I, III, IV and VI (refs 27,29). Myeloid cells play an important role in the ECM remodelling by producing matrix metalloproteinases (MMPs) that degrade collagen[30].

To investigate the effects of co-transplanted myeloid cells on the collagen content in our xenografts, we used the Sirius Red stain (Supplementary Tables 1 and 2, Fig. 2, Supplementary Figs 1 and 6A,B). Under bright field microscopy (Fig. 2, Supplementary Figs 1 and 6A,B) Sirius Red detects collagens of type (I, III and IV) and using polarized birefringent light microscopy only collagens of type I and III are detected (red/green/yellowish staining; Supplementary Fig. 6A,B)[31]. We found that, co-transplantation of monocytes slightly increased the collagen deposition in all of the grafts (Supplementary Tables 1 and 2; Fig. 2, Supplementary Figs 1 and 6A,B). The MCF-7/monocyte co-transplants on day 21 and day 90 showed robust collagen

deposition (Supplementary Table 1; red colour, Fig. 2 (day 90); red colour and green/yellow, Supplementary Fig. 6A,B (day 21 and 90)). The T47D/monocyte co-transplants showed a similar collagen deposition (Supplementary Table 2 and Supplementary Fig. 1), whereas the TN MDA-MB-231/monocyte and SUM-159/monocyte co-transplants showed a larger variation between tumours (25–75% stroma Supplementary Tables 1 and 2; red colour, Fig. 2, Supplementary Figs 1 and 2A). Interestingly, despite the high number of fibroblasts in the TN MDA-MB-231/monocyte and SUM-159/monocyte xenografts (Fig. 2 and Supplementary Fig. 1), they expressed significantly less collagens of type I and III, compared with the MCF-7/monocyte and T47D/monocyte co-transplants (Supplementary Tables 1 and 2; red/green/yellowish pictures; Supplementary Fig. 6A,B). A low expression of collagens type I and III in TNBCs, specifically, was also verified in a tissue microarray consisting of 144 human breast cancers, where a low birefringent light of Sirius Red viewed in polarized light correlated significantly to TN breast tumours (Table 1; $P=0.036$). Bright light Sirius Red staining of collagen deposition did not correlate to TNBCs, but showed a negative correlation to both tumour size ($P=0.003$) and Nottingham histologic grade (NHG) status ($P=0.002$) (Table 1); Spearman's Rho analysis using SPSS software.

The non-classical beaded filament collagen VI was expressed primarily in the TN MDA-MB-231/monocyte and SUM-159/monocyte xenografts, but also to some extent in the SUM-159 xenografts (Supplementary Tables 1 and 2; Supplementary Fig. 7A)[28]. The collagen VI deposits in the MDA-MB-231/monocyte xenografts probably came from the transplanted myeloid cells, since primary human monocytes cultured in conditioned medium from TN MDA-MB-231 and MDA-MB-468, but not MCF-7 and interestingly not SUM-159 breast cancer cells, upregulate collagen VI to a similar level as the control M2 macrophages (Supplementary Fig. 7B,C)[28]. Primary mouse fibroblasts cultured in conditioned medium from breast cancer cells, or from co-cultures of breast cancer cells and primary monocytes, did not express more collagen VI than mouse fibroblasts grown in normal medium (Supplementary Fig. 7B,C). Monocytes cultured in conditioned medium from the luminal T47D breast cancer cells also induced collagen VI, indicating that it might be a more general breast cancer cell inducing mechanism, unrelated to the TNBC subtype (Supplementary Fig. 7B,C).

These findings suggest that the tumour type will direct the myeloid cells differently in TN as compared with luminal A tumours, so that myeloid cells will produce both collagen degrading MMPs and perhaps also anti-inflammatory collagen VI in TN tumours, thus promoting their invasiveness[28]. Thus, although the TN MDA-MB-231/monocyte and SUM-159/monocyte co-transplants had more fibroblasts as judged by IHC (Supplementary Tables 1 and 2, Fig. 2 and Supplementary Fig. 1) these fibroblasts did not produce more of the classical collagens.

Primary fibroblasts are activated by monocytes in TN tumours. We next investigated what might facilitate the high number of mouse fibroblasts seen in the TN MDA-MB-231/monocyte and SUM-159/monocyte co-transplants. To this end, we performed primary mouse fibroblast migration, survival and proliferation assays *in vitro*, to compare the effect of TN/monocyte and luminal/monocyte conditioned medium.

Scratch wound assays revealed that primary mouse fibroblasts migrated equally well in supernatants from all culture conditions (Fig. 5a and Supplementary Fig. 7D), while a significant survival advantage was seen only in the primary mouse fibroblasts

Table 1 | Analysis[a] of CXCL16 and Collagen expression in a breast cancer tissue microarray.

	Sirius Red	Low birefringent Sirius Red (PL)	CXCL16 malignant cells	CXCL16 fibroblasts
TNBC				
Correlation coefficient	−0.142	0.178*	−0.025	0.231**
Sig. (2-tailed)	0.104	0.036	0.779	0.010
N	132	139	129	123
Tumour size				
Correlation coefficient	−0.263**	0.130	−0.099	−0.088
Sig. (2-tailed)	0.003	0.122	0.256	0.326
N	136	144	133	126
Node status				
Correlation coefficient	−0.028	−0.093	−0.182*	−0.057
Sig. (2-tailed)	0.759	0.294	0.048	0.546
N	122	129	119	115
NHG				
Correlation coefficient	−0.257**	0.031	−0.137	−0.098
Sig. (2-tailed)	0.002	0.715	0.117	0.277
N	136	144	133	126
Sirius Red				
Correlation coefficient	—	−0.291**	−0.035	0.032
Sig. (2-tailed)		0.001	0.692	0.723
N		136	132	125
Low birefringent Sirius Red (PL)				
Correlation coefficient	−0.291**	—	0.283**	0.220*
Sig. (2-tailed)	0.001		0.001	0.013
N	136		133	126

[a]Spearman's Rho analysis using SPSS software. *$P<0.05$; **$P<0.01$; ***$P<0.001$.
Correlation between expression levels and clinical parameters in a breast cancer tissue microarray ($N=144$) of CXCL16 in malignant cells (0–3), CXCL16 in fibroblasts (0–1), Sirius Red (0–3) or Low birefringent in polarized light (PL) Sirius Red (Birefringent in PL (0)—No birefringent in PL (1)). For scoring see 'Methods'.

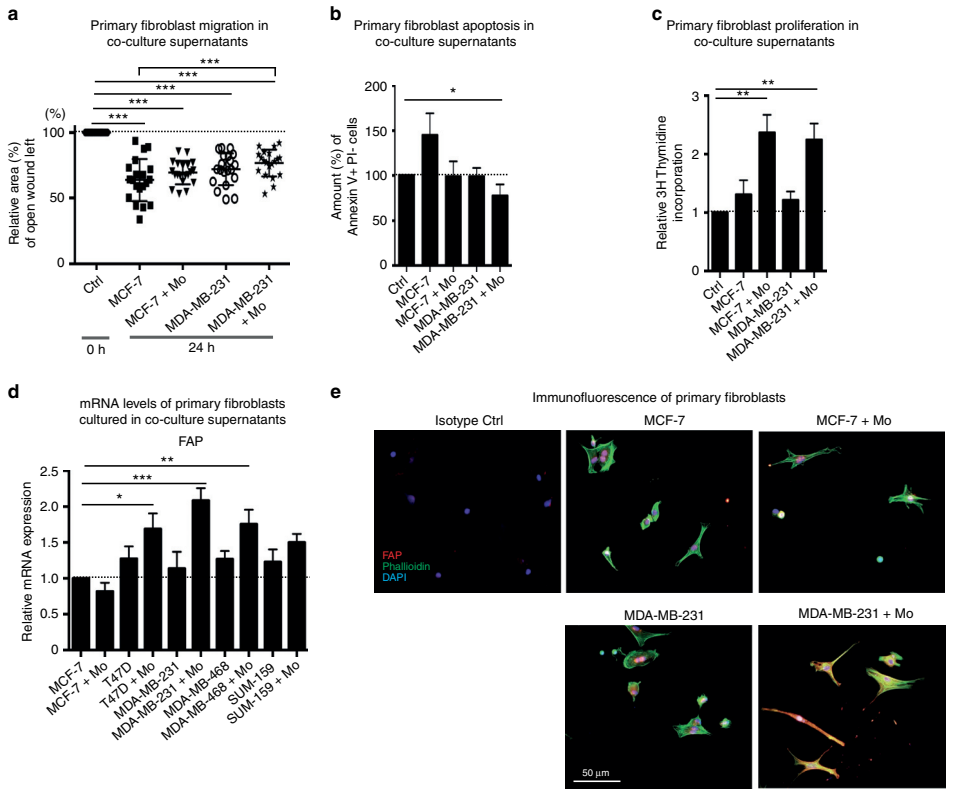

Figure 5 | Characterization of primary fibroblasts in TNBC cultures. Primary mouse fibroblasts are activated by monocytes in the TNBC context *in vitro*. (**a**) Scratch wound assays showing mouse primary fibroblast migration in supernatants derived from co-cultures of human primary monocytes (Mo) and luminal A (MCF-7) or TN (MDA-MB-231) breast cancer cells. *** = $P<0.001$ ANOVA non-parametric Kruskal–Wallis test. $N=20$. (**b**) Survival analysis of mouse primary fibroblast grown in supernatants derived from co-cultures of human primary monocytes and luminal A (MCF-7) or TN (MDA-MB-231) breast cancer cells. Annexin V staining was used to analyse the percentage of apoptotic cells. * = $P<0.05$ ANOVA. $N=10$. (**c**) Proliferation of mouse primary fibroblasts grown in supernatants derived from co-cultures of human primary monocytes and luminal A (MCF-7) or TN (MDA-MB-231) breast cancer cells, measured using a thymidine incorporation proliferation assay. ** = $P<0.01$. ANOVA Dunnett's multiple comparison test. $N=14$. (**d**) mRNA expression levels of FAP in mouse primary fibroblasts cultured in supernatants derived from co-cultures of human primary monocytes and luminal A (MCF-7 and T47D) or TN (MDA-MB-231, MDA-MB-468 and SUM-159) breast cancer cells, assessed by RT-QPCR analysis. * = $P<0.05$ ** = $P<0.01$ *** = $P<0.001$ ANOVA Dunnett's multiple comparison test. $N=4$. (**e**) Immunofluorescence of anti-fibroblast activation protein (FAP; red), phalloidin to stain actin filaments (green) and DAPI (nuclear stain; blue) in primary mouse fibroblasts cultured in supernatants derived from co-cultures of primary human monocytes and luminal A (MCF-7) or TN (MDA-MB-231) breast cancer cells. Scale bar represents 50 µm. Error bars indicate s.e.m.

cultured in TN MDA-MB-231/monocyte and SUM-159/monocyte supernatant (Fig. 5b and Supplementary Fig. 7E). Increased proliferation of mouse fibroblasts was seen in MCF-7/monocyte, MDA-MB-231/monocyte and SUM-159 or SUM-159/monocyte cultures (Fig. 5c and Supplementary Fig. 7F), but not in T47D/monocyte or MDA-MB-468/monocyte co-cultures (Supplementary Fig. 7F). These data indicate that fibroblast survival is probably the major cause of the increased presence of fibroblasts in the TN MDA-MB-231/monocyte and SUM-159/monocyte grafts, but that fibroblast proliferation also can be affected significantly by myeloid cells.

The activation status of cultured primary mouse fibroblasts was then investigated by measuring fibroblast activating protein (FAP) levels. Interestingly, we found that primary mouse fibroblasts were activated by all of the TNBC cell/monocyte supernatants, as seen by upregulation of FAP at both mRNA (Fig. 5d) and protein (Fig. 5e) levels, further corroborating our αSMA data presented in Fig. 2 and Supplementary Fig. 1. The luminal T47D/monocyte supernatants also upregulated fibroblast FAP mRNA (Fig. 5d), while fibroblast TGFβ mRNA was induced slightly only by culture in the MDA-MB-231 conditioned medium (Supplementary Fig. 7G). The low numbers of myeloid cells surviving in the luminal T47D xenografts may explain why fibroblast activation was affected only in the *in vitro* cultures of T47D/monocytes supernatants, but not in the corresponding tumours.

Fibroblasts in TN tumours produce CXCL16 specifically. In the experiments mentioned above, we showed that monocytes can promote stroma formation equally well in TN and luminal A tumours, but that the activated fibroblasts are numerous in the TN tumours. This does not explain why CD163$^+$ myeloid cells are more frequent in the stromal areas of TN breast tumours, as we published previously[4]. We therefore looked for myeloid cell chemoattractants that would be produced specifically in fibroblasts from TN breast tumours. We first isolated primary human CAFs from ER$^-$ (TN) and ER$^+$ breast tumour patient samples, cultured them *in vitro* and collected the supernatants. We subsequently performed an angiogenesis protein array and found that CXCL16, amphiregulin and tissue inhibitor of metalloproteinases (TIMP1) were expressed at high levels in the TN but not the ER$^+$ breast cancer CAF supernatants (red box; Fig. 6a). CXCL16 is known to be a T cell chemoattractant, but recently also as a myeloid cell chemoattractant. Amphiregulin is a protein involved in tumour progression and tissue inhibitor of metalloproteinases act to inhibit MMPs[32–36]. IHC of CXCL16 on primary breast tumours revealed that both ER$^+$ and TN tumour cells expressed CXCL16 (black arrow; Fig. 6b), and that fibroblasts in the TN tumour also expressed CXCL16 (red arrow; Fig. 6b).

We next evaluated the levels of secreted CXCL16 using an enzyme-linked immunosorbent assay (ELISA) on supernatants from primary CAFs and showed that CAFs isolated from TN tumours (3/4) secreted high amounts of CXCL16, while low CXCL16 was measured in CAFs isolated from ER$^+$ tumours (0/8) (Fig. 6c). IHC staining of CXCL16 in a tumour tissue microarray consisting of 144 human breast cancers showed that high fibroblast expression of CXCL16, correlated significantly to TN breast tumours ($P=0.010$), while CXCL16 expression in the malignant cells *per se* did not (Table 1). However, only a fraction of the TN tumours expressed CXCL16 in the fibroblasts (Table 2). The high fibroblast CXCL16 expression also correlated to a low birefringent light of Sirius Red ($P=0.013$; Table 1). Boyden chamber migration experiments showed that monocytes (Fig. 6d left) and, to a lesser extent, M2 macrophages (Fig. 6d, right) migrated towards CXCL16 more so than towards another chemokine, CXCL12. We also observed a significant monocyte migration towards ER$^-$ (TN) CAF supernatants (Fig. 6e). The hematoxylin eosin (HE) image (Fig. 6e, right) shows a cytospin of the migrated cells. In support of these findings, we also found that expression of *CXCL16* mRNA was induced in activated primary mouse fibroblasts cultured in TN MDA-MB-231/monocyte or MDA-MB-468/monocyte conditioned medium, but not in conditioned medium from MDA-MB-231 or MDA-MB-468 cell culture without monocytes, nor the luminal cell supernatants (Fig. 6f). The TN SUM-159/monocyte supernatant also increased *CXCL16* mRNA expression significantly, as did the SUM-159 only supernatants (Fig. 6f). Using PDX models, mouse *CXCL16* mRNA originating from infiltrating mouse cells was significantly induced in one of the TN PDX grafts, and increased in the other three TN PDX grafts, as compared with the luminal PDX graft (Fig. 6g). Finally, in the syngeneic models *CXCL16* mRNA was significantly increased in whole tumour all exon array data of 4T1.2 versus 67NR tumours (Supplementary Table 3)[25]. Since CXCL16 can attract T cells, NKT cells, myeloid cells and also fibroblast precursors, these findings are of large importance when it comes to understanding why the tumour stroma in TN breast tumours in particular, attracts immune cells.

Immunosuppressive gene expression profile in TNBCs. To extend our findings from our preclinical models to primary human breast tumours, we analysed RNAseq data from The Cancer Genome Atlas. The tumours were sub-grouped based on the PAM50 centroids and here the basal subgroup largely coincides with TN tumours. While mRNA encoding the myeloid cell marker CD11b (*ITGAM*) was expressed at equal levels in all breast tumour subgroups (Fig. 7a), mRNA of the anti-inflammatory markers *CD163* and *S100A9* were expressed at significantly higher levels in basal than luminal A breast tumours (Fig. 7a). Also, the collagen VI (*COL6A1*)/collagen I (*COL1A1*) mRNA ratio (*COL6A1/COL1A1*) was significantly higher in basal than luminal A breast tumours (Fig. 7a). Further corroborating our findings, we found that mRNAs for *CXCL16* as well as for *IL8, CSF2, MMP9, EDN1* and *CCL2* all were expressed at significantly higher levels in basal-like breast cancers as compared with luminal A tumours (Fig. 7a).

Discussion

In this study, we demonstrate that co-transplanted primary human monocytes differentiate into CD163$^+$ myeloid cells, both in luminal A and TN breast tumour grafts, but that the immunosuppressive MDSC-marker S100A9 (refs 37,38) was expressed only by the CD163$^+$ cells in the TN grafts. S100A9 expression has previously been associated with ER$^-$PR$^-$ tumours, both when expressed in the myeloid cells but also in the malignant cells *per se*[21]. Neither MDA-MB-231 nor SUM-159 cells express S100A9, thus making it possible for us to investigate the myeloid S100A9 expression, in a TN environment *in vivo*. More myeloid cells were present in the TN tumours and this was due to their increased survival and proliferation. Hence, the TN tumour environment is crucial for myeloid cells and their effect on fibroblasts. We also showed that monocytes are preferentially attracted to TN breast tumours by secreted factors, such as GM-CSF, CCL2 and IL-8, confirming the findings of Hollmén *et al.*[39] and Su *et al.*[40]. In addition, S100A9 is probably one of the major MDSC chemoattractants[16]. Even though expression of the mouse myeloid differentiation antigen Gr-1 was scarce, a potential infiltration of immature BMDCs cannot be excluded as NSG-mice still have normal numbers of immature myeloid cells[22]. Furthermore, the xenograft models used in this study represent a situation where the myeloid cells are present in the tumours from initiation and hence, these models do not address the question how these cells are recruited to the tumours or which cells of the microenvironment that arrive first. Therefore, we confirmed our data in mice grafted with TN as compared with luminal patient-derived tumour tissue (PDX), where we could see that more myeloid cells infiltrated the TN tumours, and although the infiltrating myeloid cells were located closer to the borders of the tumours, these areas did show more S100A9 expression and activated fibroblasts (αSMA) in the TN, as compared with the luminal graft. We observed a similar pattern using syngeneic mouse models of luminal (67NR) as compared with TN (4T1) breast tumours, grafted in immunocompetent mice. The effect of myeloid cells on tumour stroma formation was seen in the borders of grafts in general, and therefore might be underestimated in human tumour pathology. In our model where the myeloid cells were mixed with the tumour cells prior to injection, a clear effect was seen throughout the tumour, on both activated fibroblasts and myeloid cells *per se*. In the future, we suggest validating our findings in spontaneous breast cancer models with transplanted circulating human myeloid cells, but also in models using novel drugs against tumour-infiltrating myeloid cells.

Monocytes have been previously shown to promote metastasis but evidence is lacking on how they affect the tumour stroma[18]. We showed that the myeloid cells in both luminal A and TN tumours enhanced recruitment of fibroblasts of mouse origin, but

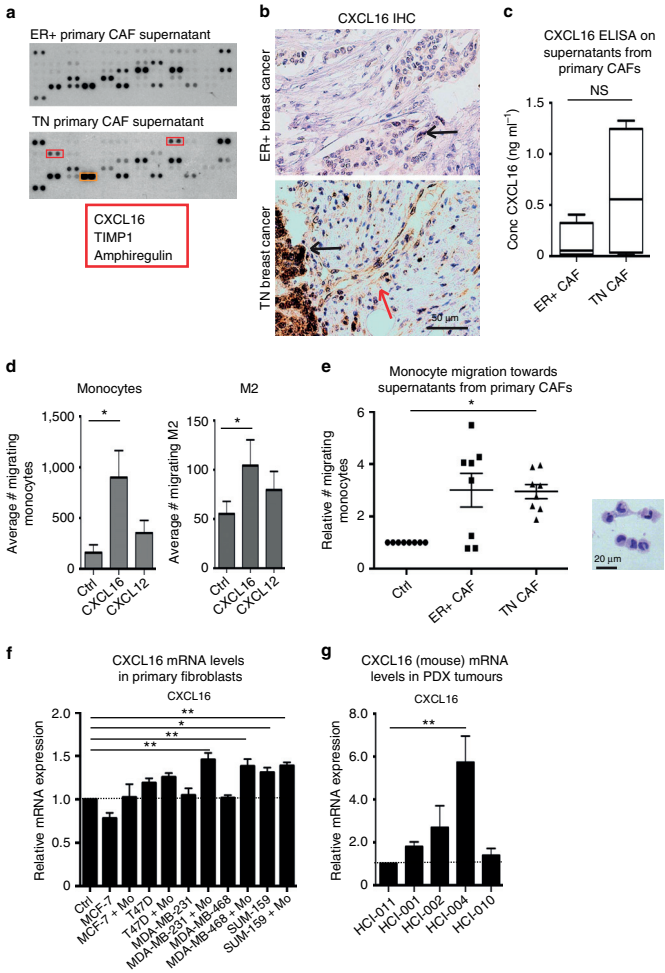

Figure 6 | CXCL16 expression is induced in fibroblasts by myeloid cells. (**a**) Human angiogenesis array proteome profiler of supernatants from primary CAFs derived from ER^+ or $ER^-PR^-Her2^-$ (triple negative; TN) tumours. (**b**) IHC of CXCL16 in human primary breast cancers. Upper panel shows ER^+PR^+ breast cancer and lower panel one TNBC. Black arrow highlights CXCL16 expressing malignant cells and red arrow, CXCL16 expressing fibroblasts. (**c**) The levels of secreted CXCL16 were measured using a human CXCL16 ELISA performed on supernatants prepared from primary human CAFs isolated from TN or ER^+ primary breast tumours. $N=4$ TN and $N=8$ ER^+. Mann–Whitney *U*-test. (**d**) Migration of primary human monocytes (left) or M2 macrophages (right) was measured using Boyden migration chambers with 8 µm pore size towards control medium (Ctrl), recombinant CXCL16 or CXCL12 as a control. $*=P<0.05$. ANOVA Dunn's multiple comparisons test. $N=5$ and $N=14$. (**e**) Migration of primary human monocytes (left) or M2 macrophages (right) was measured using Boyden migration chambers with 8 µm pore size towards control medium (Ctrl), or towards conditioned medium from primary human CAFs derived from either ER^+ or TN tumours. $*=P<0.05$ $**=P<0.01$. ANOVA Dunn's multiple comparisons test. $N=8$. The HE image (right) shows that the migrated cells are indeed monocytes and the scale bar represents 20 µm. (**f**) RT-QPCR analysis of CXCL16 levels in primary mouse fibroblasts cultured in either control medium (Ctrl) or supernatants derived from luminal A (MCF-7 and T47D) or TN (MDA-MB-231, MDA-MB-468 and SUM-159) breast cancer cells with or without co-culture with primary human monocytes (Mo). $*=P<0.05$ $**=P<0.01$. ANOVA Dunn's multiple comparisons test. $N=4$–8. Error bars indicate s.e.m. (**g**) RT-QPCR analysis of infiltrating mouse cell CXCL16 levels in human PDX tumours derived from one luminal (HCI-011; set to 1) and four TN (HCI-001, 002, 004, 010) breast tumours. $**=P<0.01$. ANOVA Dunn's multiple comparisons test. $N=6$–7. Error bars indicate s.e.m.

Table 2 | Crosstable[a] over TNBCs and CXCL16 fibroblast expression.

	CXCL16 fibroblasts (0)	CXCL16 fibroblasts (1)	Total
TNBC 0	101	7	108
1	11	4*[a]	15
Total	112	11	123

[a]χ^2 Linear by linear Association $P = 0.011$ using SPSS software. *$P < 0.05$.

promoted their activation into myofibroblasts only in the context of TN tumours. Monocytes *per se* had the potential to activate the fibroblasts in the TN tumours and to induce an alternative collagen (collagen VI) formation. Hence, monocytes can promote stroma formation in both luminal A and TNBCs, but the type of stroma potentiated by the myeloid cells might depend on the tumour type. It is intriguing to note that in 3 out of 4 xenograft-groups, the monocyte co-transplanted tumours were smaller in size, a finding that might be explained by a different ECM in these tumours and the levels of MMPs (for example, MMP9) that can dictate the thickness of collagen deposition and also the orientation of collagen fibres[41]. Collagen VI, which is induced by TGFβ in macrophages, is not preferentially degraded by MMPs. It has also been shown to be a fibroblast mitogen and suggested to be involved in drug resistance, tumour progression and myeloid cell recruitment[29,42,43].The beaded microfilament structure of collagen VI is also important for anchoring cells to the ECM and may be important for anchoring myeloid cells[28]. Undoubtedly, more research on the role for different collagens in tumour progression is warranted.

CXCL16 has previously been shown to attract T cells, bone marrow-derived fibroblast precursors and to potentiate fibrosis and myofibroblast activation in renal fibrosis[34,35,44]. In this study we show that monocyte-induced fibroblast activation involves expression of the chemoattractant CXCL16 in a TN context primarily. This observation is supported by our findings that primary human CAFs from TN breast tumours specifically expressed CXCL16 and that a high CXCL16 expression in fibroblasts correlates with a TN breast tumour type in a breast cancer tissue microarray. The CXCL16 expression levels varied, and not all TN breast tumours expressed CXCL16 in their fibroblasts, an observation that might be explained by the heterogeneity within the TN subgroup and indeed when analysed using the TCGA breast cancer RNAseq data, only the basal breast cancer subgroup showed a significantly higher expression level of *CXCL16*. We also found that CXCL16 could be expressed by the malignant cells of both luminal A and TN tumours, a finding that might explain why $CD163^+/GRN^+$ myeloid cells are located in the tumour nests of luminal A tumours[4]. In light of this, we suggest that CXCL16 may be viewed as a monocyte and fibroblast chemoattractant expressed in human breast tumours. In addition to this, also GM-CSF (ref. 45) and CXCL4 (refs 46,47) are important for fibroblast recruitment or activation. Similarly, Su *et al.* and Hollmén *et al.* reported on a positive feedback loop between breast cancer cells and macrophages in mesenchymal-like and TNBCs[39,40].

In summary, the findings from our study indicate that myeloid cells are recruited preferentially to TN breast tumours where they become skewed to immunosuppressive myeloid cells ($CD163^+$ $S100A9^+$), activate CAFs and induce expression of CXCL16 in CAFs that in turn can recruit more myeloid cells and fibroblasts, but also T cells (Fig. 7b). This is the first study to address the particular effects of myeloid cells on tumour stroma, and also specifically with regards to breast cancer subtype. These findings are of particular importance for the design of trials concerning novel drugs being developed against tumour-infiltrating myeloid cells. In addition, our data add valuable information to aid management decisions concerning which drugs to be used in different breast cancer subtypes and also to predict drug resistance since anti-PD1/L1 resistant tumours might develop immune resistance in the presence of infiltrating anti-inflammatory myeloid cells. Our data suggest that patients with TNBCs, in particular, might benefit from treatment with novel immunomodulatory drugs or chemotherapeutics that target immunosuppressive cells.

Methods

Ethics statement. Permission for the study was obtained from the Regional Ethics Committee at Lund University. For permission to conduct human research, the following ethics permits were obtained: Dnr 2010/477, Dnr 2012/689, Dnr 2014/669, Dnr 445/07 and Dnr 2009/658. The participating patients provided written informed consent or for the tumour tissue microarray had the option to withdraw. The NSG models (approvals M249-09 and M69/11) and nude mice (approval M149/14), were approved by regional ethics committee for animal research at Lund University, Sweden. The PDX experiments were reviewed and approved by the University of California, San Francisco Institutional Animal Care and Use Committee (IACUC). The syngeneic, mouse breast cancer models, were approved by the Animal Experimentation and Ethics Committee (AEEC) of the Peter MacCallum Cancer Center, Australia.

Isolation of primary human monocytes. Leucocytes were isolated within 2–3 h of blood collection from healthy blood donors by leucocyte depletion filtration performed according to a previously published method[48] or from blood collected in EDTA tubes from healthy blood donors. First, peripheral blood mononuclear cells were prepared using Ficoll-Paque PLUS gradient centrifugation, then monocytes were isolated by magnetic cell sorting using the Monocyte Isolation Kit II, according to the manufacturers' instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) with antibodies against CD3, CD7, CD16, CD19, CD56, CD123 and glycophorin A.

Animal procedures. Female 8-week-old NSG-mice (NOD.Cg-*Prkdc(scid)Il2rg(tm1Wji)*/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 (approvals M249-09 and M69/11). MCF-7, MDA-MB-231, T47D or SUM-159 human breast cancer cell lines (1×10^6 cells, 5×10^6 for T47D cells) were injected alone or in combination with primary human monocytes (1×10^6 cells) that were pre-stimulated with interleukin-10 (IL-10) 10 ng ml^{-1} for 30 min in a total volume of 100 µl Hanks' balanced salt solution to enhance survival[49], on the right flank. Mice were monitored twice weekly. Tumours were excised either 21 days (MDA-MB-231, T47D, SUM-159 and MCF-7) or 21 and 90 days (MCF-7) after injection, before being fixed in 4% paraformaldehyde and embedded in paraffin. For each experiment, 5–10 mice were used in each group. Similar results were obtained with MCF-7 grafts on day 21 and 90; results for day 90 are shown in Figs 1 and 2 and for day 21 in Supplementary Fig. 6.

NMRI-Nude mice (8-week-old) (The Jackson Laboratory Maine, USA) were housed in a controlled environment and all procedures were approved by the regional ethics committee for animal research (approval M149/14). Primary mouse fibroblasts were isolated by dissecting the ears of nude mice, cutting them into small pieces and treating overnight with collagenase type I (17100-017, Thermo Scientific, MA, USA) together with Dulbecco's modified Eagle medium (DMEM) high glucose supplemented with penicillin/streptomycin and 1% minimum essential medium Eagle (MEM) at 37 °C. The following day a single cell suspension was prepared, filtered through a 70 µm pore filter, washed and cultured in HAMs F12 medium supplemented with penicillin/streptomycin and 10% FBS. Subsequently, non-adherent cells were washed away.

Patient-derived xenografts. Breast cancer PDXs were generated from one luminal (HCI-011) and four TN (HCI-001, HCI-002, HCI-004, HCI- 010) breast cancers serially passaged in NOD/SCID mice as described previously[50,51]. Tumour fragments were placed into cleared inguinal fat pads of pre-pubescent NOD/SCID mice, grown to 20–25 mm and subsequently dissected and stored by freezing in 90% FBS and 10% dimethylsulfoxide until used or fixed in paraffin for IHC. Total RNA was extracted according to the manufacturers' instructions using RNeasy Plus Mini kit (Qiagen, Hilden, MD, USA) and iScript Reverse Transcription Supermix for RT-QPCR (BIO-RAD) was used for cDNA synthesis. RT-QPCR was performed using iTAG Universal SYBR Green Supermix (BIO-RAD).

Syngeneic mouse breast cancer models. Syngeneic primary tumours that were generated previously by injection of 1×10^5 luminal (67NR) or TN (4T1.13) cells in the fourth inguinal fat pad of 8–10-week-old female BALB/c mice[25] were used for IHC.

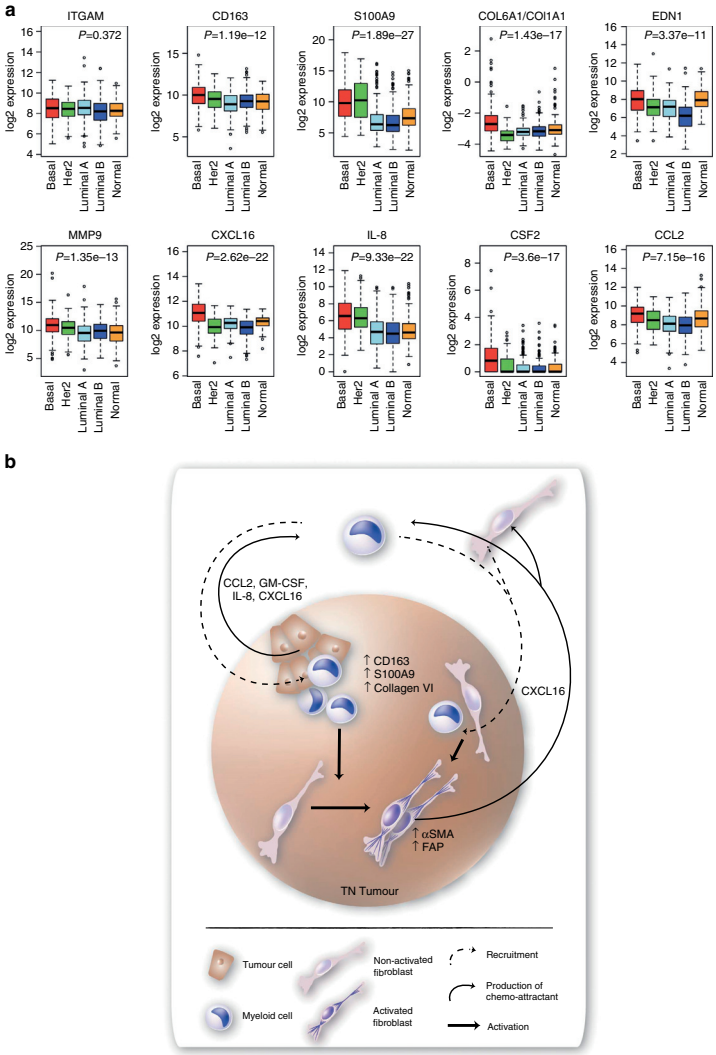

Figure 7 | Gene expression profile in human breast cancers. Human basal breast cancers have an immunosuppressive gene expression profile. (**a**) Box plots showing log2 gene expression levels of indicated genes in molecular breast cancer subtypes, using TCGA breast cancer RNAseq data. *P* values were calculated using a *t*-test comparing levels between basal-like tumours and luminal A tumours. The middle line demonstrates the median, the box illustrates the interquartile range, and the whiskers indicate the most extreme data point that is not $>1.5\times$ the interquartile range away from the box. Data points beyond these values are individually shown. (**b**) Schematic to model the effects exerted by myeloid cells on stroma formation in TN breast tumours. Myeloid cells are recruited to TN breast tumours by proteins including CCL2, GM-CSF, IL-8, S100A9 and CXCL16, where they are induced to express CD163 and the immunosuppressive factors S100A9 and collagen VI. Furthermore, in a TN environment, the myeloid cells activate CAFs and induce expression of CXCL16 by the fibroblasts, which, in turn, can recruit more myeloid cells and fibroblasts. The activated stroma in combination with the presence of anti-inflammatory myeloid cells, will render the TN tumours a more aggressive behaviour.

Cell culture. The human breast cancer cell lines MDA-MB-231, MDA-MB-468, MCF-7 and T47D were obtained from ATCC, and cultured in 10% FBS RPMI-1640 supplemented with penicillin/streptomycin. The SUM-159 cell line was produced by Professor S. Ethier and were cultured in F-12 HAM's medium supplemented with 5% FBS, 1 μM L-Glutamine, 1 μg ml^{-1} hydrocortisone (BD BioScience, San Diego, CA, USA) and 5 μg ml^{-1} insulin (Novo Nordisk A/S, Mâlöv, Denmark) and penicillin/streptomycin. The cell lines were routinely tested and found negative for mycoplasma. Conditioned medium from all cell lines was collected at subconfluency and human primary monocytes were cultured for 7–14 days in the conditioned medium (OptiMEM supplemented with 1% penicillin/streptomycin or RPMI-1640 supplemented with 10% FBS and penicillin/streptomycin) or using only medium with 10 ng ml^{-1} rhGM-CSF as control. Human primary M2 macrophages were cultured in OptiMEM supplemented with penicillin/streptomycin and 10 ng ml^{-1} rhGM-CSF for 5 days, after which 20 ng ml^{-1} rhIL-4 was added for another 2 days. All media and supplements were purchased from Thermo Scientific (Logan, UT, USA).

TMA and immunohistochemistry. The breast cancer cohort consists of 144 patients diagnosed with invasive breast cancer at Skåne University Hospital, Malmö, Sweden, between 2001 and 2002. The cohort and TMA have previously been described in detail[19,52,53]. CXCL16 cytoplasmic expression was estimated in the malignant cells (intensity: 0 = negative, 1 = weak, 2 = moderate, 3 = strong intensity) and in the stromal fibroblasts (intensity 0 = negative-weak, 1 = strong intensity). Sections (4 μm thick) of the paraffin embedded tumours were mounted onto glass slides and deparaffinized, prior to antigen retrieval using the PT-link system (DAKO, Glostrup, Denmark) and staining in a Autostainer Plus (DAKO) with the EnVisionFlex High pH-kit (DAKO). All histological sections were counterstained with HE. All primary antibodies used for IHC are shown in Supplementary Table 4 (specificity; clone; dilution; company). Sirius Red staining was performed using in house methods. Cytospins were prepared from monocytes or non-enzymatic cell dissociation buffer-collected (Sigma Aldrich) M2 cultures that were air-dried.

Cytokines and other reagents. All recombinant human cytokines were obtained from R&D Systems, and the following concentrations were used in all experiments: 10 ng ml^{-1} GM-CSF, 20 ng ml^{-1} IL-4, 100 ng ml^{-1} CXCL12 and 100 ng ml^{-1} CXCL16. For flow cytometry, the following reagents were used (all from BD Biosciences): CD14 clone M5E2, HLA-DR clone G46-6, CD90, EpCAM, CD11b, CD34 annexin V and 7AAD.

The human angiogenesis array Proteome Profiler kit (R&D systems) was used to analyse the soluble mediators according to the manufacturer's protocol. Analysis of CXCL16 in supernatants collected from primary human CAFs was performed using a human specific CXCL16 ELISA kit (R&D Systems) according to the manufacturer's instructions.

CAF isolation. Primary human tumours were dissected, minced into smaller pieces and treated overnight with collagenase at 37 °C. The next day, single cell suspensions were prepared and subsequently seeded into large flasks containing HAM/F-12 medium supplemented with 10% FBS and penicillin/streptomycin. After 12 h the non-adherent cells were washed away and the adherent cells were continuously cultured in HAM/F-12 medium supplemented with 10% FBS and penicillin/streptomycin. The medium was changed every other day to remove non-adherent cells until large groups of fibroblasts became apparent. Collected supernatants were stored at −80 °C.

Boyden chamber migration assays. Human primary monocytes or M2 macrophages were allowed to migrate through Costar Transwell Permeable Support 8.0 μm (pore size) 24-well plates (Corning; Sigma Aldrich) towards the conditioned medium of breast cancer cells (cultured under serum free conditions), or towards the chemokines CXCL12 or CXCL16 (100 ng ml^{-1}).

Scratch wound assays. For the scratch wound assays, freshly prepared primary mouse fibroblasts were seeded directly into 6-well plates. Once confluent, a pipette tip was used to scratch the fibroblast monolayer with a total of two scratches per well. To start the assay, the wells were rinsed and conditioned medium applied. The area of the open wound was analysed after 24 h at 37 °C. Cells were collected for quantitative real-time PCR (RT-QPCR) and supernatants were collected for ELISA analyses.

Quantitative real-time PCR. Total RNA was extracted according to the manufacturers' instructions using RNeasy Plus kit (Qiagen, Hilden, MD, USA) for fibroblasts or Trizol (Invitrogen, Thermo Scientific) for monocytes. Random hexamers and the M-MuLV reverse transcriptase enzyme (Thermo Scientific) were used and RT-QPCR was performed in triplicate using Maxima SYBR Green/Rox (Thermo Scientific) and analysed on the Mx3005P QPCR system (Agilent Technologies). The relative mRNA expression was normalized to *ACTB*, *HPRT* and *GAPDH* and calculated using the comparative Ct method[54]. For primers see Supplementary Table 5.

Thymidine incorporation. Primary human monocytes or primary mouse fibroblasts were cultured in different breast cancer conditioned media and allowed to proliferate for 24 h. [methyl-^{3}H] thymidine (1 μCi) was added for 18 h, and incorporation was determined in a Microbeta Counter (Perkin & Elmer; MA, USA).

Gene expression profile analysis. TCGA breast cancer RNAseq data (http://cancergenome.nih.gov/) were downloaded on 30 January 2015. Data were log2 transformed following addition of 1 to each value using R (3.1.1). Breast cancer subtypes were classified using the PAM50 centroids[55] after centring the data around the median.

Gene expression data for the syngeneic tumours were obtained from the previous analysis described in Johnstone *et al.*[25] and deposited into the Gene Expression Omnibus (G.E.O.) with Accession No. GSE42272 (http://www.ncbi.nlm.nih.gov/geo/).

Statistical analyses. Statistics by non-parametric Mann–Whitney *U* Wilcoxon test, ANOVA (multiple comparisons when indicated in the Figure legends) or student *t*-test as indicated. SPSS, Graph Pad Prism or R (3.1.1) software was used for statistical analyses.

Data availability. The TCGA breast cancer RNAseq data referenced during the study are available in a public repository from the TCGA website (http://cancergenome.nih.gov/). Gene expression data for the syngeneic tumours were obtained from the previous analysis described in Johnstone *et al.*[25] and deposited into the Gene Expression Omnibus (G.E.O.) with Accession No. GSE42272 (http://www.ncbi.nlm.nih.gov/geo/). All the other supporting the findings of this study are available within the article and its Supplementary Information Files or from the corresponding author (K.L.) upon request.

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Acknowledgements

The results are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. We thank Ms Elise Nilsson and Mrs Kristina Ekström-Holka for professional technical skills in preparation of IHC. We also thank Drs Susan Evans Axelsson, Kristofer Ahlqvist and Giacomo Canesin for extra help in the animal house. This work was generously supported by grants from the Swedish Research Council, The Swedish Cancer Society, Kocks Foundation, Österlunds Foundation, Gunnar Nilsson Cancer Foundation, MAS Cancer Foundation, Åke Wibergs Foundation, The National Cancer Institute, USA (R01 CA180039 and U01 CA199315 to Z.W.) and finally the SCAN-B project (http://www.med.lu.se/scan_b) and the Mrs Berta Kamprad foundation. Elevate Scientific, www.elevatescientific.com, Lund, Sweden, was used for linguistic corrections and improvements.

Author contributions

R.A. performed the majority of experiments. C.B., C.H. and S.M. performed the xenograft NSG *in vivo* experiments and took part in designing the study initially together with K.L. D.B. and S.P. were responsible for the design and execution of *in vivo* experiments. As clinical pathologists, B.T. and M.E.J. were responsible for the fresh human tumour material and for guiding the scoring of the IHC and verifying all histological stains. C.H., K.S. and Z.W. were responsible for the PDX models. R.L.A. was responsible for the syngeneic models. S.P.E. was responsible for the SUM-159 cells and for critical scientific input. K.J. was responsible for the clinical breast cancer tumour tissue microarray and analyses. C.L. was responsible for analysing RNAseq data, for significant input in both data analysis and in writing the manuscript. K.L. designed all the experiments, wrote the manuscript, as well as interpreted and analysed the data. All authors read and approved the manuscript and all were involved in revising the manuscript.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

Competing financial interests: The authors declare no competing financial interests.

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How to cite this article: Allaoui, R. *et al.* Cancer-associated fibroblast-secreted CXCL16 attracts monocytes to promote stroma activation in triple-negative breast cancers. *Nat. Commun.* **7,** 13050 doi: 10.1038/ncomms13050 (2016).

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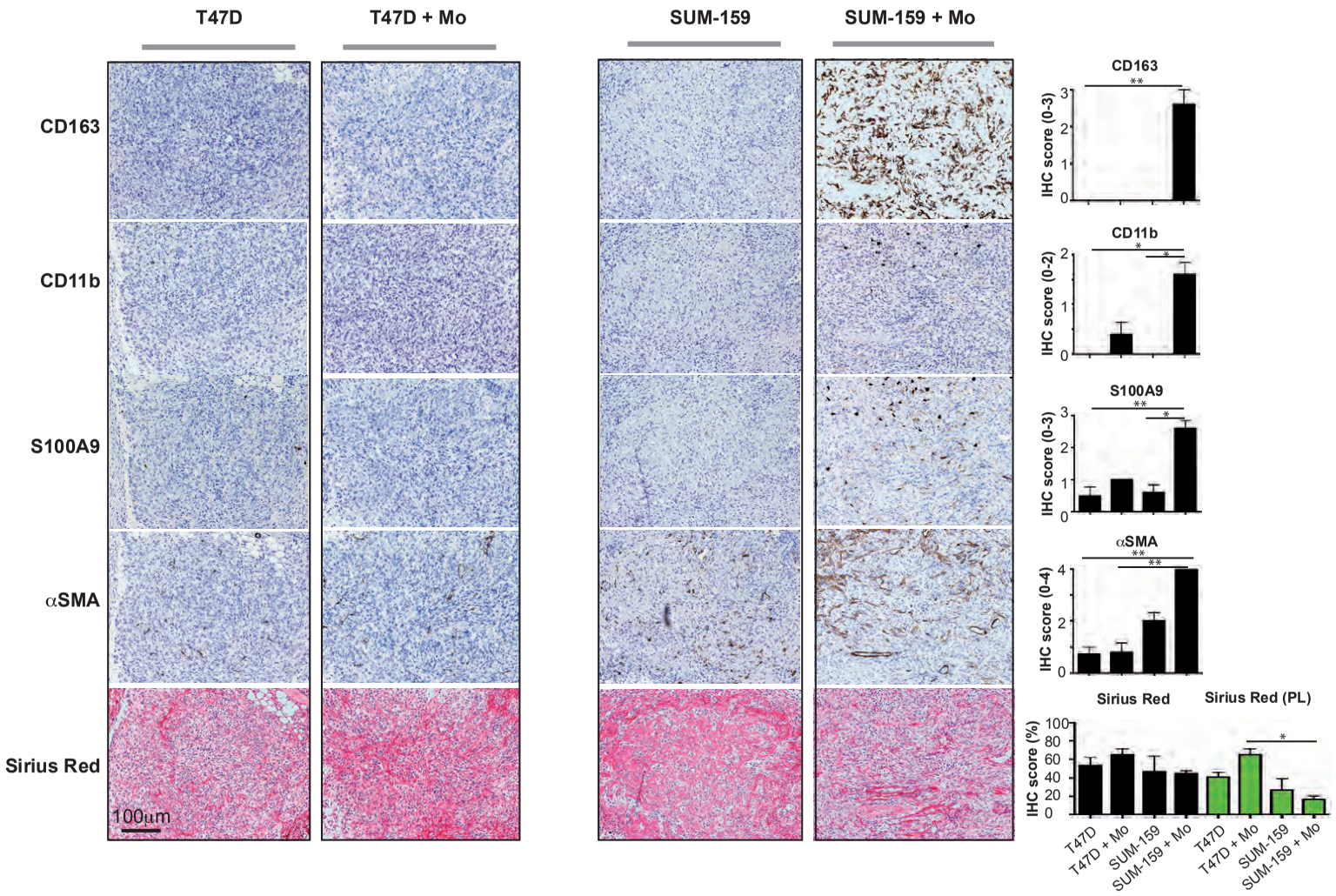
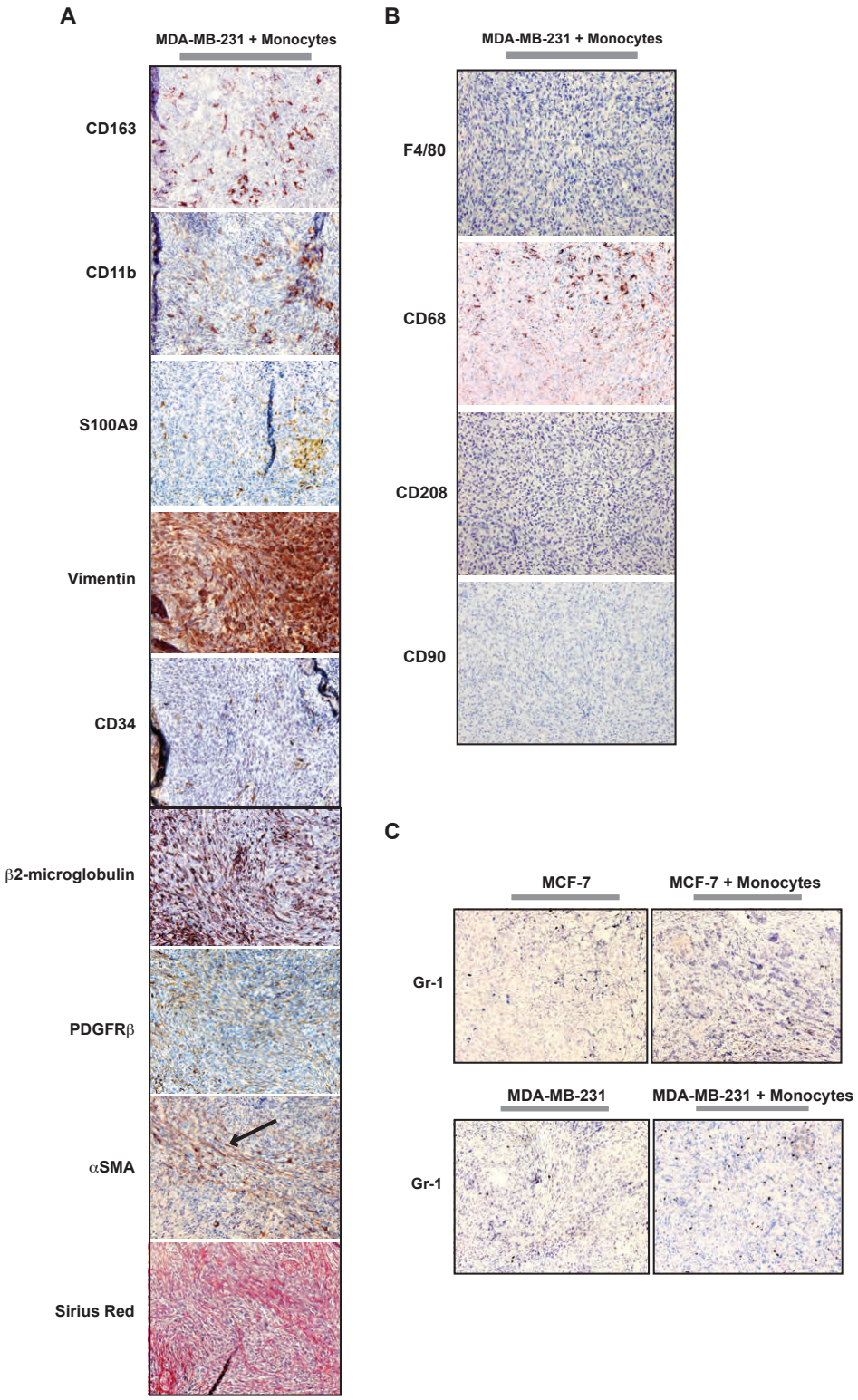
Fig 1


T47D
T47D + Mo
SUM-159
SUM-159 + Mo
CD163
CD11b
S100A9
αSMA
Sirius Red
100µm
CD163
IHC score (0-3)
CD11b
IHC score (0-2)
S100A9
IHC score (0-3)
αSMA
IHC score (0-4)
Sirius Red
Sirius Red (PL)
IHC score (%)
T47D
T47D + Mo
SUM-159
SUM-159 + Mo

Supplementary Figure 1. *Immunohistochemistry of xenografts*

Tumor xenografts consisting of triple negative (TN) SUM-159 breast cancer cells co-transplanted with primary human monocytes, express more myeloid-related, immunosuppressive and activated fibroblast markers than luminal A T47D / monocyte xenografts. The xenografts were grown in highly immunodeficient NSG-mice (see Material and methods), and sections from the tumors were stained with myeloid (CD163, CD11b, S100A9) and the activated fibroblast marker αSMA. The two cell lines chosen are negative for S100A9 [1]. Immunohistochemistry was performed using the indicated antibodies. All histological sections were counterstained with HE. N=5 mice were analyzed for each group; Grafts were analyzed on day 21. The histograms to the right show the mean value for each IHC score with statistical analysis. IHC scores are shown in Supplementary Table 2. *=$p<0.05$, **=$p<0.01$ ANOVA non-parametric Kruskal Wallis test. N=5. Error bars indicate SEM.

Fig 2


A
MDA-MB-231 + Monocytes
CD163
CD11b
S100A9
Vimentin
CD34
β2-microglobulin
PDGFRβ
αSMA
Sirius Red
B
MDA-MB-231 + Monocytes
F4/80
CD68
CD208
CD90
C
MCF-7
MCF-7 + Monocytes
Gr-1
MDA-MB-231
MDA-MB-231 + Monocytes
Gr-1

Supplementary Figure 2. *Immunohistochemistry of xenografts*

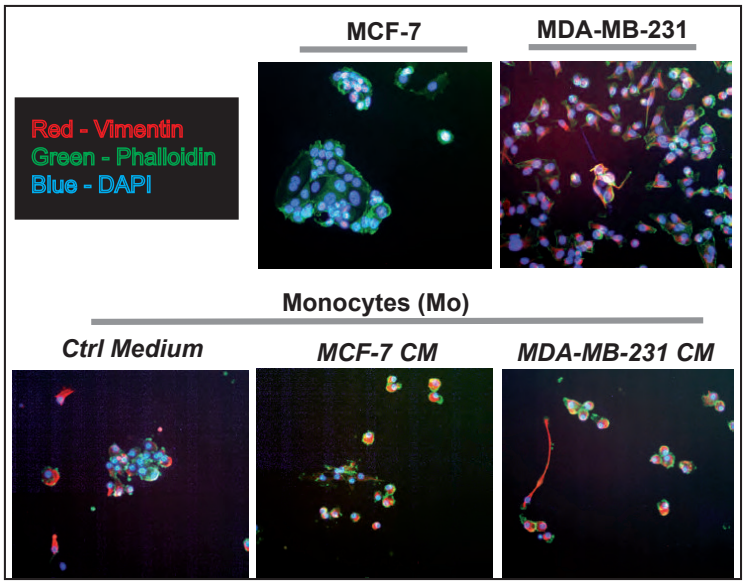
(A) Xenografts consisting of triple-negative (TN) MDA-MB-231 breast cancer cells co-transplanted with primary human monocytes, into NSG mice. One graft representing a *low* myeloid cell take is shown for the TN MDA-MB-231 / monocyte co-transplant group.

(B) Xenografts consisting of triple-negative (TN) MDA-MB-231 breast cancer cells co-transplanted with primary human monocytes, into NSG mice and stained for murine macrophages (F4/80), human macrophages (CD68) human myeloid dendritic cells (mDCs; CD208) and human fibroblasts (CD90).

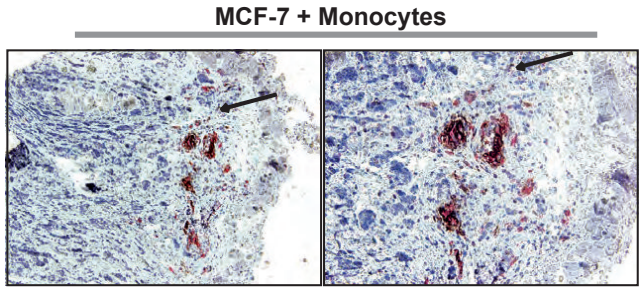
(C) Xenografts consisting of luminal A (MCF-7) and triple-negative (TN) MDA-MB-231 breast cancer cells transplanted with or without primary human monocytes, into NSG mice and stained for the mouse myeloid marker, Gr1. All histological sections were counterstained with HE. N=5 for each group.

Fig 3

A

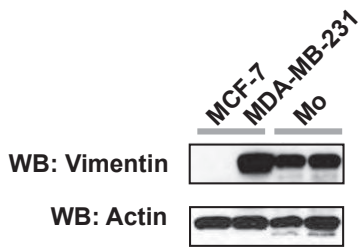


B



CD11b – Red
Vimentin – Brown

C

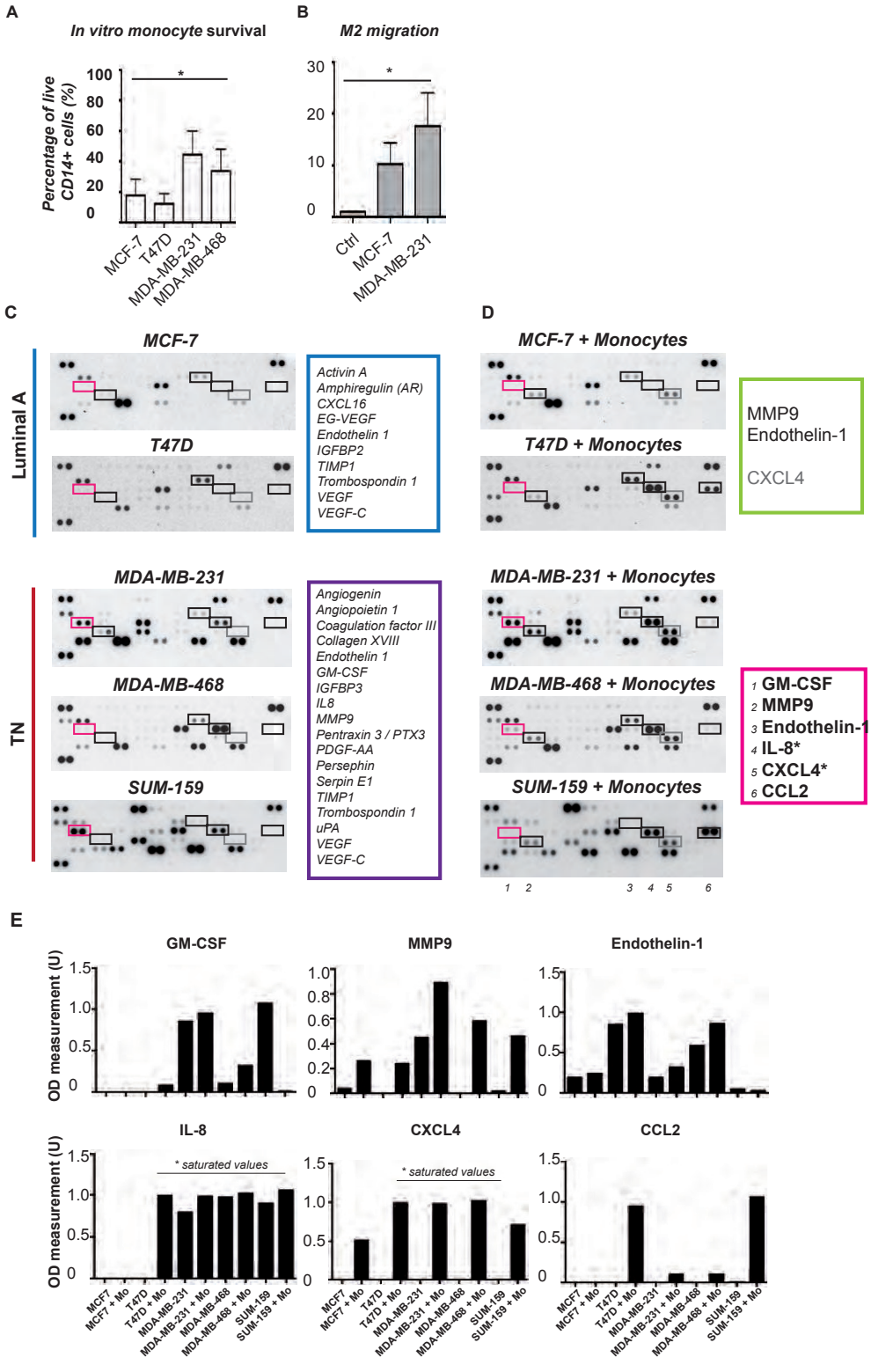


Supplementary Figure 3. *Vimentin is expressed by myeloid cells*

(A) Immunofluorescence of primary human monocytes cultured with breast cancer cell conditioned medium or under control conditions (only GM-CSF) and stained for Vimentin (red), phalloidin (to stain actin filaments; green) and DAPI (nuclear stain; blue). MCF-7 and MDA-MB-231 breast cancer cells were used as negative and positive controls, respectively.

(B) Double staining IHC of CD11b and vimentin in xeno-transplants from MCF-7 / monocytes tumors as indicated. Black arrows show staining with vimentin but not CD11b.

(C) Western blot (WB) of vimentin expression in MCF-7 and MDA-MB-231 breast cancer cells or human primary monocytes (Mo) isolated from samples from two healthy blood donors. Actin is used as a loading control.


A
In vitro monocyte survival
Percentage of live CD14+ cells (%)
100
80
60
40
20
0
*
MCF-7
T47D
MDA-MB-231
MDA-MB-468
B
M2 migration
30
20
10
0
*
Ctrl
MCF-7
MDA-MB-231
C
Luminal A
MCF-7
T47D
Activin A
Amphiregulin (AR)
CXCL16
EG-VEGF
Endothelin 1
IGFBP2
TIMP1
Trombospondin 1
VEGF
VEGF-C
TN
MDA-MB-231
MDA-MB-468
SUM-159
Angiogenin
Angiopoietin 1
Coagulation factor III
Collagen XVIII
Endothelin 1
GM-CSF
IGFBP3
IL8
MMP9
Pentraxin 3 / PTX3
PDGF-AA
Persephin
Serpin E1
TIMP1
Trombospondin 1
uPA
VEGF
VEGF-C
D
MCF-7 + Monocytes
T47D + Monocytes
MDA-MB-231 + Monocytes
MDA-MB-468 + Monocytes
SUM-159 + Monocytes
1 2 3 4 5 6
MMP9
Endothelin-1
CXCL4
1 GM-CSF
2 MMP9
3 Endothelin-1
4 IL-8*
5 CXCL4*
6 CCL2
E
GM-CSF
MMP9
Endothelin-1
IL-8
CXCL4
CCL2
OD measurement (U)
* saturated values
MCF7
MCF7 + Mo
T47D
T47D + Mo
MDA-MB-231
MDA-MB-231 + Mo
MDA-MB-468
MDA-MB-468 + Mo
SUM-159
SUM-159 + Mo

Supplementary Figure 4. *Effect of breast cancer cells on monocytes*

(A) Survival of isolated human primary monocytes in breast cancer cell conditioned medium, grown for 7 days, was assessed. 7AAD and $CD14^+$staining was performed to analyze the content of live monocyte/macrophages in each culture. *=$p<0.05$ ***=$p<0.001$. ANOVA. N=5. Error bars indicate SEM.

(B) Boyden chamber migration assay of primary human M2 macrophages migrating towards control medium or breast cancer cell conditioned medium. *=$p<0.05$ ANOVA. N=8. Error bars indicate SEM.

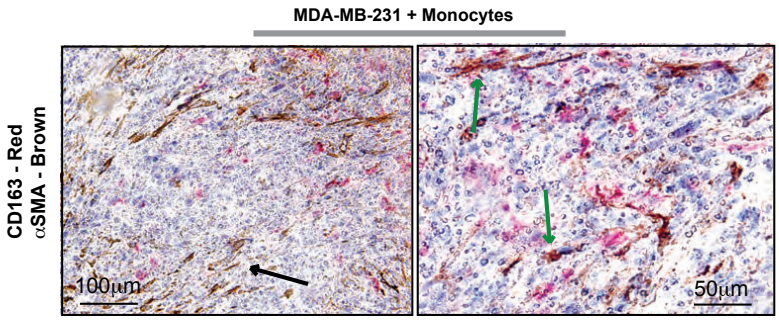
(C) Human angiogenesis array proteome profiler of supernatants from luminal A (MCF-7 and T47D) or triple negative (TN) (MDA-MB-231, MDA-MB-468 and SUM-159) breast cancer cells before monocyte co-culture. The factors in the blue box are expressed typically in luminal A breast cancer cells, and the factors in the purple box are expressed typically in TN breast cancer cells.

(D) Human angiogenesis array proteome profiler of supernatants from co-cultures of human primary monocytes and luminal A (MCF-7 and T47D) or TN (MDA-MB-231, MDA-MB-468 and SUM-159) breast cancer cells. The factors in the green and pink boxes are specifically upregulated upon co-culture with monocytes, with the criteria if upregulated in both cultures of luminal A or TN breast cancer/monocyte co-cultures, respectively. The star (*) indicates saturated values. The numbers (1-7) below the boxes indicate corresponding dots with each factor.

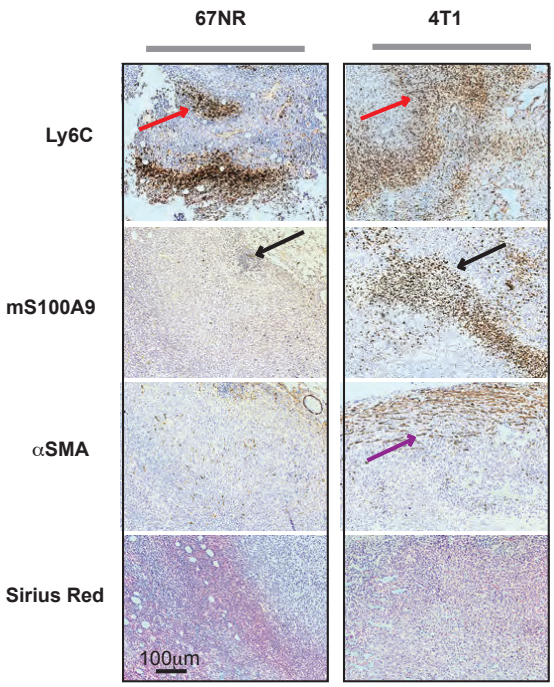
(E) The histograms represent the OD values for each factor in relation to the reference dots A1-2 (upper left corner) for each filter. N=2.

Fig 5

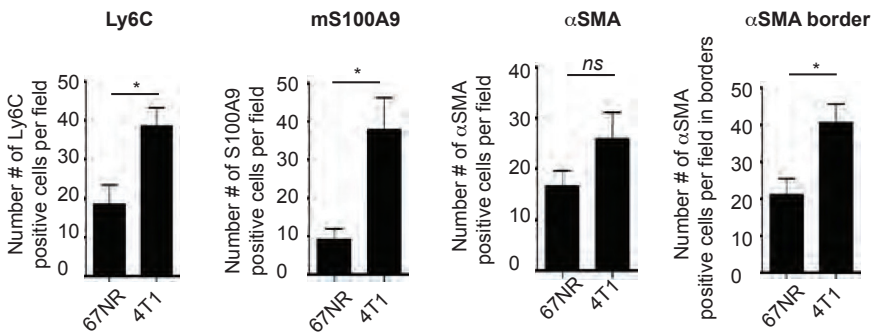
A


MDA-MB-231 + Monocytes
CD163 - Red
αSMA - Brown
100μm
50μm

B


67NR
4T1
Ly6C
mS100A9
αSMA
Sirius Red
100μm

C


Ly6C
mS100A9
αSMA
αSMA border
Number # of Ly6C positive cells per field
Number # of S100A9 positive cells per field
Number # of αSMA positive cells per field
Number # of αSMA positive cells per field in borders
*
ns
0
10
20
30
40
50
67NR
4T1

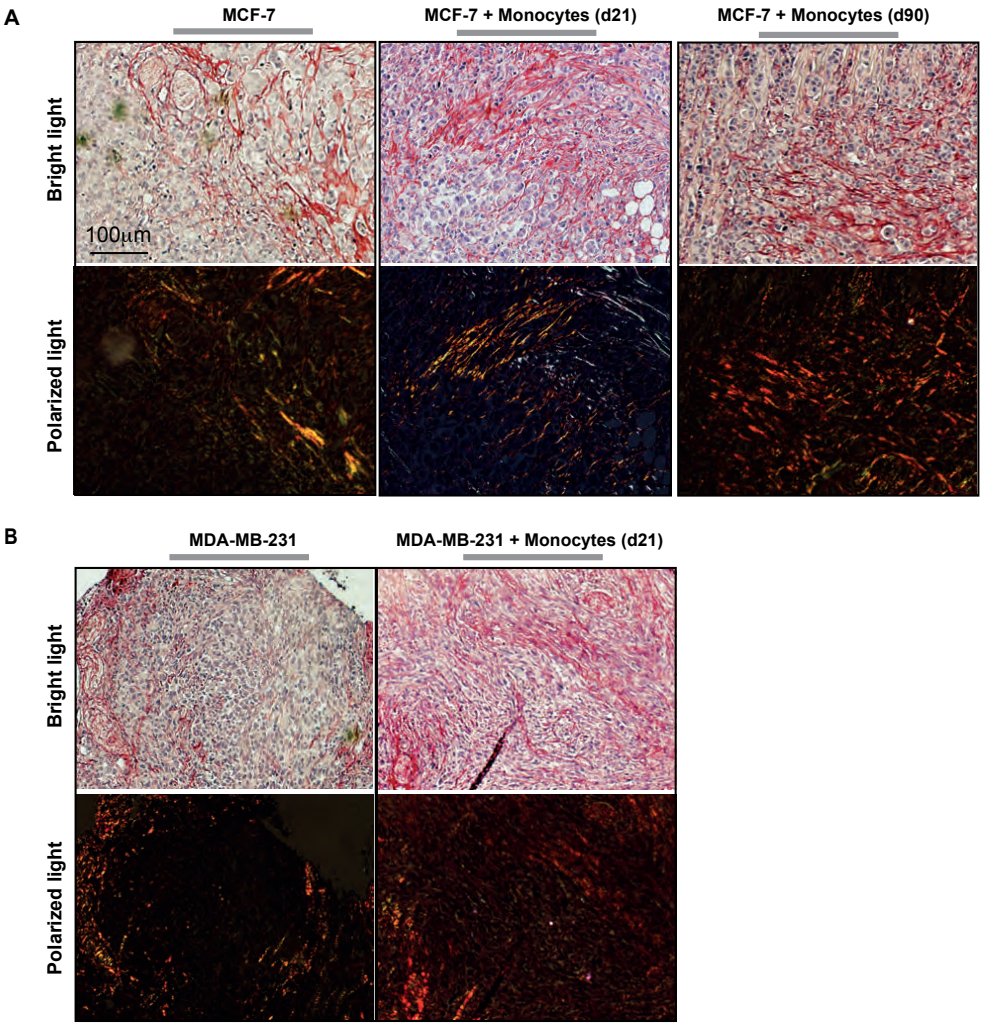
Supplementary Figure 5. *Immunohistochemistry of xenografts and syngeneic tumors*

(A) Double immunohistochemical staining of CD163 and αSMA in the MDA-MB-231 / monocytes xenografts. Black arrows show staining with only αSMA and green arrows show double staining.

(B) Syngeneic mouse tumors consisting of luminal A (67NR) or TN (4T1.13) breast cancer cells transplanted into BALB/c mice. The TN 4T1.13 grafts express more myeloid-related (Ly6C), immunosuppressive (S100A9) and activated fibroblast (αSMA) markers than the luminal 67NR tumors. Sections from the tumors were stained with myeloid (Ly6C and S100A9) and the activated fibroblast marker αSMA. Immunohistochemistry was completed using the indicated antibodies. All histological sections were counterstained with HE.

(C) The histograms show the mean value for each IHC score with statistical analysis. For scoring five fields were counted per staining. *=$p<0.05$ Mann-Whitney U-test. Error bars indicate SEM.

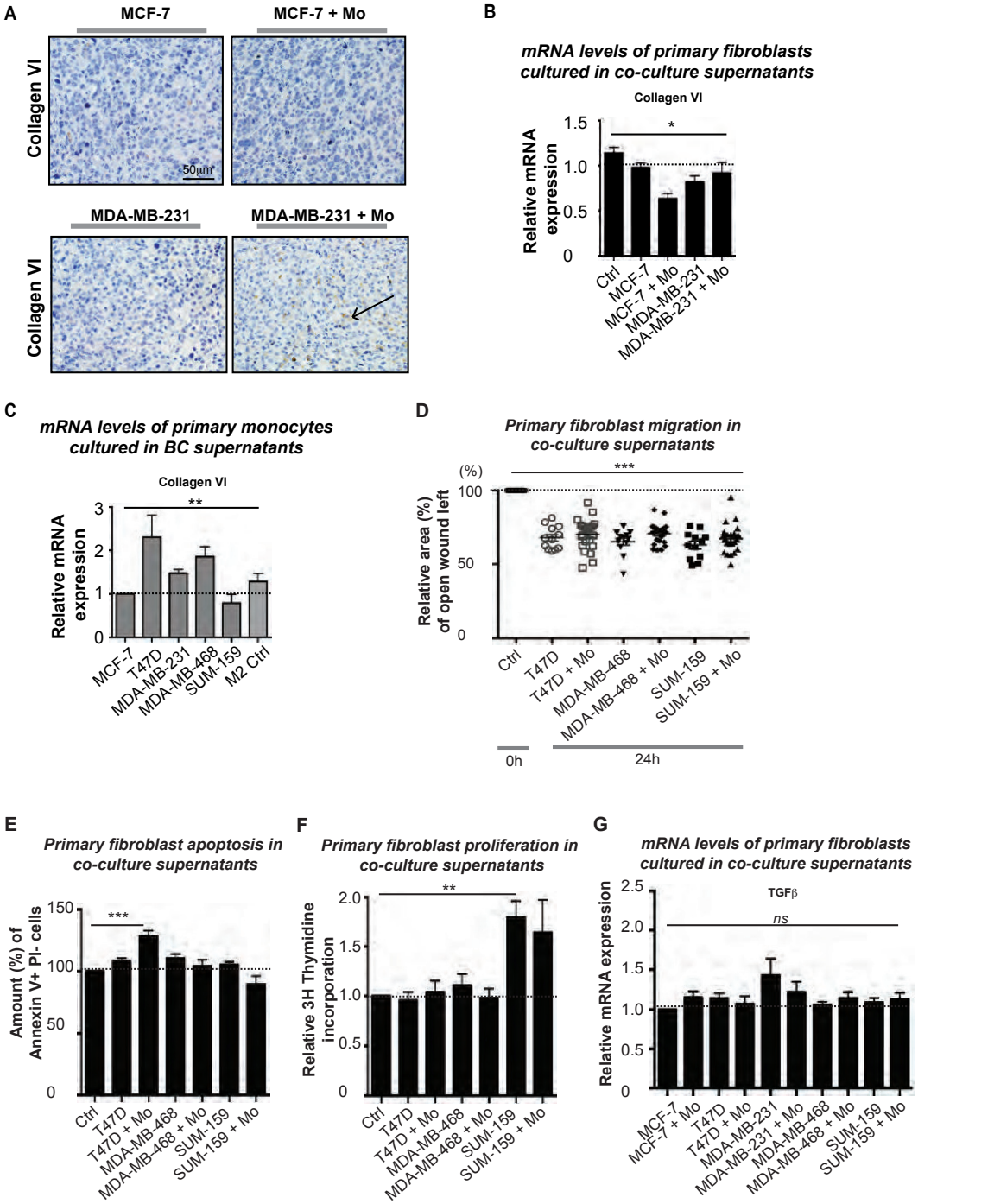
Fig 6


A
MCF-7
MCF-7 + Monocytes (d21)
MCF-7 + Monocytes (d90)
Bright light
Polarized light
100µm
B
MDA-MB-231
MDA-MB-231 + Monocytes (d21)
Bright light
Polarized light

Supplementary Figure 6. *Sirius Red staining of xenografts*

(A) Sirius Red staining of the stroma (top row; bright light microscope; red) and classical collagen bundles (bottom row; polarized light microscope; red/green/yellowish stain) on; MCF-7 or MCF-7 / monocytes on day 21 and day 90, as indicated.

(B) Sirius Red staining of the stroma (top row; bright light microscope; red) and classical collagen bundles (bottom row; polarized light microscope; red/green yellowish stain) on; MDA-MB-231 or MDA-MB-231 / monocytes xenografts. (N=5 for each group) on day 21.


A
MCF-7
MCF-7 + Mo
Collagen VI
50µm
MDA-MB-231
MDA-MB-231 + Mo
Collagen VI
B
mRNA levels of primary fibroblasts cultured in co-culture supernatants
Collagen VI
Relative mRNA expression
1.5
1.0
0.5
0
*
Ctrl
MCF-7
MCF-7 + Mo
MDA-MB-231
MDA-MB-231 + Mo
C
mRNA levels of primary monocytes cultured in BC supernatants
Collagen VI
Relative mRNA expression
3
2
1
0
**
MCF-7
T47D
MDA-MB-231
MDA-MB-468
SUM-159
M2 Ctrl
D
Primary fibroblast migration in co-culture supernatants
(%)
100
50
0
***
Relative area (%) of open wound left
Ctrl
T47D
T47D + Mo
MDA-MB-468
MDA-MB-468 + Mo
SUM-159
SUM-159 + Mo
0h
24h
E
Primary fibroblast apoptosis in co-culture supernatants
Amount (%) of Annexin V+ PI- cells
150
100
50
0
***
Ctrl
T47D
T47D + Mo
MDA-MB-468
MDA-MB-468 + Mo
SUM-159
SUM-159 + Mo
F
Primary fibroblast proliferation in co-culture supernatants
Relative 3H Thymidine incorporation
2.0
1.5
1.0
0.5
0
**
Ctrl
T47D
T47D + Mo
MDA-MB-468
MDA-MB-468 + Mo
SUM-159
SUM-159 + Mo
G
mRNA levels of primary fibroblasts cultured in co-culture supernatants
TGFβ
ns
Relative mRNA expression
2.5
2.0
1.5
1.0
0.5
0
MCF-7
MCF-7 + Mo
T47D
T47D + Mo
MDA-MB-231
MDA-MB-231 + Mo
MDA-MB-468
MDA-MB-468 + Mo
SUM-159
SUM-159 + Mo

Supplementary Figure 7. *Collagen IV expression in TNBC xenografts and cultures*

Collagen VI is expressed by myeloid cells in a triple-negative breast tumor context.

(A) Xenografts of luminal A MCF-7 or TN MDA-MB-231 breast cancer cells, alone (left) or with primary human monocytes (Mo; right) in NSG-mice. Immunohistochemistry was performed using antibodies to collagen VI. Black arrow indicate collagen VI expression in TN / monocyte grafts. All histological sections were counterstained with HE.

(B) Collagen VI mRNA expression levels measured by RT-QPCR in primary mouse fibroblasts grown in breast cancer / monocyte co-culture supernatants. *=$p<0.05$ ANOVA. N=4.

(C) Collagen VI mRNA expression levels measured by RT-QPCR in human myeloid cells cultured in breast cancer supernatants. Primary human M2 macrophages = positive control*=$p<0.05$ ANOVA. N=4. Error bars indicate SEM.

(D) Scratch wound assays showing mouse primary fibroblast migration in supernatants derived from co-cultures of human primary monocytes (Mo) and luminal A (T47D) or TN (MDA-MB-468 and SUM-159) breast cancer cells. ***=$p<0.001$ ANOVA non-parametric Kruskal Wallis test. N=20.

(E) Survival analysis of mouse primary fibroblasts grown in supernatants derived from co-cultures of human primary monocytes and luminal A (T47D) or TN (MDA-MB-468 and SUM-159) breast cancer cells. Annexin V staining was performed to analyze the percentage apoptotic cells. ***=$p<0.001$ ANOVA Dunn's multiple comparison test. N=10.

(F) Proliferation of mouse primary human fibroblasts grown in supernatants derived from co-cultures of human primary monocytes and luminal A (T47D) or TN (MDA-MB-468 and SUM-159) breast cancer cells, measured using a thymidine incorporation proliferation assay. **=$p<0.01$ ANOVA Dunn's multiple comparison test. N=14.

(G) mRNA expression levels of TGFβ in mouse primary fibroblasts cultured in supernatants derived from co-cultures of human primary monocytes and luminal A (MCF-7 and T47D) or TN (MDA-MB-231, MDA-MB-468 and SUM-159) breast cancer cells, assessed by RT-QPCR analysis. ns=non-significant ANOVA Dunn's multiple comparison test. N=4-8.

Supplementary Table 1.

Histological and immunohistochemistry scores of xenografts consisting of luminal A MCF-7 or triple-negative (TN) MDA-MB-231 (231) breast cancer cells, alone or co-transplanted with primary human monocytes (Mo), in NSG mice

	MCF-7 ($1x10^6$cells)	MCF-7+Mo ($1x10^6$ + $1x10^6$)	231 ($1x10^6$)	231+Mo ($1x10^6$ + $1x10^6$)
Size (mm) [1]	3	X	1	2
	3+2[2]	2	1.5	1
	3+4[2]	2.5	4	1
	3	2+1[2]	5	2
	4	2	3	4
CD11b (0-2) [1]	0	X	0	1
	0	1	0	1
	0	*1*	0	1
	0	0	0	2
	0	1	0	2
CD68 (0-3) [3]	0	X	0	1
	0	0	0	1
	0	0	0	0
	0	0	0	1
	0	0	0	1
CD163 (0-3) [1]	0	X	0	1
	0	0	0	1
	0	0	0	1
	0	0	0	3
	0	1	0	3
S100A9 (0-3) [1]	0	X	0	2
	0	0	0	2
	0	0	0	2
	0	0	0	2
	0	0	0	2
Vimentin (0-2) [1]	0	X	2	2
	0	1	2	2
	0	1	2	2
	0	1	2	2
	0	1	2	2
αSMA (0-4) [1]	1	X	2	3
	1	2	3	3
	1	1	2	3
	1	2	2	3
	2	1	2	4
β2-microglobulin (%) [1]	25	X	50-75	50-75
	10	25-50	25	>75
	10	25-50	10	>75
	25-50	75	10	50
	25-50	50-75	25	75
PDGFRβ (0-1) [1]	1	X	1	1
	1	1	1	1
	1	1	1	1
	1	1	1	1
	1	1	1	1
Sirius Red (%) [1]	50	X	25-50	75-100
	50	50-75	25	25-50
	50	50-75	25	50-75
	50	50-75	25-50	10-25
	50	75-100	25	10-25
Sirius Red (%) PL [1]	10	X	0-10	25
	25	25	0-10	0-10
	25	50	0-10	10
	25	50	0-10	0-10
	25-50	75-100	0-10	0-10

Collagen VI (0-1) [4]	0	X	0	1
	0	0	0	1
	0	0	0	1
	0	0	0	1
	0	0	0	1
CD34 (0-2) [1]	2	X	2	2
	0	1	2	2
	0	0	2	0
	1-2	1-2	2	2
	1	2	2	2

[1] For statistics see Fig. 1, Fig. 2, Fig. 3 and Supplementary Fig. 1.

[2] Two tumors

[3] Statistics for CD68 not shown in Figures: (MDA-MB-231 + Mo) CD68 expression as compared to (MCF-7 + Mo); *$p<0.05$ (t-test)

[4] Statistics for Collagen VI not shown in Figures: (MDA-MB-231 + Mo) Collagen VI expression as compared to (MCF-7 + Mo); **$p<0.01$ (t-test)

[x] No tumor

PL = polarized light

Supplementary Table 2.

Histological and immunohistochemistry scores of xenografts consisting of luminal A T47D or triple-negative (TN) SUM-159 breast cancer cells, alone or co-transplanted with primary human monocytes (Mo), in NSG mice

	T47D ($5x10^6$cells)	T47D+Mo ($5x10^6$ + $1x10^6$)	SUM-159 ($1x10^6$)	SUM-159+Mo ($1x10^6$ + $1x10^6$)
***Size (mm)*[1]**	4	1.5	3	5
	3	1.5	5	7
	X	1	3	6.5
	4	2	2	5
	4	2	3	5
***CD11b (0-2)*[1]**	0	0	0	1
	0	0	0	2
	X	0	0	2
	0	1	0	2
	0	1	0	1
***CD163 (0-3)*[1]**	0	0	0	3
	0	0	0	3
	X	0	0	3
	0	0	0	3
	0	0	0	1
***S100A9 (0-3)*[1]**	0	1	1	2
	1	1	0	3
	X	1	1	3
	0	1	1	3
	1	1	0	2
***αSMA (0-4)*[1]**	1	0	2	4
	1	1	1	4
	X	2	2	4
	0	0	2	4
	1	1	3	4
***Sirius Red (%)*[1]**	67	75	25	50
	50	75	75-100	25-50
	X	75	10	50
	25	50	75-100	50
	75	50	25	25-50
***Sirius Red (%) PL*[1]**	50	50	10-25	10-25
	25-50	75	75	10
	X	75	10	25
	25-50	50	25	10-25
	50	75	0-10	10
***Collagen VI (0-1)*[2]**	0	0	0	1
	0	0	1	1
	X	0	0	1
	0	0	1	1
	0	0	1	1

[1] For statistics see Supplementary Fig. 1 and Fig. 3.

[2] Statistics for Collagen VI not shown in Figures: (T47D + Mo) Collagen VI expression as compared to (SUM-159 + Mo); **p<0.01 (t-test)

[x] No tumor

PL = polarized light

Supplementary Table 3. Gene expression of ACTA2, Ly6C, S100A9 and CXCL16 in mouse TNBC 4T1.2 tumors compared to mouse luminal 67NR tumors.

Gene	Gene Name	Whole tumor gene array data (4T1.2 vs 67NR) (Fold change)	Adjusted P value
ACTA2	Alpha smooth muscle actin	3.51	3.34E-03
Ly6C	Lymphocyte antigen 6 complex	1.89	0.788
S100A9	S100 Calcium binding protein A9	7.03	1.75E-04[1]
CXCL16	Chemokine (C-X-C-Motif) Ligand 16	2.74	1.73E-02

[1] Already published data [2]

Supplementary Table 4.

Antibodies used for immunohistochemistry (specificity[1]; clone; dilution; distributor)

anti-CXCL16 (specific for human; ab101404 dilution 1:100; Abcam)

anti-CD11b (specific for human; clone #EP1345Y dilution 1:100; Abcam)

anti-CD163 (specific for human; clone #10D6 dilution 1:250; Novocastra)

anti-CD68 (specific for human; dilution 1:1500; DAKO)

anti-vimentin (clone #V9 dilution 1:1000; Dako)

anti-αSMA (recognizes both mouse and human origin; clone #1A4 dilution 1:1000; Dako)

anti-human S100A9 (specific for human; calgranulin B clone #H90 dilution 1:2000; Santa Cruz)

anti-mouse S100A9 (specific for mouse; ab105472 dilution 1:100; Abcam)

anti-β2microglobulin (specific for mouse; sc-8361 dilution 1:100; Santa Cruz)

anti-PDGFRβ (clone #3169 dilution 1:100; Cell Signaling)

anti-Collagen VI (recognizes both mouse and human origin; clone #H-200 dilution 1:250; Santa Cruz)

anti-HLA-ABC (specific for human; Ab70328 dilution 1:2000; Abcam)

anti-CD34 (specific for mouse; clone #MEC14.7 dilution 1:800; Santa Cruz)

anti-F4/80 (specific for mouse; clone #Cl:A3-1 dilution 1:2000; Abcam)

anti-DC-LAMP (specific for human; CD208; clone #101E1.01 dilution 1:1000; Dendritics)

anti-CD90 (specific for human; clone #EPR3132 dilution 1:250; Abcam)

anti-Ly6C (specific for mouse; ab15627 dilution 1:100; Abcam)

anti-Gr1 (specific for mouse; clone #RB6-8C5; Nordic Biosite)

---

[1] *Specificity (mouse vs human) tested for all antibodies*

Supplementary Table 5. Primers used in Quantitative real-time PCR

GENES	FORWARD	REVERSE
Mouse ACTB	CTCTGGCTCCTAGCACCATGAAGA	CATGATGCTTGATCACATGTCTCG
Mouse HPRT	CAAGCTTGCTGGTGAAAAGGAC	GTCAAGGGCATATCCTACAACAAA
Mouse GAPDH	TGCACCACCAACTGCTTAG	GATGCAGGGATGATGTTC
Mouse alpha-SMA	ACTGGGACGACATGGAAAAG	GTTCAGTGGTGCCTCTGTCA
Mouse TGF-B	GGATACCAACTATTGCTTCAGCTCC	AGGCTCCAAATATAGGGGCAGGGTC
Mouse FAP	ACTGGGTGTATATGAAGTTGAGGAC	TTCTTCATCAATGAAACCCATTT
Mouse CXCL16	product number: 100-25636, Bio-Rad	
Mouse COL6A1	CCACAGGGTGACCAAGGAAG	ACCTCGGTATCCTTTAGGTCCAA
Human ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA
Human GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Human SDHA	TGGGAACAAGAGGGCATCTG	CCACCACTGCATCAAATTCATG
Human YWHAZ	ACTTTTGGTACATTGTGGCTTCAA	CCGCCAGGACAAACCAGTAT
Human UBC	ATTTGGGTCGCGGTTCTTG	TGCCTTGACATTCTCGATGGT
Human COL6A1	ACCGACTGCGCTATCAAGAA	TCGGTCACCACAATCAGGTA

Supplementary References

1 Bergenfelz, C. *et al.* S100A9 expressed in ER(-)PgR(-) breast cancers induces inflammatory cytokines and is associated with an impaired overall survival. *Br J Cancer* **113**, 1234-1243, doi:10.1038/bjc.2015.346 (2015).

2 Johnstone, C. N. *et al.* Functional and molecular characterisation of EO771.LMB tumours, a new C57BL/6-mouse-derived model of spontaneously metastatic mammary cancer. *Dis Model Mech* **8**, 237-251, doi:10.1242/dmm.017830 (2015).

Printed by Media-Tryck, Lund 2017 NORDIC ECOLABEL, 3041 0903

LUND UNIVERSITY
Faculty of Medicine

Department of Translational Medicine,
Cancer Immunology, Malmö
Lund University, Faculty of Medicine
Doctoral Dissertation Series 2017:72
ISBN 978-91-7619-452-2
ISSN 1652-8220