# Exploring the Potential for More Effective Cancer Treatment: Investigations on Tumour Immunity and Cancer Stem Cells in Breast Cancer and Melanoma

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Lisa Speigl

aus Böblingen

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*This thesis is dedicated to my parents Gitta and Helmut Speigl and to my aunt Edith Steudle, who with their many years of support built the foundation of this work.*

*In loving memory of Gitta Speigl*

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# Zusammenfassung

Zielsetzung dieser Doktorarbeit war es, bestimmte derzeitig bestehende Hürden die einer effektiven Kebsbehandlung im Wege stehen, näher aus zwei verschiedenen Blickwinkeln zu untersuchen: Kebsstammzellen und Tumorimmunität.

Aufgrund ihrer Resistenz gegenüber konventionellen Krebstherapien wird angenommen, dass Kebsstammzellen eine wesentliche Hürde bei der effektiven Bekämpfung von Krebs darstellen, die es derzeit noch zu überwinden gilt. Im malignen Melanom ist die Existenz und Identifizierung von Krebsstammzellen nach wie vor unter anderem aufgrund vieler kontroverser Studien nicht vollständig aufgeklärt. Um zu einem besseren Verständnis hierzu beizutragen, wurde in dieser Arbeit die Expression eines Panels bestimmter Krebsstammzellmarker (ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin) sowohl *in vitro* in Melanomzelllinien als auch *in situ* in Melanomtumorgewebe, sowie in gesunden Kontrollproben untersucht. Es konnte gezeigt werden, dass die meisten der getesteten Krebsstammzellmarker von der Mehrzahl der Melanomzellen sowohl *in vitro* als auch *in situ* exprimiert wurden, allerdings wurden sie auch innerhalb der getesteten Kontrollen von differenzierten, nicht-malignen Zellen exprimiert, was darauf hindeutet, dass diese Marker nicht spezifisch für Krebsstammzellen sind. Des Weiteren sollte in dieser Arbeit die klinische Aussagekraft von ausgewählten Krebsstammzellmarkern in Melanomtumorgewebe bestimmt werden. Die Ergebnisse zeigten, dass hohe Expressionslevel des Markers ABCG2 mit einem fortgeschrittenen klinischen Stadium assoziiert waren und dass sowohl eine erhöhte ABCG2- als auch eine erhöhte CD133-Expression mit einer schlechteren Überlebenszeit von Melanompatienten korrelierte. Diese beiden Marker könnten daher zukünftig als potentielle therapeutische Targets für das maligne Melanom weiter validiert werden.

Trotz der jüngsten Expansion in der Verwendung von Immuntherapien bei vielen Krebsarten ist diese Form der Therapie immer noch keine Standardbehandlung für Patienten mit Brustkrebs. Um einen Beitrag dazu zu leisten, eine mögliche klinische Implementierung von Immuntherapie bei Brustkrebs zukünftig erleichtern zu können, war eine weitere Zielsetzung dieser Arbeit, das Immunsystem von Brustkrebspatienten näher zu charakterisieren. Hierbei sollten potentielle neue

therapeutische Angriffpunkte sowie prognostische Indikatoren identifiziert werden. In diesem Kontext wurde zum einen das Immunsystem von Brustkrebspatienten mit dem gesunder Frauen verglichen und zum anderen die Einflussnahme verschiedener Therapieansätze ermittelt. Hierfür wurden in einer ersten Studie sowohl myeloische als auch lymphatische Immunzellen im peripheren Blut von Brustkrebspatientinnen mittels multiparametrischer Durchflusszytometrie untersucht. Zudem wurden funktionale Tests durchgeführt, um zugrundeliegende Mechanismen besimmter Immunfunktionen zu identifizieren. Die Ergebnisse zeigten, dass Brustkrebspatientinnen im Vergleich zu gesunden, gleichaltrigen Frauen ein erhöhtes Level myeloischer Suppressorzellen und T-Zellen in früheren Differenzierungsstadien im peripheren Blut aufweisen. Darüber hinaus zeigten die funktionalen Tests, dass zirkulierende myeloische Zellen von Brustkrebspatienten die autologe T-Zell-Proliferation stärker unterdrückten als die entsprechenden Zellen von gesunden Frauen, was teilweise durch reaktive Sauerstoffspezies vermittelt wurde. In einer weiteren Studie wurde das periphere Immunsystem in Brustkrebspatienten, welche verschiedene chemotherapeutische Ansätze erhielten, auf Assoziationen mit der Patientenprognose untersucht. In dieser Studie konnte gezeigt werden, dass die Level von zirkulierenden T-Zellen in Patienten, die mit hoch-dosierter Paclitaxelel-Chemotherapie behandelt wurden, mit der Überlebenszeit der Patienten korrelierten. Diese Assoziation konnte hingegen nicht in Patienten mit hoch-dosierter Cyclophosphamid-Behandlung nachgewiesen werden. Entsprechende Korrelationen mit zirkulierenden myeloischen Zellen wurden in dieser Brustkrebskohorte ebenfalls identifiziert. Zusätzlich wurden in einer dritten Studie Tumor-infiltrierende T-Zellen und myeloische Zellen von Brustkrebspatienten untersucht, um deren Relevanz in Bezug auf die Patientenprognose zu ermitteln. Die Ergebnisse dieser Studie zeigten, dass Brustkrebspatienten mit einer höheren Anzahl an T-Zellen im Tumorgewebe klinisch fitter waren und eine längere Überlebenszeit aufwiesen. Im Gegensatz wiesen Patienten mit höheren Leveln an granulozytischen Zellen in der Tumormasse eine geringere klinische Gesundheit auf. Zusammenfassend zeigen die Ergebnisse dieser drei Studien, dass die identifizierten „Immunsignaturen“ sowohl im zirkulierenden Blut als auch in der Tumormasse von Brustkrebspatienten herangeogen werden können, um Einblicke in potentielle neue Biomarker zu gewinnen, welche bei der Entwicklung von neuen Immuntherapien für diese Krankheit assistieren könnten.

# Summary

This thesis aimed to better understand the current hurdles preventing more effective cancer treatment. This was investigated from two angles, cancer stem cells (CSCs) and tumour immunity.

CSCs possess the capacity of self-renewal and the ability to give rise to progeny with the potential to proliferate and differentiate. As such, these cells are able to differentiate into different lineages and clones that make up the tumour mass and as such are claimed to represent a major form of resistance to conventional therapeutic approaches which must be overcome in order to achieve better cancer treatments. In melanoma, the identification and characterization of CSCs remains incomplete, with many studies reporting conflicting findings. To bridge this gap in understanding, the expression of markers which identify CSCs in melanoma on a putative basis (ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin) was studied *in vitro* in cell lines, *in situ* in tissues and in healthy control samples. It was observed that CSC markers were expressed by the majority of melanoma cells *in vitro* and *in situ*, but they were also found on healthy differentiated tissues and cells, indicating that they are not specific markers for cancer stem cells. Furthermore, the clinical role of selected CSC markers was investigated in melanoma tissues, revealing that high levels of ABCG2 were associated with advanced clinical stage, while higher expression of both ABCG2 and CD133 correlated with poorer patient survival. These data pave the way for the validation of these moieties as therapeutic targets for melanoma in future.

Despite the recent expansion in the use of immunotherapy for many cancer types, it is still not a standard treatment for breast cancer. To assist in the clinical implementation of immunotherapy in breast cancer, this investigation sought to characterise the immune systems of breast cancer patients with the aim of identifying therapeutic targets and prognostic indicators in the following settings 1) patients compared to healthy women, and 2) under treatment with different forms of therapy. To do this, the frequencies of myeloid and lymphoid immune cells at different stages of differentiation were investigated in the peripheral blood of breast cancer patients using multiparametric flow cytometry. Functional *in vitro* assays were additionally employed to investigate mechanisms of immune function. The results revealed that

compared with healthy women of the same age, breast cancer patients have significantly elevated frequencies of cells with a myeloid-derived suppressor cell phenotype in the blood as well as higher levels of T cells at earlier stages of differentiation. Furthermore, functional testing showed that myeloid cells from breast cancer patients more potently suppressed autologous T cell proliferation than cells from healthy women, which was found to be partly mediated by the release of reactive oxygen species. The peripheral immune system was then investigated for association with breast cancer prognosis in patients under different therapeutic settings. This study found that the levels of circulating T cells in patients treated with high-dose paclitaxel-containing therapy correlated with patient survival. By contrast, patients treated with high-dose cyclophosphamide-containing therapy showed no such associations. Correlations with the level of circulating myeloid cells were also found. Considering these results for peripheral blood, T cells and myeloid cells were then investigated in the tumour mass for their relevance to patient outcome. The results of this study showed that patients with higher levels of intra-tumoural T cells were clinically fitter and experienced longer breast cancer-specific survival. In contrast, high relative levels of granulocytic cells were found in patients with poorer clinical health. Collectively, the results of these studies show that immune alterations in the blood and tumours of breast cancer patients may be used to gain insight into new prognostic biomarkers that could assist in developing new immunotherapies for this disease.

# Abbreviations

| | |
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| ABCG2 | ATP-binding Cassette sub-family G, member 2 |
| ALDH1A1 | Aldehyde Dehydrogenase 1 family, member A1 |
| ARG-1 | Arginase 1 |
| BCSS | Breast Cancer Specific Survival |
| CD | Cluster of Differentiation |
| CSCs | Cancer Stem Cells |
| DC | Dendritic Cell |
| pDCs | plasmacytoid Dendritic Cells |
| mDCs | myeloid Dendritic Cells |
| ER | Estrogen Receptor |
| FI | Fluorescence Index |
| GA | Geriatric Assessment |
| HER2 | Human Epidermal Growth Factor Receptor 2 |
| HLA | Human Leukocyte Antigen |
| HLA-DR | Human Leukocyte Antigen – antigen D Related |
| IFN | Interferon |
| KPS | Karnofsky Performance Score |
| LOFS | Leuven Oncogeriatric Frailty Score |
| MDSCs | Myeloid Derived Suppressor Cells |
| MHC | Major Histocompatibility Complex |
| mMDSCs | monocytic Myeloid Derived Suppressor Cells |
| PBMC | Peripheral Blood Mononuclear Cell |
| PFS | Progression Free Survival |
| PR | Progesterone Receptor |
| ROS | Reactive Oxygen Species |

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|---|---|
| SOD | Superoxide Dismutase |
| STAT | Signal Transducer and Activator of Transcription |
| TEMRA | Terminally differentiated Effector Memory cells re-expressing CD45RA |
| TGF-β | Transforming Growth Factor beta |
| Th | T Helper |
| TIL | Tumour-Infiltrating Leukocytes / Lymphocytes |
| Treg | Regulatory T-cell |
| TNF | Tumour Necrosis Factor |

# List of publications in the thesis

## Accepted papers

**Publication 1:**

**Speigl, L.,** Janssen, N., Weide, B., Pawelec, G. and Shipp C. Prognostic impact of the putative cancer stem cell markers ABCG2, CD133, ALDH1A1 and CD44V7/8 in metastatic melanoma. Br J Dermatol. 2017 Nov;177(5):1447-1449. doi: 10.1111/bjd.15194. Epub 2017 Jul 9.

**Publication 2:**

**Speigl L.,** Burow H., Bailur J.K., Janssen N., Walter C.B., Pawelec G., Shipp C. CD14+ HLA-DR-/low MDSCs are elevated in the periphery of early-stage breast cancer patients and suppress autologous T cell proliferation. Breast Cancer Res Treat. 2018 Apr;168(2):401-411. doi: 10.1007/s10549-017-4594-9. Epub 2017 Dec 11.

**Publication 3:**

Lafrenie, R.*, **Speigl, L.***, Buckner, C., Pawelec, G., Conlon M. and Shipp C. The frequency of immune cell subtypes in peripheral blood correlates with outcome for metastatic breast cancer patients treated with high dose chemotherapy. Clin Breast Cancer. 2019 May 27. pii: S1526-8209(19)30015-1. doi: 10.1016/j.clbc.2019.05.002. [Epub ahead of print] (* = equal contribution)

**Publication 4:**

**Speigl, L.,** Grieb, A., Janssen, N., Hatse, S., Brouwers, B., Smeets, A., Floris G., Bailur J.K., Kenis C., Neven, P., Wildiers, H., Pawelec, G. and Shipp, C. Low levels of intra-tumoural T cells in breast cancer identify clinically frail patients with shorter disease-specific survival. J Geriatr Oncol. 2018 Nov;9(6):606-612. doi: 10.1016/j.jgo.2018.03.021. Epub 2018 Apr 21.

## Manuscripts

**Manuscript 1:**

**Speigl, L.,** Janssen, N., Weide, B., Sinnberg, T., Pawelec, G. and Shipp C. Putative cancer stem cell markers are frequently expressed by melanoma cells in vitro and in situ but are also present in benign differentiated cells.

# Personal contribution

**Publication 1 and Manuscript 1:**

I contributed to…

- Experimental establishment and performance (measurement and evaluation of fluorescence intensities and cell counts in melanoma tissue and culturing and evaluation of protein expression in melanoma cell lines)
- Collection and assembly of data
- Data analysis and interpretation
- Manuscript preparation, writing and editing

**Publication 2 and Publication 3:**

I contributed to…

- Experimental establishment and performance (recruitment of patient cohorts, isolation of PBMCs, phenotypic evaluation and analysis of blood PBMCs from breast cancer patients and functional assays including inhibitor tests)
- Collection and assembly of data
- Data analysis and interpretation
- Manuscript preparation, writing and editing

**Publication 4:**

I contributed to…

- Experimental establishment and performance (measurement and evaluation of immune cell counts in breast tissue)
- Collection and assembly of data
- Data analysis and interpretation
- Manuscript preparation, writing and editing

# List of publications not embedded in the thesis

**Publication 5:**

Janssen, N., Fortis, S.P., **Speigl, L.**, Haritos, C., Sotiriadou, N. N., Sofopoulos, M., Arnogiannaki, N., Stavropoulos-Giokas, C., Dinou, A., Perez, S., Pawelec, G., Baxevanis, C. N., and Shipp, C. Peripheral T cell responses to tumour antigens are associated with molecular, immunogenetic and cellular features of breast cancer patients. Breast Cancer Research and Treatment (2017) 161(1):51-62.

**Publication 6:**

Janssen N., **Speigl L.**, Pawelec G., Niessner H., Shipp C. Inhibiting HSP90 prevents the induction of myeloid-derived suppressor cells by melanoma cells. Cell Immunol. 2018 May;327:68-76. doi: 10.1016/j.cellimm.2018.02.012. Epub 2018 Feb 21.

# Introduction

## Cancer

Cancer is one of the leading causes of deaths accounting for approximately 13% of all deaths worldwide each year. It is presently a major cause of mortality, especially in developed countries. In 2015, about 90.5 million people were living with cancer, being diagnosed at a rate of about 14.1 million new cases each year and causing 8.8 million deaths [1, 2]. The main characteristic of cancer is the formation of cells with uncontrolled growth that have the potential to invade to other parts of the body. Benign cancers are not invasive, whereas malignant cancers develop a metastatic potential and can invade adjacent parts of the body and spread to distant organs which is the major cause of death. The development of cancer is a multistep event typically consisting of genetic mutations in several different processes controlling cell behaviour. Such genomic instability contributes to the genetic diversity that triggers tumour growth. However, mutations can also be caused by many different factors, including physical, chemical or biological sources such as radiation or viruses, but also lifestyle [3, 4]. Typical mutations mostly comprise gain-of-function in proto-oncogenes and/or tumour suppressor genes resulting in a loss of function. As mostly changes in multiple genes are required to transform a normal cell into a tumour cell, an accumulation of mutations can ultimately result in cells being insensitive to death and anti-growth signals while at the same time retaining self-sufficiency for endless proliferation. Moreover, tumours establish a so-called (tumour) microenvironment consisting of physical and cellular components conducive to cancer growth (due to metabolic reprogramming of tumour cells and persistent inflammation). This represents a formidable barrier to the functioning of immune cells, thereby impairing the host's anti-tumour immune response [5]. Taken together, the main characteristics of cancer cells have consequently been summarised as the following eight hallmark features: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, reprogramming energy metabolism, evading immune destruction, inducing angiogenesis and activating invasion and metastasis [6]. Despite significant advances in the understanding of cancer and consequently in the treatment and management of cancer patients there is still no single or effective "cure" for cancer. The current therapies are still, on the whole, ineffective at curing

long-term disease. This is presumably due to the complex and multifaceted nature of cancer that allows it to adapt and overcome the commonly employed forms of single or dual therapy.

# Overcoming the hurdles of effective cancer therapy

## Part I: The alleged root of cancer: Cancer Stem Cells

### *Melanoma*

Malignant melanoma is the most aggressive form of skin cancer and one of the deadliest cancers in its metastatic form. It is estimated to be responsible for about 160,000 - 200,000 new cases and more than 65,000 deaths worldwide each year with the highest incidence rates in Australia and New Zealand. Most malignant melanomas are caused by heavy sun exposure in light-skinned populations [7-9]. Many melanomas begin with the proliferation of structurally normal melanocytes which first develop the feature of aberrant growth and subsequently acquire the ability to proliferate within the layer of epidermis (radial growth phase). This is followed by the vertical growth phase, where the lesions invade the dermis and can also extend into sub-cutaneous fat. A successful spread of cells to other areas of the skin and distant organs leads to metastatic melanoma [10, 11]. Prognostic features of malignant melanoma comprise the Clark model which describes the transformation from normal melanocytes to malignant melanoma on a histological level and the tumour thickness in millimeters measured by Breslow's depth [12, 13]. The progression of melanoma involves four clinical stages. For patients with disease stages I and II the 5-year survival rates range between 89–95% (I) and between 45–79% (II) and the melanoma can be removed by surgical excision. Patients with regional metastases (stage III) have a 5-year survival rate of 24–70%, whereas survival rates for patients with distant metastases (stage IV) range between only 7–19%. The latter two stages require systemic treatments including chemo-, radio- and/or immunotherapy [14, 15]. Consequently, metastatic melanoma is difficult to treat and despite a number of recent therapeutic advances, especially in the field of immunotherapy, a diagnosis of metastatic melanoma nowadays still foreshadows a poor long-term prognosis for the majority of patients [16-19].

### *Cancer stem cells and melanoma*

Factors believed to be associated with failure of current conventional cancer therapies include the so-called Cancer Stem Cells (CSCs) [20, 21]. CSCs share common features with corresponding tissue stem cells, such as self-renewal capacity and the ability to give rise to progeny with the potential to proliferate and differentiate. As such, CSCs possess the capacity to differentiate into various lineages and clones that make up the tumour mass and have therefore been proposed as the driving force behind tumorigenesis and the “seeds” of metastases [20]. CSCs also appear to possess the essential property of self-protection, for example through the activity of transport proteins which confer resistance to cancer drugs [20, 22, 23], while it remains to be seen if the latest immunotherapeutic drugs used to treat melanoma are capable of successfully eliminating tumourigenic CSCs. Furthermore, it has been proposed that these latest immunotherapies may even facilitate the selection of therapy-resistant CSCs [21]. Due to the roles that CSCs play in tumour biology there is consequently a great deal of interest in targeting them for clinical treatment, but the identification and roles of CSCs in melanoma remains incompletely understood [24-30]. Thus, an accurate description of CSCs as well as an understanding of their clinical relevance in different cancer types is incomplete, but were this to be achieved new avenues for cancer treatment might be opened. This in turn may provide a basis for more successful therapeutic approaches which could be developed through novel combinations of existing drugs or through entirely novel agents capable of specifically targeting tumourigenic CSCs which combat the root of cancer itself.

A number of studies has attempted to better understand the nature of CSCs in melanoma. However, major differences regarding their functional properties and expression patterns were reported [24-26, 28, 30-33]. This might be due to the use of different techniques and sample types (human, animal) across studies, but also to not testing sufficient numbers of samples.

In melanoma, some of the most commonly investigated CSC markers comprise the proteins ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin (biological functions and properties of these proteins are summarized in Supplemental Material 1 of Manuscript 1 in the appendix of this thesis). Given that markers of CSCs play roles in the pathological processes of cancer cells such as therapeutic resistance and cellular proliferation, this implies that they may also be

relevant to disease progression and patient clinical status. Indeed, a number of studies has investigated the clinical roles of CSC markers and shown that they are markers of poor prognosis across diverse cancer types. ABCG2, CD133, ALDH1A1 and CD44v7/8 have been shown to be associated with poor survival in different cancer types [34-41]. Few studies, however, have investigated these proteins in melanoma patients; it has been shown that CD133, CD44v7/8 and ALDH1A1 [42-44] are present in clinical melanoma samples, but at the time of this work their relevance to patient outcome was not yet known.

## Part II: Cancer immune system interactions

### *Breast cancer*

Breast cancer is the most common invasive cancer in women worldwide comprising 22.9% of invasive cancers in women and 16% of all female cancers. It is estimated that more than 1.7 million new cases of breast cancer occurred among women worldwide in 2012. In developed countries, breast cancer is the second most common cause of cancer-related deaths after lung cancer among women [45, 46]. The incidence of breast cancer varies around the world. In general, developed countries have the highest incidence rates and life time risk of breast cancer. All of the reasons accounting for this are not yet fully known, yet it is likely that lifestyle and reproductive factors play a large role [47, 48]. Many factors are known to increase the risk of developing breast cancer. Primary risk factors are age and being of female gender. For example, breast cancer incidence and death rates dramatically increase with age with up to a fivefold increase in risk for women above 65 years. Especially since populations in developed countries continue to increase in life expectancy and show a greater proportion of older adults, the incidence of older patients developing (breast) cancer will continue to rise [49]. These biological and sociological factors will result in a flood of elderly breast cancer patients in future, placing significant additional strain on health services. However despite these clear trends, older patients are nevertheless under- represented in clinical trials, limiting the applicability of many treatments [50]. Age-associated decline impairs the tolerability of conventional therapies, and a lack of therapeutic options specifically tailored for this patient group making their clinical management arguably more difficult [51]. Furthermore, older adults are a highly heterogeneous population in terms of physical

health, a factor which by itself can determine patient outcome. For example, frailty is common in the elderly and can influence to what extent a patient tolerates anti-tumour treatment [52]. Apart from gender and age, another non-modifiable risk factor for breast cancer are family inherited mutations in breast cancer susceptibility genes [53, 54]. However, a lot of risk factors for breast cancer are modifiable and due to lifestyle including e.g. obesity, higher levels of certain hormones, dietary factors radiation, exposure to certain chemicals and lack of exercise [46, 55-58]. The vast majority of breast cancers originate from the breast tissue epithelium lining the lobules (glands for milk production) or ducts (connecting the lobules to the nipple) and are classified as ductal or lobular carcinoma. Most breast carcinomas are invasive and infiltrate into the surrounding breast tissue. The classification of breast cancer comprises different factors: the tumour grading describing the differentiation degree of the cancer cells, the receptor status and the clinical TNM staging categorising the size of the tumor (T), whether or not it has spread to the lymph nodes (N) (stages 1 – 3) or whether it has metastasised (M) (stage 4). Breast cancer cells may or may not express the hormone receptors estrogen receptor (ER) and progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2), a growth-promoting protein [59-62]. All of these factors influence prognosis and treatment response of a patient. In general, breast cancer has a relatively good prognosis with 5-year survival rates of more than 90% for stages 1 / 2 and 72% for stage 3. Only patients with distant metastases (stage 4) tend to have a poorer outcome with a five year survival rate of about 22% [63]. Treatment of breast cancer depends on various factors including the stage and biological characteristics of the cancer. Mainstay treatments are surgery, which may be followed by chemotherapy or radiation, or both [64, 65] and in women with metastatic spread systemic therapy is the main treatment option. To date, systemic treatments comprising chemotherapy, hormonal therapy and targeted therapy have been shown to be highly effective in prolonging life [66, 67]. Targeted drugs work by binding specific molecules more common or active in cancer cells such as the growth-promoting protein HER2. Multiple medications for the treatment of this subtype, such as monoclonal antibodies directly targeting this protein but at the same time also triggering destruction by the immune system are now approved [68-70]. However, despite these steady advances in the treatment of breast cancer, overall clinical outcomes still remain suboptimal, especially for late-stage patients. There might also be a potential to employ immunotherapies for breast cancer patients in the future. However, in contrast to

melanoma and other types of cancer [71-74], immunomodulatory or cellular immunotherapy is not yet a routine form of treatment for breast cancer.

### *Facets of pro- and anti- tumour immunity*

Among other features, the microenvironment of tumours is characterised by inflammation, which is caused by immune effector cells infiltrating the tumour. Interactions between the immune system and cancer are complex and multifaceted and can result in either tumour suppression, tumour promotion, or both. On the one hand, inflammatory effector cells such as cytotoxic CD8+ T cells recognise and actively interfere with the development of the tumour. Their presence has been taken as evidence that the host is not ignorant of the developing cancer - a process referred to as “immune surveillance”, which may be sufficient to eliminate the tumour [75]. CD8+ T cells recognise peptides presented by tumour cells on MHC Class I molecules and become activated. Their activation and differentiation can result in controlling cancer growth by mechanisms including secretion of cytokines (primarily TNF-α and IFN-γ) with anti-tumoural effects, as well as direct cytotoxicity depending on the release of cytotoxic granules or via Fas/FasL interactions resulting in apoptosis of tumour cells. In addition, also CD4+ Th1-mediated adaptive immunity is important for mounting an effective anti-tumour immune response. On the other hand, immune cells may also contribute to tumour progression. This may be a consequence of mechanisms by which the tumour re-programs immune cells so that they suppress anti-tumour immune functions resulting in the tumour escape from immune control (“immune evasion”) [5, 76-78]. Examples for such tumour-promoting, immunosuppressive cell types are regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) [76, 79].

MDSCs are a relatively recently-recognised heterogeneous group of activated immature myeloid cells that negatively regulate anti-tumour immune-responses by active suppression, as their name suggests. They comprise a morphological mixture of granulocytic and monocytic cells but lack the expression of cell-surface markers associated with fully differentiated monocytes, macrophages or dendritic cells (DCs) [80]. To date, a clear phenotype to definitely describe MDSCs has not been identified. However, several combinations of different markers are currently used as phenotypic indicators for MDSCs. In humans, for example, MDSCs can be defined as the CD14+ CD11b+ CD33+ CD15+ phenotype or cells that express the CD33

marker. Also, they lack the expression of markers of mature myeloid and lymphoid cells and usually lack or down-regulate the major histocompatibility complex (MHC) class II molecule HLA-DR [81, 82]. MDSCs can be distinguished into two lineages: granulocytic MDSCs with a Lin-HLA-DR-CD33+ or CD11b+CD14-CD15+ phenotype and monocytic MDSCs expressing CD14+HLA-DRneg/low or CD11b+CD14+HLA-DRneg/low. To date, great diversity in MDSC phenotypes has been described in different human cancers, but it remains unknown whether this diversity is due to different induction mechanisms between cancer types or just the different surface markers examined by the investigators [79]. MDSCs are some of the most important players mediating tumour immunosuppression. Major mechanisms used by MDSCs to suppress beneficial immune responses include impairing T cell function such as through the release of reactive oxygen species (ROS), production of suppressive soluble molecules such as TGFβ or through arginine starvation by expression of ARG1. Also the signal transducer and activator of transcription 3 (STAT-3) has been shown to be involved in MDSC suppression [83-86].

### *Investigating the immune system in breast cancer: peripheral vs. intra-tumoural immunity*

There is an increasing number of studies investigating the role of **circulating immune populations** in cancer patients. Circulating MDSCs in patient blood have been shown to be associated with patient prognosis in a range of cancer types including breast cancer [79, 87-90]. In the latter, the investigated circulating MDSC phenotypes comprised mostly less well-defined MDSC phenotypes (those which might express monocytic or granulocytic markers but were not tested) or focused on immature/undifferentiated phenotypes (those that were negative for monocytic and granulocytic markers) [87, 91] and included one study on CD15+CD16low granulocytic MDSCs [88]. All reported an increase of the investigated MDSC phenotype in breast cancer patients and one study an association with clinical cancer stage and treatment response [87]. To date, few studies have examined the clinical role of circulating MDSCs of monocytic origin (in particular the phenotype CD14+HLA-DR-) in breast cancer patient blood [90, 92]. The main issue of the investigation of MDCSs in the periphery in breast cancer patients is certainly the inconsistency of employed markers and the fact that nearly all studies lack the employment of functional assays, a point worth considering given that mere

phenotypic characterisation is not necessarily sufficient to distinguish between MDSCs and other non-suppressive myeloid cells. In contrast to the negative prognostic impact of circulating MDSCs, the presence and quantity of certain circulating T cell populations, especially of the CD8+ cytotoxic compartment, have been associated with a more favorable outcome in breast cancer patients. For example, it was shown that breast cancer patients possessing circulating CD8+ T cells which are reactive to the HER2 antigen *in vitro* experience a better prognosis than patients without these tumour-reactive T cells [93, 94]. Generally, it needs to be taken into consideration that the majority of studies on peripheral immune signatures in breast cancer has focused on later / more progressed stages of disease, where immune changes are more profound. Interestingly, studies monitoring changes in the peripheral immune system in breast cancer patients particularly at earlier disease stages are rare to date [95, 96].

While certain different populations of circulating immune cells have been shown to correlate with favorable (e.g. cytotoxic T cells) or worse (e.g. MDSCs) patient prognosis in various cancer types, including breast cancer, an even more direct proof of cancer immune surveillance is the direct **infiltration of tumours by leukocytes**, especially lymphocytes, which are, as mentioned above, capable of directly recognising and killing tumour cells. They are referred to as “Tumour-infiltrating leucocytes”, or “Tumour-infiltrating lymphocytes”, in the case of lymphocytes (TILs), the latter being one of the most important anti-tumour factors identified to date. High levels of TILs, predominantly CD8+ cytotoxic but also Th1 CD4+ T cells have been shown to correlate with delayed disease progression and as a good prognostic factor in various human cancer types including breast cancer [97-101]. In comparison, tumour-infiltrating myeloid cells are less intensively studied and their role remains unclear. Frequencies and phenotypes of different tumour-infiltrating MDSCs have been evaluated in a number of cancer types [86, 102-104], including breast cancer [91]. However, in contrast to circulating MDCSs, very little is known about their clinical significance to date. A better understanding of such aspects may increase accuracy of predicting patient survival and lead to a more complete understanding of the factors associated with disease progression.

# Objectives of the thesis

This work aimed to better understand hurdles preventing effective cancer treatment. Studies embedded in this thesis included work on metastatic melanoma, an extremely aggressive type of cancer, and breast cancer which is the leading cause of death in women.

**Part I: The alleged root of cancer: Cancer Stem Cells**

The characterisation of CSCs in solid tumours including melanoma is still imperfect and remains incomplete, but an accurate view of them may pave the way for new cancer therapies. This work attempted to perform a comprehensive assessment of the most commonly studied CSC markers in melanoma, namely ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin. The aim was to test this comparatively large panel of putative markers in a high number of melanoma samples (including tissue and cell lines) using multiple experimental techniques in a variety of exclusively human sample types. The ultimate aim was to resolve the discrepancies between the large number of conflicting studies and to test the notion of these markers as being specific for CSCs. The specificity of these markers was examined by comparison of tumour samples with appropriate non-malignant differentiated control sample types. Secondly, because CSCs play roles in the pathological processes of cancer cells such as therapeutic resistance and cellular proliferation this implies that they may also be relevant to patient clinical features. Therefore, the aim was to uncover the prognostic impact of four of the above-mentioned putative CSC markers (CD133, ABCG2, ALDH1A1 and CD44v7/8) in melanoma tissue, because few studies have investigated these proteins in melanoma patients and their prognostic relevance is not yet known. This may contribute valuable information to the understanding of CSC markers in melanoma and may lead to a clearer definition of their clinical roles, which could open the door for new therapeutic targets and more effective cancer therapies.

**Part II: Cancer-immune system interactions**

There is growing interest in exploring the potential use of immunotherapy in for breast cancer patients. Because breast cancer comprises a heterogeneous collection of diseases, identifying the patient groups which will benefit from particular forms of immunotherapy will be of key importance. But also identifying the barriers which reduce the efficacy of immunotherapy will be required to more accurately design effective treatment strategies. Since the use of blood-based biomarkers rather than tumour tissue biomarkers provides a less invasive approach which also allows longitudinal follow-up, the aim was to assess circulating populations of myeloid and lymphoid immune cells in a cohort of female breast cancer patients, with particular emphasis on cells with suppressor phenotypes such as monocytic MDSC-like (mMDSC) cells. This may highlight which populations of immune cells could be targeted for effective immunotherapy in particular patient subgroups. Furthermore, the goal was to perform functional assays with circulating myeloid cells (including mMDSCs) from these breast cancer patients to assess their ability to suppress activation and proliferation of circulating autologous T cells including uncovering the employed mechanism to suppress T cells. The ultimate aim of this approach was to identify disease-associated alterations in breast cancer patients and to uncover suppressive mechanisms used by circulating myeloid cells, which together may provide valuable information for targeted immunotherapy approaches in future.

In addition to determining disease-associated immune alterations in breast cancer patients an additional objective was to assess the prognostic impact of the above-mentioned myeloid and lymphoid circulating immune populations. To date there are few studies investigating such associations for circulating leukocytes in breast cancer in contrast to tumour-infiltrating leucocytes, which have been widely shown to be a prognostic indicator in breast cancer. Therefore, the goal was to determine possible associations of peripheral immune populations with clinical outcome in a second cohort exclusively consisting of late-stage breast cancer patients. This cohort underwent treatment with either high-dose cyclophosphamide- or paclitaxel-based chemotherapy. Because chemotherapy is still a mainstay treatment for breast cancer (cyclophosphamide and paclitaxel are two commonly used standard agents for metastatic breast cancer) it was sought to identify if pre-treatment immune profiles could be used to predict clinical outcome in patients treated with these drugs, and if

there are differences associated with treatment type. This information may be valuable for the individualization of breast cancer patient management in future.

As age is one of the risk factors associated with breast cancer development, it is not surprising that breast cancer incidence rises dramatically with age. Therefore, adapting cancer therapies, particularly emerging immunotherapies, to elderly patients will become increasingly important in the near future. Despite this, older patients are nevertheless under-represented in clinical trials, limiting the applicability of many treatments. This work therefore additionally aimed at developing an understanding of the clinical importance of the immune system in a cohort of exclusively elderly breast cancer patients (≥70 years). In contrast to the two breast cancer cohorts above which focused on immune populations present in the circulation of patients, the aim of this third breast cancer study was to assess tumour tissue-based populations of myeloid and lymphoid immune cells in a cohort of elderly female breast cancer patients. Two intra-tumoural major immune subsets in breast tissues were examined, namely T cells and granulocytic cells. Clinical follow-up was available for this cohort, and the main aim was to examine if the levels of these two cell compartments are important for patient prognosis. Furthermore, in such studies, levels of intra-tumoural T cells and granulocytes should also be examined for possible associations with patient clinical frailty and fitness, because the global health status of older (breast) cancer patients influences their clinical course. However, this has rarely been accomplished to date, and little is known regarding the influence of the immune system on the global health of elderly cancer patients, and vice versa. By combining the measurement of clinical health and immune features, this study attempted to identify more accurate methods of assessing patient status and frailty which may be important to improve patient management and identify new therapeutic targets for elderly patients with breast cancer.

# Results and Discussion

## Part I: The alleged root of cancer: Cancer Stem Cells

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### Manuscript 1: Putative cancer stem cell markers are frequently expressed by melanoma cells in vitro and in situ but are also present in benign differentiated cells

### Publication 1: Prognostic impact of the putative cancer stem cell markers ABCG2, CD133, ALDH1A1 and CD44V7/8 in metastatic melanoma

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***Results***

In order to better understand the role of CSCs in melanoma this study aimed to 1) improve characterisation of CSCs by performing a comprehensive assessment of a panel of putative CSC markers, and 2) investigate the clinical impact of these CSC (markers). Both of these aims combined may contribute valuable information towards new therapeutic treatment strategies to more effectively combat the "root" of cancer.

To achieve these goals, in a first step 40 established melanoma cell lines were screened for their expression of the seven most commonly studied putative CSC markers in melanoma ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin by using flow cytometry. This showed all 40 cell lines to be positive for the markers ALDH1A1, CD271 and Nestin, around half of the cell lines expressed ABCG2 and three were positive for CD133 while only four expressed the CD44 splice variant isoform 7/8 and none was positive for CD44v10. We observed that these proteins were expressed similarly on all cells in the population and not on a subpopulation of positive cells expressing the markers. Investigating relationships between the expression of CSC markers revealed correlations between Nestin and ALDH1A1 ($p < 0.0001$, $r = 0.5988$) and ABCG2 ($p = 0.0193$, $r = 0.364$) expression. Interestingly, comparing the rare primary- with metastatic-derived cell lines, revealed no marked differences in the expression of the seven CSC markers, while similar

results were also obtained when comparing the established cell lines with early-passage cell strains (n = 4).

To investigate the potential environmental influence on CSC marker expression, established melanoma cell lines were additionally cultured under conditions designed to better reflect the tumour microenvironment (i.e. 2% $O_2$ and pH 6.7, here designated “experimental culture conditions”), because standard *in vitro* cell culture conditions (20% $O_2$ and neutral pH medium) are hyperoxic and do not accurately reproduce *in vivo* tumour growth conditions This showed that, compared with the conventional *in vitro* culture model, experimental culture conditions slowed growth in all melanoma cell lines ($p < 0.0001$) and reduced viability in the majority ($p < 0.01$). Altered expression of the CSC markers was also found in the experimental culture model, leading to either up- or down-regulation of the proteins. These expression changes between the conventional and experimental culture models were found to correlate with improved melanoma cell line viability in the cases of CD271 and Nestin ($p = 0.0063$ and $p = 0.0258$, respectively). Improved cell growth was observed in the cell lines up-regulating ALDH1A1 expression in the experimental model ($p = 0.0168$).

The next step in this study was to validate the results obtained *in vitro* with melanoma cell lines by comparing them with melanoma tumour tissue. To achieve this, the expression of four selected CSC markers - ALDH1A1, ABCG2, CD133 and CD44v7/8 - was examined *in situ* in an equal number of metastatic melanoma tissue deposits (n = 40). When comparing tissue with established melanoma cell lines, the four putative CSC markers showed different expression patterns *in situ*. CD44v7/8 and ABCG2 expression was more commonly observed in tissue compared with cell lines, whereas ALDH1A1 and CD133 were expressed less frequently in melanoma deposits compared cell lines. Consistent with the results in cell lines, all CSC markers were commonly expressed in the majority of melanoma cells *in situ* rather than on distinct subpopulations (exceptions were found in some ALDH1A1-expressing tissues).

To further validate these markers as genuine CSC markers, their expression on benign differentiated cells was examined in order to test their specificity as CSC markers. To achieve this, melanoma tissues and cell lines were compared to human dermal fibroblasts, primary human epidermal melanocytes from two different sources

and normal human skin in the case of CD133 and ABCG2. The results demonstrate that these seven putative CSC markers are not specific for cancer or normal stem cells, because they could be detected (either at comparable or occasionally even higher levels than in malignant cell types) in all tested benign differentiated cell types.

Given that the majority of investigated melanoma tissue specimens were positive for the four investigated putative CSC markers (ALDH1A1, ABCG2, CD133 and CD44v7/8), this cohort of late-stage melanoma patients was examined in more detail with emphasis on clinical relevance. It was found that melanoma cells within tissue specimens expressed higher levels of putative CSC markers than stromal cells for all four proteins. Correlations between the levels of ALDH1A1, ABCG2, CD133 and CD44v7/8 were also assessed in these tumour tissues. This analysis showed that ABCG2 protein levels were positively correlated with CD133 and CD44v7/8. Next, the clinical features stage, M category, progression time (time from stage III to IV and from diagnosis to stage IV), age and gender were assessed for associations with ALDH1A1, ABCG2, CD133 and CD44v7/8 expression levels. This analysis showed that patients with stage IV disease had tumours with higher levels of ABCG2 than those with stage III melanoma. Univariate survival analysis revealed that ABCG2 and CD133 but not ALDH1A1 or CD44v7/8 correlated with survival ($p = 0.0003$ and 0.0210, respectively). Multivariate survival analysis considering the patient features of age, gender, disease stage and M category showed ABCG2 ($p = 0.017$) but not CD133 to be an independent prognostic factor.

## *Discussion*

This study was performed to better understand the nature of CSCs in human melanoma, which was achieved by surveying a panel of CSC markers (CD271, ALDH1A1, Nestin, ABCG2, CD133, CD44v7/8 and CD44v10) in a large number of samples *in vitro* and by including multiple differentiated non-malignant cell types. Four of these markers (ABCG2, ALDH1A1, CD44v7/8 and CD133) were additionally investigated in an equal number of melanoma tissues *in situ*, thereby investigating marker- and sample-dependent differences that may exist.

The differences observed between cell lines and tissues in this study may be associated with selection for melanoma tumours or cells which are able to grow *in vitro* as an established cell line. The growth requirements in this artificial environment

are likely to differ substantially from those *in vivo*; thus the fraction of melanoma tumours, or individual cells within a tumour, which are able to survive surgical excision, processing and subsequent growth as a monolayer (under hyperoxic conditions) appears to select for melanoma cells or for tumours with a particular profile of CSC marker expression. It is perhaps less likely that these changes occurred during *in vitro* culture, unless they occur very early, because we observed similar results for early-passage and established cell lines. Collectively, these results suggest that the bulk of melanoma cells express similar levels of CSC markers. Contrary to a number of published studies, it was surprising that in the majority of cases, the seven tested markers did not show distinguishable sub-populations of positive and negative cells. Many prior studies which have shown CSCs to be expressed in only a small proportion of all melanoma cells used freshly resected tumour cells that have undergone enzymatic digestion. This treatment has been shown to reduce the frequency of detected tumour cells expressing CSC markers [32]. In contrast, the present work examined formalin-fixed tissue samples, or used cell lines that had undergone brief treatment with a more gentle detachment method than commonly-used trypsin, thereby potentially explaining the observation in our study which shows that CSC markers are commonly expressed in melanoma. Despite the fact that widespread expression of these proteins was observed in melanoma cell lines and tissues, it is important to note these markers are only putative and therefore could be non-specific or possibly even irrelevant for the identification of CSCs [105]. This question was addressed by testing their expression by benign differentiated cell types of related origin (primary human melanocytes, human dermal fibroblasts and normal human skin) which was aimed at revealing the specificity of these markers for CSCs. Because it was found that benign differentiated cells express these proteins as well, this weakens the proposition that the markers are specific for CSCs and leaves open the possibility that more accurate CSC markers in melanoma may still be discovered. In support of this idea, expression of ALDH1, CD44 variants, Nestin and CD133 by benign differentiated cell types was also shown in previous reports [42, 43, 106]. Because in a few cases, it was possible to compare primary- with metastatic-derived cell lines, it is unlikely that the observed results are due to dissemination of phenotypic monoclonal metastatic CSCs from a heterogeneous primary tumour.

The next objective of this study was to uncover the clinical role of the four CSC markers which were investigated in melanoma tissue (ABCG2, CD133, ALDH1A1 and CD44v7/8). The results of this clinical investigation revealed that tumours expressing high levels of ABCG2 were more likely to be of advanced stage and that these patients experienced worse survival in multivariate analysis, while patients with high levels of CD133 on their tumours also showed poor survival but in univariate analysis only. In contrast, no prognostic value for the expression of ALDH1A1 or CD44v7/8 could be detected. This was perhaps due to the testing of specific isoforms of these proteins, which does not rule out that other members of these protein families may be clinically relevant in melanoma. These four putative CSC markers examined in this study have been widely investigated in other cancer types, but to the best of my knowledge it was reported here for the first time that they are relevant in metastatic melanoma. The concept of CSCs implies that successful cancer therapy will depend on effectively eliminating these cells. As it becomes clear from all results discussed previously, much remains to be established concerning the characterisation of and possible therapeutic intervention against CSCs, but based on these results and those from other studies it is evident that putative markers of CSCs are relevant to patient outcome, which implies that they may also be clinically useful. Similar to the results reported here, the majority of prior studies shows that ABCG2 and CD133 are markers of poor surviving patients in other cancer types including esophageal, non-small cell lung, colorectal ovarian, glioma and liver cancer [34-38, 107] suggesting that they play similar roles across different cancer histologies. However, not all studies reported findings in line with this notion, and despite the studies implicating CD133 as a marker of poor prognosis, it was associated with better survival in glioblastoma [108]. ABCG2 was shown to be associated with survival in non-small cell lung cancer [35], but another study showed that it was not prognostically relevant [109]. Other investigations in ovarian and breast cancer also report that ABCG2 is not related to patient prognosis [110, 111]. It is therefore evident that the roles of CSC markers cannot be assumed but require investigation in each clinical context, whereby this study provides a new contribution by examining melanoma. Considering the proposed roles of ABCG2 and CD133 as markers of CSCs, it seems likely that their association with poor patient survival is mediated through stem-like processes. Resistance to drugs is a common feature of CSCs and ABCG2 has been shown to be a key protein involved in conferring drug resistance to cancer cells including melanoma [22, 23]. This implicates ABCG2 as a potential

mediator of therapeutic resistance in melanoma patients, and may also be relevant for immunotherapies that prime T cells against tumour cells because ABCG2 has a wide range of substrates and is associated with caspase-mediated apoptosis [112], the mechanism of cell death mediated by cytotoxic T cells. CD133 appears to play stem-like roles that differ from those of ABCG2; CD133 has been shown to be crucial for the growth and metastasis of melanoma cells [113], which may be related to the finding here that it is associated with poor survival. Aside from this, these two proteins perform other cellular functions which could also be related to their association with patient survival. For example, ABCG2 is not only involved in regulating intracellular levels of drugs, but also in regulating cellular levels of other molecules such as lipids. Given the important role that lipids play in the metabolism of cancer cells [114, 115], regulation of the uptake of extracellular molecules such as lipids may be another process by which ABCG2 plays its negative role in melanoma. Furthermore, the expression of ABCG2 and CD133 was shown to correlate in this study, suggesting that they may act in concert to assist the pathological processes of cancer cells.

Because the results of this investigation also show that melanoma cells express higher levels of these proteins compared with neighbouring stromal cells *in situ*, this suggests that their functions are also up-regulated in melanoma cells, indicating a possible causal link between their high levels and poor patient survival. Despite the finding that these markers were expressed by non-cancerous cells, the observation that melanoma cells expressed these proteins at higher levels than surrounding stromal cells suggests that therapies against them may show preferential activity against tumour cells and therefore fewer off-target side effects.

# Part II: Cancer immune system interactions

## Publication 2: CD14+ HLA-DR-/low MDSCs are elevated in the periphery of early-stage breast cancer patients and suppress autologous T cell proliferation

### *Results*

To better understand the influence that breast cancer exerts on the immune system, the aim of this study was to compare the profile of the peripheral immune system between breast cancer patients and healthy individuals. The study additionally aimed to uncover mechanisms of immune suppression mediated by certain myeloid cells in breast cancer.

To compare the immune systems between breast cancer and healthy individuals, both myeloid and lymphoid immune cell populations were measured in the peripheral blood of 40 female breast cancer patients and 25 healthy women using multi-colour flow cytomentry. The characterization of myeloid cells included monocytes, mDCs and pDCs (16 populations), while the lymphoid populations assessed consisted of effector and memory T cells in both the CD4+ and CD8+ compartments (62 populations, comprising a spectrum of cell types from early to late stages of differentiation).

Initially, clinical features of breast cancer patients were investigated for associations with the above-mentioned circulating immune populations. Certain tumour characteristics such as pathological tumour size (pT), tumour grade, HER2 status, oestrogen (ER) and progesterone (PR) receptor expression and patient age were considered. This analysis showed a number of correlations between T cell populations and breast cancer patient clinical features. For example, patients with larger tumours tended to have higher levels of earlier differentiated CD4+ T cell populations ($p < 0.01$), while CD4+ phenotypes at later differentiation stages were present at lower levels in these patients ($p < 0.01$). In addition, it was observed that a number of later differentiated populations of CD8+ T cells were negatively associated with hormone receptor expression and a number of inverse correlations between patient age and the level of CD8+ T cells including naïve CD8+ T cells ($p = 0.0001$)

and central memory phenotypes (p = 0.0167) was also observed. The fact that no relationships could be detected between age and tumour characteristics (pT, tumour grade, HER2, ER and PR), suggests that the associations between them and leukocyte levels are independent of age.

Next, the goal was to examine whether the levels of circulating lymphoid and myeloid cell populations differed between breast cancer patients and healthy control age-matched women. The results of this approach revealed that the frequencies of cells with the mMDSC suppressor phenotype (CD14+ HLADR−/ low) was significantly higher in breast cancer patients when assessing their levels as a percentage of total leukocytes (CD45+), and also relative to CD14+ monocytes (p = 0.0084 and p = 0.0105, respectively). Noteworthy was that this was also true for early-stage patients in clinical stages 1 and 2 (n = 33) (p = 0.0116 and p = 0.0151), indicating that these differences can already be detected in earlier stages of disease. These observed differences appeared to be specific for cells with the mMDSC suppressor phenotype, because no such differences in the levels of CD14+ monocytes, mDCs or pDCs between breast cancer patients and healthy women could be detected. Populations of lymphoid cells were also present at different levels between patients and controls. While the frequencies of circulating CD4+ and CD8+ T cells showed no difference between breast cancer patients and healthy women, the relative frequencies of several earlier differentiated T cell populations were elevated in breast cancer patients (p = 0.004), which was again also the case when only considering early-stage patients (p = 0.0026). In contrast, later differentiated T cells lacking the expression of CD45RA tended to be lower in breast cancer patients than healthy women (p = 0.0466).

Because the previously mentioned results revealed that cells with an mMDSC phenotype (CD14+ HLA-DR−/low) were elevated in breast cancer patients, their suppressive capacity was then examined. In order to model immune suppression *in vivo*, the suppressive potential of equivalent numbers of isolated CD14+ cells was compared between breast cancer patients and healthy women. Because CD14+ HLA-DR−/low mMDSCs, but not the total levels of CD14+ monocytes were elevated in breast cancer patients, it was asked whether these cells with an mMDSC phenotype from breast cancer patients had greater suppressive capacity. To

determine the suppressive properties of circulating CD14+ HLA-DR−/low mMDSCs from patients, isolated CD14+ cells were co-cultured with autologous CD14-depleted PBMCs. CD14-depleted PBMCs were labelled with the proliferation marker CFSE and stimulated with CD3/CD28 beads, and the degree of proliferation by CD4+ and CD8+ T cells measured by flow cytometry after five days.

Following the culture period, consistent suppression of CD4+ and CD8+ T cell proliferation by CD14+ myeloid cells from breast cancer patients was found ($n = 5$) ($p < 0.0001$). To investigate whether this suppressive capacity by circulating myeloid cells was specific to breast cancer patients, the suppressive capacity of myeloid cells from healthy age-matched women was also determined ($n = 4$). This revealed that myeloid cells from healthy women could also suppress proliferating T cells, but the suppressive capacity was less pronounced when compared to breast cancer patients ($p = 0.0037$). To investigate the mechanism(s) potentially responsible for the suppressive capacity of CD14+ myeloid cells from breast cancer patients, these cells were treated with inhibitors targeting different suppressive pathways in myeloid cells namely TGF-β, ROS and STAT-3. It was observed that inhibition of ROS partially restored T cell proliferation ($p < 0.001$), while also weak effects for anti-TGFβ ($p = 0.01$) and anti-STAT-3 ($p = 0.001$) were found. Compared with untreated cultures, those treated with the ROS inhibitor restored CD8+ T cell proliferation by 131% and CD4 73% on average. Treatment with anti- TGFβ and anti-STAT-3 only led to a 34% (CD8) and 11% (CD4) and 44% (CD8) and 31% (CD4) proliferation increase compared with untreated cultures, respectively.

## *Discussion*

This study was performed to gain an indication as to which immune cells could potentially be targeted for more effective immunotherapy in breast cancer. To achieve this, the identification of immune differences in breast cancer patients compared with healthy age-matched women on both a phenotype and functional level was considered an important first step. Characterising such “signatures” might provide a good resource of identifying potential targets for immunotherapy. Due to public health campaigns and medical advances, breast cancer is now typically diagnosed at an early stage of disease, which is why it was additionally examined if immune perturbations occur early in disease development because such studies in

early-stage breast cancer are rare [95, 96]. The finding that CD14+HLA-DR-/low mMDSCs are elevated in early-stage breast cancer patients suggests immune suppression early in the development of breast cancer, which may help protect tumour cells from immune attack. Interestingly, total monocyte frequencies were not found to differ between breast cancer patients and healthy women, indicating that the pool of CD14+ myeloid cells in breast cancer to be selectively driven towards MDSC differentiation, thereby leaving other populations of myeloid cells unaffected. Indeed, levels of other myeloid cells, such as DCs, were also not found to be different in breast cancer patients and controls. The results of this work also showed that CD14+ myeloid cells from breast cancer patients are potent suppressors of autologous T cell proliferation, likely reflecting the finding that CD14+ HLA-DR−/low mMDSC phenotype cells are present at higher levels in these patients. The potential association between CD14+ HLADR−/ low mMDSCs and immune suppression is supported by other studies showing the suppressive features of this particular mMDSC phenotype in a number of other cancer types [116-118]. It was observed that inhibiting ROS partially restored immune suppression by CD14+ myeloid cells, implicating it as a suppressive mechanisms used by breast cancer mMDSCs, as previously shown in other cancer types [119-121]. It was additionally found that levels of early differentiated T cells were elevated in breast cancer patients compared with healthy women. Higher levels of these cells indicates potential for the immune system to recognize novel or newly arising tumour antigens present in the tumour and thus to mount an immune response against tumour cells. This anti-tumour potential might be counter-balanced by the simultaneous finding of elevated CD14+ HLA-DR−/low mMDSCs in these patients, which may suppress the activity of beneficial T cells, for example by preventing their differentiation. Indeed, elevated levels of mMDSCs as well as more immature T cells were found to be elevated in breast cancer patients. This association suggests that mMDSCs may impair the maturation of T cells in cancer patients, which is supported in a prior study of our working group where it was observed that patients with tumour antigen-reactive T cells experienced greater clinical benefit if they also had low MDSC levels [93]. In contrast to T cells in the periphery, T cells infiltrating the tumour (TILs) have frequently been shown to be associated with favourable prognoses in breast cancer [95, 100, 101, 122]. The relationship between peripheral and intra-tumoural TILs in breast cancer is not yet known, and thus it cannot be judged whether the herein observed altered levels of circulating T cells in breast cancer patients relates to the

presence of T cells in the tumour. It is conceivable that higher levels of T cells in the blood may act to support the maintenance of intratumoural T cells, but such associations remain to be confirmed. However, it should certainly be considered that immune cells in the periphery may play different roles to those infiltrating the tumour.

## Publication 3: The frequency of immune cell subtypes in peripheral blood correlates with outcome for metastatic breast cancer patients treated with high dose chemotherapy

### *Results*

In contrast to the previous study in earlier stage breast cancer, this investigation sought to identify peripheral immune features in late-stage breast cancer patients that relate to patient outcome and response to therapy. This was studied in a cohort of 88 female breast cancer patient treated with two different high-dose chemotherapy regimens: high-dose cyclophosphamide-containing chemotherapy (patient Group 1, n = 51) and high-dose paclitaxel-containing chemotherapy (patient Group 2, n = 37).

Both myeloid and lymphoid peripheral immune cell populations (this included monocytes, mDCs, pDCs as well as effector and memory CD4+ and CD8+ T cells) were measured using multi-colour flow cytomentry. Experimental data were analyzed for the entire patient cohort, and to identify treatment dependent differences, separately for patients in Group 1 (cyclophosphamide-containing chemotherapy) or Group 2 (paclitaxel-containing chemotherapy). Clinical endpoints were progression-free survival (PFS) and breast cancer specific mortality (BCSS).

The clinicopathological characteristics showed that patients in Group 1 and Group 2 were very similar with regard to age, hormone receptor status, and sites of metastasis. The clinical outcome was also found to be very similar, and there were no differences in PFS or BCSS between the groups, suggesting that the efficacy of the different therapies were equivalent.

The examination of circulating myeloid cell populations revealed that higher levels of mMDSCs (CD14+HLA-DR-/low) were associated with longer BCSS, however this was only found in patients treated with cyclophosphamide-containing chemotherapy (Group 1) ($p = 0.04$). The frequency of monocyte populations (CD14+HLA-DR+) was

also found to correlate with BCSS for the entire cohort of patients as well as patients in Group 1 (but not Group 2) ($p < 0.05$). A higher frequency of mDCs (CD14-HLA-DR+CD11c+CD123-CD16+) correlated with longer survival both for the whole group of patients ($p = 0.019$) and for patients in Group 2 ($p = 0.023$). In line with this, a higher frequency of pDCs (CD14-HLA-DR+CD11c-CD123+), also indicated longer BCSS for patients in Group 2 ($p = 0.036$).

Circulating lymphoid populations were also observed to correlate with survival. In the whole cohort of patients, several T cell phenotypes were associated with survival. This included T cell populations ranging from less mature naïve cells to more mature memory phenotypes. Separating the entire patient cohort according to treatment type revealed that the associations were almost exclusively restricted to patients in Group 2, as they were not found in Group 1 patients. A large number of T cell populations correlated with prognosis in Group 2, for example, higher frequencies of CD4+ and CD8+ central memory T cells (CD4/8+CD95+CD45RA-CD27+CD28+) in the peripheral blood showed better prognosis ($p = 0.0003$, $p = 0.011$, respectively), whereas higher levels of CD4+ and CD8+ naïve T cell phenotypes (CD4/8+CD95-CD45RA+ CD27+CD28+) were found in patients with shorter survival times ($p = 0.027$ and $p = 0.002$, respectively). Moreover, it was observed that a higher frequency of CD95-expressing CD4+ and CD8+ T cells was associated with better prognosis for patients in Group 2 ($p = 0.0002$ and $p = 0.002$, respectively).

## *Discussion*

The results from this study showed that the relative levels of certain monocyte, dendritic cell, and CD4+ and CD8+ T cell subtypes correlated with BCSS in metastatic breast cancer patients treated with high-dose chemotherapy. These associations were dependent on the chemotherapy regimen used to treat the patients. Monocyte populations could predict prognosis for patients treated with high-dose cyclophosphamide-based chemotherapy (Group 1), while differences in certain CD4+ and CD8+ T cell phenotypes predicted prognosis for patients treated with high-dose paclitaxel-based chemotherapy (Group 2). Apart from the different treatments received, patients in Group 1 and Group 2 had very similar clinical features, such as age, hormone receptor status, and clinical outcome following treatment. Because the blood for this study was drawn prior to treatment with chemotherapy, the results represent the baseline frequency of the immune populations. As such, the type of

therapy received is an essential factor that should be considered on an individual basis in order to predict patient outcome using immune phenotyping.

Previous studies have shown that different types of chemotherapy have differential effects on specific populations of immune cells. For example, treatment with doxorubicin or paclitaxel was shown to eliminate MDSCs in a mouse model [123, 124], while treatment of patients with paclitaxel can lead to a reduction of peripheral MDSCs [125] and an improvement of immune responses [126]. In contrast, cyclophosphamide treatment can increase MDSCs [127, 128] and strongly decrease the level of both cytotoxic and T helper cells [129]. Differences in the effects of the varying chemotherapy treatments can be complex and hard to define. For example, paclitaxel treatment eliminates MDSCs which could minimise their impact post-treatment, but has minimal effects on T cells which allow the effects of baseline T cell levels to remain prognostic. In support of this, a higher frequency of CD14+HLA-DR- MDSCs in this study was unexpectedly associated with superior survival for patients treated with high-dose cyclophosphamide. This finding is different from previously published results under other conditions where higher levels of MDSCs in breast cancer patients prior to different treatments were associated with poorer prognosis [93, 130, 131]. These discrepancies might be explained by the different treatment protocols employed (dosage, schedule and drug), but also variations in the approach used to identify MDSCs between studies such as different surface marker antibody panels and software analysis methods. Furthermore, prior studies looking at granulocytic MDSCs may be limited by the use cryopreserved samples which deplete granulocytic MDSCs [132]. It should also be considered that the levels of MDSCs in the circulating blood may not necessarily relate to their levels in tumour tissue [132]. Therefore, circulating MDSC frequencies may not be fully predictive of the anticipated immunosuppressive pro-tumor effect. In this study, the frequency of MDSCs did not correlate with outcome for patients treated with high-dose paclitaxel containing chemotherapy in contrast to high-dose cyclophosphamide treatment.

The results of this study showed that in addition to myeloid cells, the levels of circulating lymphocytic immune cells also indicated patient prognosis. For example, a high frequency of naïve CD4+ and CD8+ T cells was associated with a relatively poor prognosis for patients with metastatic breast cancer treated with high-dose paclitaxel-containing chemotherapy. Naïve T cells have not yet been exposed to antigen [133] and therefore are not anticipated to be involved in mediating anti-tumor effects. In

contrast, a high frequency of previously antigen-exposed effector/memory CD4+ and CD8+ T cells was found to be associated with improved prognosis, consistent with the notion that these cells promote anti-tumour activity. This finding is consistent with previous reports, where an increased frequency of more mature (CD95+) tumour-infiltrating cells was also shown to be associated with improved prognosis [134].

## Publication 4: Low levels of intra-tumoural T cells in breast cancer identify clinically frail patients with shorter disease-specific survival.

### *Results*

In contrast to Publication 2 and Publication 3 where myeloid and lymphoid immune populations were assessed in the circulating blood of breast cancer patients, in this study, myeloid and lymphoid immune cells were assessed in the tumour tissues of breast cancer patients. To achieve this, a cohort of 58 elderly breast cancer patients was examined for tumour-infiltrating CD3+ (T-cells) and CD15+ (granulocytic) cells using fluorescence microscopy. To better compare with the previous studies in this thesis, it was initially aimed to test for monocytic (CD14+) myeloid cells. However, due to poor performance of the different antibodies tested, granulocytic cells were chosen to represent the myeloid compartment because they can also differentiate into MDSCs.

The results of this study showed that breast tumours of this elderly patient cohort are commonly infiltrated by both CD3+ (T-cells) and CD15+ (granulocytic) cells: 93% of breast tissues contained T cells (mean cell count of 17, range 0 – 108), while granulocytic cells occurred less commonly but still in 64% of tumours and at substantially lower abundance (mean 1, range 0 – 11). There was no difference in the level of infiltration by CD3+ or CD15+ cells between the tumour centre and invasive front.

It was also investigated whether the degree of tumour infiltration by CD3+ and CD15+ cells was associated with tumour features in elderly breast cancer patients. This revealed that patients with higher tumour grade had tumours that were more heavily infiltrated by CD3+ cells ($p = 0.0321$), while patients with higher levels of CD3+ cells in the tumour had lower or absent expression of oestrogen and

progesterone receptors (p = 0.0236 and 0.0367, respectively). In contrast, tumour infiltration by CD15+ cells showed the opposite relationship for PR expression (p = 0.0208). In addition, the global health status of elderly breast cancer patients was measured using different performance measures including the Balducci method and more detailed methods assessing clinical frailty, such as the Karnofsky performance scale (KPS), G8 geriatric assessment and the Leuven Oncogeriatric Frailty Score (LOFS) [135]. Because in a prior study [30] performed by our working group it was found that the levels of leukocytes in blood relate to the clinical health of elderly breast cancer patients, the present work further investigated these findings by examining whether immune features in the tumour tissues of elderly patients are also associated with patient health or performance. Examining the levels of tumour-infiltrating CD3+ and CD15+ cells in the context of patient performance measures revealed a number of associations: patients with a higher abundance of tumour-infiltrating CD3+ cells were fitter according to G8 geriatric assessment (p = 0.0060), performance status measured by KPS (p = 0.0372) and showed better and superior status according to the LOFS (p = 0.0187). The opposite trend was found for infiltration of CD15+ cells: patients with tumours that were more densely infiltrated by total levels of granulocytic cells were less fit according to LOFS (p = 0.0474), in line with previous work from our group which found higher levels of granulocytic cells in blood to be inversely associated with LOFS [30]. Importantly, selecting patients who showed high levels of T cells in combination with low levels of granulocytes emphasised these relationships by revealing a relatively homogeneous group of patients with high LOFS scores (p = 0.0006). Because these results show that the clinical health of elderly breast cancer patients is reflected by the immune state within the tumour, it was then asked whether the levels of leukocytes in tumour tissue are also informative for patient prognosis. Correlating levels of CD3+ cells and CD15+ cells with patient survival showed higher total levels of intra-tumoural T cells in patients with longer disease-specific survival up to five years following diagnosis (p = 0.0444) in univariate analysis. In contrast to T cells, granulocytic cells, irrespective of location in the tumour, were not associated with patient clinical course. Overall survival which considers all causes of death was not found to be associated with levels of intra-tumoural leukocytes.

### *Discussion*

Examining the tumours of elderly breast cancer patients for infiltration by certain immune cells revealed that both CD3+ and CD15+ cells were commonly found within these breast tumours, which suggests that breast cancer in elderly patients may be generally immunogenic. Additionally, the immune parameters measured here were found to be clinically relevant; patients with higher tumour grade had greater numbers of intra-tumoural CD3+ cells compared with patients with lower grade tumours. Higher CD3 levels also negatively correlated with the expression of oestrogen and progesterone receptors, in line with prior studies showing that hormone receptor-negative breast tumours are more heavily infiltrated by TILs [136, 137]. Although immune cell infiltration in breast tumours is well-recognised, little is known regarding the relationship between the immune system and the fitness of older cancer patients. The results reported here suggest that the state of the immune system may influence the functioning of older breast cancer patients, or vice versa. A number of associations between the level of intra-tumoural CD3+ and CD15+ leukocytes and patient clinical health or performance were identified, in turn proposing immune features as potential biomarkers for the clinical health of cancer patients. Patients with higher levels of intra-tumoural CD3+ T cells were fitter and had higher patient performance status according to their KPS, LOFS and G8 scores. In contrast, high levels of CD15+ granulocytic cells in the tumour were found in patients with inferior health status according to LOFS. Collectively, these results suggest that better patient health and functioning is linked to high levels of intra-tumoural T cells, whereas the presence of granulocytic cells appears to be associated with poorer health in older patients. However, it remains to be clarified whether the intra-tumoural immune status leads to better clinical health, or if superior clinical health results in improved immune status. Increasing age is associated with elevated output of myeloid cells and reduced output of naïve T cells, the latter primarily due to thymic involution [138, 139]. Accordingly, patients with tumours which reflected these typical age-associated immune alterations (low intra-tumoural T cells and high myeloid cells) had inferior health. In contrast, patients who showed an opposite relationship – i.e. tumours with higher levels of T cells or lower levels of granulocytic cells, tended to show better health scores. Consequently, these findings may suggest that the immune system in older non-fit patients is a potential therapeutic target in which immunostimulatory drugs could assist in combatting the tumour or result in a higher

level of patient fitness. The immune differences found between fitter and frail patients may be related to differing capacities for effective immune surveillance. For example, patients with better-functioning immune systems may retain more functionally-active T cells, reflected here by greater ability to migrate to the tumour site. On the other hand, the characteristic accumulation of myeloid cells associated with age could indicate elevated levels of MDSCs, which suppress beneficial immune responses including those against tumour cells [79]. Therefore, an accumulation of granulocytic MDSCs in the tumour may result in suppressed anti-tumour immune responses, sequentially relating to a suppressed immune system and impaired health. In accordance with the finding that high relative levels of intra-tumoural T cells are present in patients with superior health, it was also found that longer-surviving patients had tumours with a higher abundance of T cells, which is consistent with previous studies showing this correlation for TILs in general [140-142]. Collectively, these results suggest that intra-tumoural T cells are important for elderly breast cancer patient prognosis and overall performance.

# Conclusions and outlook

### Part I: The alleged root of cancer: Cancer Stem Cells

**Publication 1 and Manuscript 1** of this thesis showed that the expression of CSC markers can differ depending on the nature of the sample type, which may provide some explanation for the large numbers of conflicting studies previously reported in melanoma. It was observed that the investigated putative CSC markers are commonly expressed in both melanoma cell lines and tissue, and that they are associated with important features such as viability or cell growth of melanoma cells. Unlike many prior studies in the field, the inclusion of differentiated non-malignant samples alongside malignant samples in this work allowed me to investigate the specificity of these markers to identify CSCs. This revealed widespread expression of these proteins in non-malignant cells, thereby questioning their value as CSC markers. However, to the best of my knowledge this work is the first to demonstrate that the putative CSC markers ABCG2 and CD133 are negative prognostic factors in melanoma, which may (despite their presence in non-malignant cells) set the stage for their validation as novel therapeutic targets for malignant melanoma patients.

Future work should focus on validating these results in larger patient cohorts, followed by preclinical targeting studies both *in vitro* and *in vivo,* and then subsequent translation into clinical trials which will also consider possible side effects due to the non-specificity of the proteins for melanoma cells.

## Part II: Cancer immune system interactions

In **Publication 2** of this thesis it was found that compared with healthy women, systemic immune alterations in breast cancer patients occur early in disease development. The finding that CD14+ HLA-DR−/low mMDSCs were elevated in breast cancer patients supports the potential use of strategies targeting this population of cells in breast cancer patients. Because inhibiting ROS alleviated the suppressive capacity of breast cancer CD14+ myeloid cells, antioxidant treatment approaches could be utilized in breast cancer immunotherapy. However, since inhibiting ROS could not completely restore T cell proliferation, this suggests that a combination of other mechanisms may contribute to immune suppression, or that ROS inhibition was incomplete. Further functional assays exploring a wider spectrum of candidate suppressive pathways may uncover more information regarding the mechanisms used by these cells to exert immune suppression in breast cancer. In conclusion, developing a more detailed picture of interactions between disease-associated factors and their effects on different immune cell populations (particularly MDSCs) will be crucial for the development of novel effective immunotherapeutic approaches. Moreover, the results obtained here require validation in a second larger independent cohort with clinical follow-up to reveal whether these observed immune signatures could potentially be used as biomarkers, and if they are also important for patient prognosis.

**Publication 3** investigated a cohort of late-stage breast cancer patients treated with high-dose chemotherapy to identify immune cell populations in the circulating blood which may act as prognostic markers. It was found that the levels certain immune cell populations, including monocytic cells, dendritic cells, CD4+ and CD8+ T cells correlated with patient prognosis, but that this was highly dependent on the type of chemotherapy received. This study therefore identified therapy-specific prognostic indicators which could be utilised as potential biomarkers to predict patient outcome. The information derived from this study may therefore be valuable for the improvement of personalised management of breast cancer patients in future: such

pre-treatment biomarkers could be used to employ the “best fitting” therapy for breast cancer patients would receive on an individual basis. These results may possibly also apply to other cancer types as well as to different treatment types, such as other chemotherapy regimens, targeted therapies or immunotherapy. It will be crucial to better define such interactions in future to more effectively employ currently available cancer therapies.

**Publication 4** of this thesis demonstrated that certain populations of tumour-infiltrating leukocytes are associated with the fitness and prognosis of elderly breast cancer patients. To the best of my knowledge, the results presented here are the first to reveal that the intra-tumoural immune profile is associated with patient health and performance parameters. These findings closely link the intra-tumoural immune state in breast cancer with patient health and survival, suggesting that the immune system plays important roles in maintaining the overall functioning and survival of older individuals with (breast) cancer. This might be achieved by more effective immune surveillance mechanisms in longer-surviving patients or those with higher functional status, in which the immune system is better able to control chronic or acute infections in addition to providing more effective protection against cancer. As for all previously reported results, this study requires validation in a larger independent cohort. This would allow a more robust investigation additionally considering other known prognostic factors in a multivariate analysis model including factors like tumour stage and treatment type.

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# Appendices

## Accepted papers and submitted manuscripts

**Publication 1:**

**Speigl, L.,** Janssen, N., Weide, B., Pawelec, G. and Shipp C. Prognostic impact of the putative cancer stem cell markers ABCG2, CD133, ALDH1A1 and CD44V7/8 in metastatic melanoma. British Journal of Dermatology. 2017 Nov;177(5):1447-1449. doi: 10.1111/bjd.15194. Epub 2017 Jul 9.

**Publication 2:**

**Speigl L.,** Burow H., Bailur J.K., Janssen N., Walter C.B., Pawelec G., Shipp C. CD14+ HLA-DR-/low MDSCs are elevated in the periphery of early-stage breast cancer patients and suppress autologous T cell proliferation. Breast Cancer Res Treat. 2018 Apr;168(2):401-411. doi: 10.1007/s10549-017-4594-9. Epub 2017 Dec 11.

**Publication 3:**

Lafrenie, R.*, **Speigl, L.***, Buckner, C., Pawelec, G., Conlon M. and Shipp C. Frequency of immune cell subtypes correlates with outcome for patients with metastatic breast cancer treated with high dose chemotherapy. Clin Breast Cancer. 2019 May 27. pii: S1526-8209(19)30015-1. doi: 10.1016/j.clbc.2019.05.002. [Epub ahead of print]

* = Lafrenie, R. and Speigl, L. contributed equally to this work

**Publication 4:**

**Speigl, L.,** Grieb, A., Janssen, N., Hatse, S., Brouwers, B., Smeets, A., Floris G., Bailur J.K., Kenis C., Neven, P., Wildiers, H., Pawelec , G. and Shipp, C. Low levels of intra-tumoural T cells in breast cancer identify clinically frail patients with shorter disease-specific survival.. J Geriatr Oncol. 2018 Nov;9(6):606-612. doi: 10.1016/j.jgo.2018.03.021. Epub 2018 Apr 21.

**Manuscript 1:**

**Speigl, L.,** Janssen, N., Weide, B., Sinnberg, T., Pawelec, G. and Shipp C. Putative cancer stem cell markers are frequently expressed by melanoma cells in vitro and in situ but are also present in benign differentiated cells. *(submitted)*

# Research letter

## Prognostic impact of the putative cancer stem cell markers ABCG2, CD133, ALDH1A1 and CD44V7/8 in metastatic melanoma

DOI: 10.1111/bjd.15194

Dear Editor, Despite recent therapeutic advances, a diagnosis of metastatic melanoma still foreshadows a grim prognosis for the majority of patients.[1,2] So-called cancer stem cells (CSCs) are believed to be associated with the failure of current conventional cancer therapies.[3] CSCs share common features with corresponding tissue stem cells, such as self-renewal capacity and the ability to give rise to progeny with the potential to proliferate and differentiate. Thus, therapeutically targeting CSCs may eliminate the root cause of the cancer; however, this would require the accurate identification of markers distinguishing CSCs from normal cells. The expression of candidate CSC markers is associated with a poor prognosis in a number of cancer types,[4–8] but their clinical significance remains unclear, and to the best of our knowledge there have been no clinical studies in melanoma. Here, we aimed to determine the clinical significance of four molecules identifying putative CSCs: CD133, ABCG2, ALDH1A1 and CD44v7/8.

Using fluorescence microscopy we determined the expression of CD133, ABCG2, ALDH1A1 and CD44v7/8 in formalin-fixed, paraffin-embedded metastatic lesions from 40 patients [24 men, 16 women; 15 patients with stage III melanoma, 25 patients with stage IV melanoma; mean age 63 years (range 39–89); survival time < 5 years (see Table 1 for a detailed description of the patient cohort)] treated at Tübingen University Hospital's dermatology department (ethics approval number 017/2016BO2). The following antibodies were used to detect CSC markers in melanoma tissues: ALDH1A1 rabbit monoclonal [lot: GR41450-6; clone: EP1933Y (Abcam, Cambridge, U.K.)], CD133 rabbit polyclonal (lot: X13030523; Fitzgerald, Acton, MA, U.S.A.), ABCG2 mouse monoclonal [lot: D15KF02234; clone: BXP-21 (Biolegend, San Diego, CA, U.S.A.)] and CD44v7/8 mouse monoclonal [lot: 051114; clone: VFF-17 (Bio-Rad, Hercules, CA, U.S.A.)]. When comparing expression of these proteins between melanoma and stromal cells, the Melan A mouse monoclonal antibody [lot: 00060204; clone: A103 (Dako, Hamburg, Germany)] was used to confirm the identity of melanoma cells in a subset of samples (n = 8, data not shown). The secondary antibodies Alexa Fluor 488 donkey antirabbit IgG (H+L) and Cy3 donkey antimouse IgG (H+L) (both from Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) were used for all experiments. Optimum staining conditions were determined for all antibodies individually. Tissues were stained according to a previously described protocol but using the following antigen retrieval method.[9,10] Fluorescence intensity of each tissue was assessed by comparison with a control tissue (mounted to the same slide and stained with secondary antibody only) to give a fluorescence index (fold-increase in stained tissue over control tissue). An average of 12 images per tissue covering the entire tumour were captured at ×20 magnification. Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Inc., La Jolla, CA, U.S.A.) and SPSS version 24 (IBM, Ehningen, Germany).

We observed that the majority of tissue specimens were positive for ALDH1A1 (n = 28), ABCG2 (n = 32) and CD133 (n = 38), while CD44v7/8 was expressed less frequently (n = 15) (Fig. 1a). In general, we observed similar expression of these proteins by all tumour cells within a tissue. Representative tissues stained for each protein are shown in

**Table 1** Clinical characteristics of the patient cohort

| Factor | Patients (n = 40) |
|---|---|
| Age (years) | |
| < 63 | 20 (50) |
| > 63 | 20 (50) |
| Sex | |
| Male | 24 (60) |
| Female | 16 (40) |
| Melanoma stage | |
| III | 15 (38) |
| IV | 25 (63) |
| Melanoma category | |
| M1a or b | 8 (20) |
| M1c | 17 (43) |
| NR | 15 (38) |
| Pretreatment | |
| None | 12 (30) |
| Chemotherapy | 19 (48) |
| Immunotherapy | 22 (55) |
| Radiation | 9 (23) |
| Metastasis location | |
| Trunk | 10 (25) |
| Extremity | 20 (50) |
| Head and neck | 7 (18) |
| NA | 3 (8) |

Data are n (%). NR, not relevant; NA, not available.

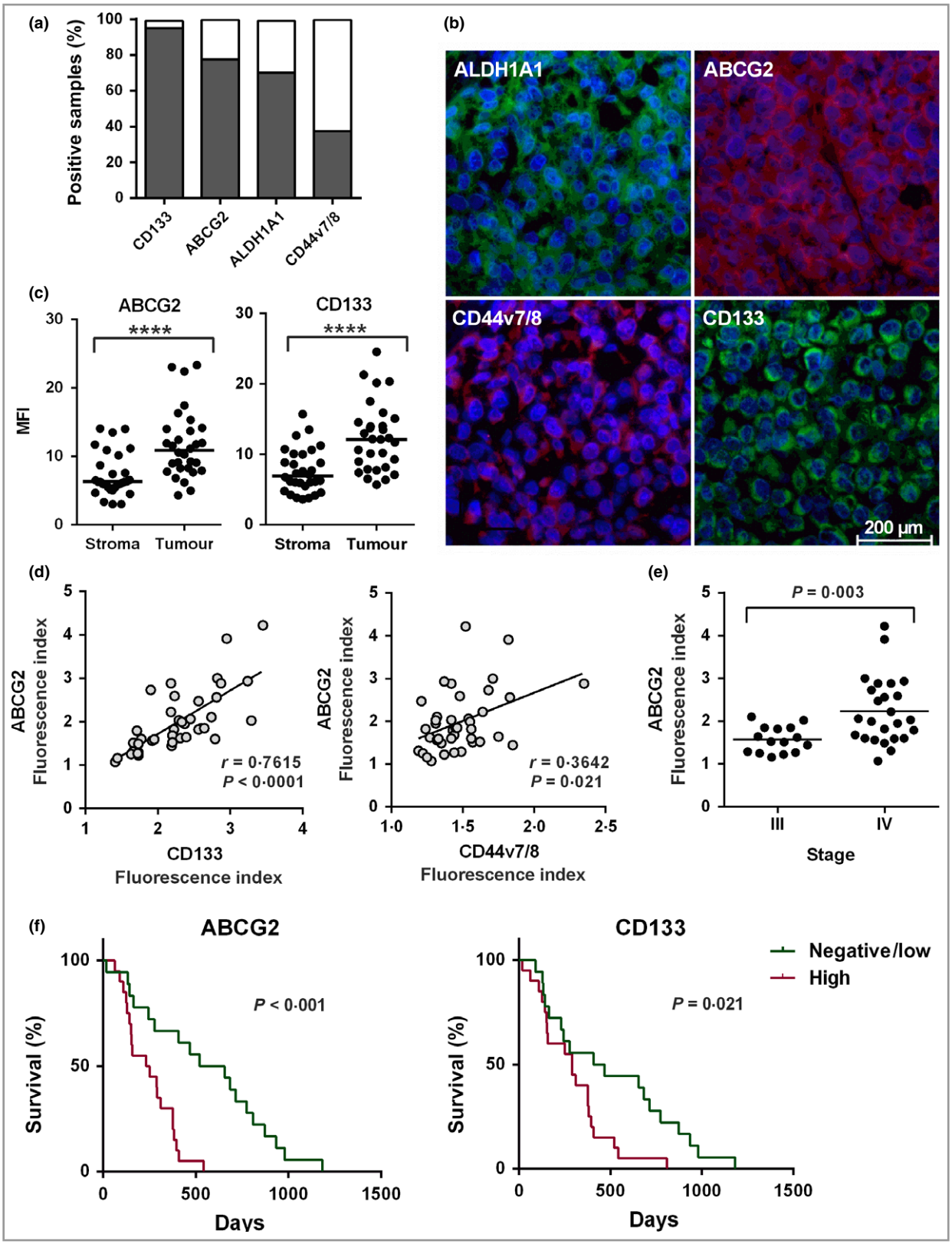


Figure 1(b). Of note was that compared with stromal cells, melanoma cells expressed higher levels of all four proteins (Fig. 1c, selected results shown). Furthermore, we found that expression of the CSC marker ABCG2 correlated with CD133 ($P < 0·0001$) and CD44v7/8 ($P = 0·021$) in melanoma tissues (Fig. 1d). We confirmed coexpression of ALDH1A1 with

**Fig 1.** Cancer stem cell (CSC) markers are widely expressed in melanoma tissues and correlate with patient clinical features. (a) Melanoma tissues commonly express CD133, ABCG2, ALDH1A1 and CD44v7/8. (b) Representative images (×40 magnification) of tissues positive for each of the CSC markers investigated. (c) Melanoma cells show higher expression of CSC markers than surrounding nonmelanoma cells (ALDH1A1 and CD44v7/8 not shown) (****$P < 0{\cdot}0001$). (d) ABCG2 expression correlates with the levels of CD133 and CD44v7/8. (e) Higher levels of ABCG2 were found in patients with stage IV melanoma than in those with stage III melanoma. (f) Kaplan–Meier survival analysis shows that patients with high levels of ABCG2 and CD133 experience worse prognosis [cohort divided into 'high' and 'low' based on the median fluorescence index (MFI)].

CD44v7/8 and ABCG2 with CD133 (data not shown) (owing to the experimental set-up other combinations of coexpression were not possible to test directly). We then evaluated expression levels of ALDH1A1, ABCG2, CD133 and CD44v7/8 for association with clinical features, including stage, M category, progression time (stage III–IV and diagnosis to stage IV), age and sex. We found that patients with stage IV disease had tumours with higher levels of ABCG2 than those with stage III melanoma ($P = 0{\cdot}003$) (Fig. 1e), while no other clinical correlations were identified. We then sought to determine if these proteins were relevant for patient survival. Using the Kaplan–Meier method, univariate analysis showed that ABCG2 (log-rank $P < 0{\cdot}001$) and CD133 (log-rank $P = 0{\cdot}021$) but not ALDH1A1 or CD44v7/8 correlated with survival of these patients with metastatic melanoma (Fig. 1f). Multivariate survival analysis considering patient age, sex, disease stage and M category showed ABCG2 ($P = 0{\cdot}017$) to be an independent prognostic factor.

The concept of CSCs implies that successful cancer therapy will depend on eliminating these cells, but current therapies mostly remain ineffective at achieving this. ABCG2 and CD133, found here to correlate with patient survival, may make useful therapeutic targets that show preferential activity against tumour cells; they were expressed by essentially every melanoma cell in the tumour and were found at higher levels in melanoma cells than in surrounding nonmelanoma cells. To the best of our knowledge this is the first report to demonstrate that the CSC markers ABCG2 and CD133 are negative prognostic factors in melanoma, which may set the stage for their validation as novel therapeutic targets.

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*Departments of* [1]*Internal Medicine II and* [2]*Dermatology, University Hospital Tübingen, Tübingen, Germany*
[3]*School of Science and Technology, College of Arts and Science, Nottingham Trent University, Nottingham, U.K.*
*Correspondence: G. Pawelec and C. Shipp*
*E-mails: graham.pawelec@uni-tuebingen.de; mrchristophershipp@gmail.com*

L. Speigl[1]
N. Janssen[1]
B. Weide[2]
G. Pawelec[1,3]
C. Shipp[1]

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CLINICAL TRIAL

# CD14+ HLA-DR−/low MDSCs are elevated in the periphery of early-stage breast cancer patients and suppress autologous T cell proliferation

**Lisa Speigl[1] · Helen Burow[2] · Jithendra Kini Bailur[1,3] · Nicole Janssen[1] · Christina-Barbara Walter[2] · Graham Pawelec[1,4,5,6] · Christopher Shipp[1,7]**

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## Abstract

**Purpose** Despite the recent expansion in the use of immunotherapy for many cancer types, it is still not a standard treatment for breast cancer. Identifying differences in the immune systems of breast cancer patients compared to healthy women might provide insight into potential targets for immunotherapy and thus may assist its clinical implementation.

**Methods** Multi-colour flow cytometry was used to investigate myeloid and lymphoid populations in the peripheral blood of breast cancer patients ($n = 40$) and in the blood of healthy age-matched women ($n = 25$). We additionally performed functional testing to identify immune suppressive mechanisms used by circulating CD14+ myeloid cells from breast cancer patients.

**Results** Our results show that breast cancer patients have significantly elevated frequencies of cells with the monocytic myeloid-derived suppressor cell (mMDSC) phenotype CD14+ HLA-DR−/low compared with healthy women ($p < 0.01$). We also observed higher levels of earlier differentiated T cells and correspondingly lower levels of T cells in later stages of differentiation ($p < 0.05$). These disease-associated differences could already be detected in early-stage breast cancer patients in stages 1 and 2 ($n = 33$ of 40) ($p < 0.05$). Levels of circulating T cells correlated with certain clinical features and with patient age ($p < 0.05$). Functional tests showed that CD14+ myeloid cells from breast cancer patients more potently suppressed autologous T cell proliferation than CD14+ cells from healthy women ($p < 0.01$). Subsequent investigation determined that suppression was mediated in part by reactive oxygen species, because inhibiting this pathway partially restored T cell proliferation ($p < 0.01$).

**Conclusion** Our results highlight the potential importance of cells with mMDSC phenotypes in breast cancer, identifiable already at early stages of disease. This may provide a basis for identifying possible new therapeutic targets to enhance anti-cancer immunity.

**Keywords** Breast cancer · MDSCs · T cells · Periphery · ROS

**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s10549-017-4594-9) contains supplementary material, which is available to authorised users.

✉ Christopher Shipp
mrchristophershipp@gmail.com

[1] Department of Internal Medicine II, University Hospital Tübingen, Waldhörnlestraße 22, 72072 Tübingen, Germany

[2] Department of Obstetrics and Gynecology, University Hospital Tübingen, Calwerstraße 7, 72076 Tübingen, Germany

[3] Present Address: Yale Cancer Center, Yale University School of Medicine, 333 Cedar St, New Haven, USA

[4] John van Geest Cancer Research Centre, Nottingham Trent University, College Dr, Clifton, Nottingham NG11 8NS, UK

[5] Division of Cancer Studies, King's College London, Strand, London WC2R 2LS, UK

[6] Health Sciences North Research Institute, 41 Ramsey Lake Road, Sudbury, ON, Canada

[7] Present Address: Natural and Medical Sciences Institute (NMI) at the University of Tübingen, Markwiesenstraße 55, 72770 Reutlingen, Germany

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## Introduction

Breast cancer is one of the leading causes of death in women worldwide [1], but despite steady advances in treatment and the promise of more effective immunotherapies, clinical outcomes remain suboptimal. In contrast to lung, melanoma and other types of cancer [2–5], immunomodulatory or cellular immunotherapy is not yet a routine form of treatment for breast cancer. Nonetheless, it is evident that the immune system does indeed play an important role in disease progression. This assertion is largely based on the results of studies which have shown a very close link between parameters of the immune system and the prognosis of breast cancer patients [6, 7]. Consequently, there is now growing interest in exploring the potential use of immunotherapy in treating breast cancer—early results from clinical trials evaluating immunotherapeutic agents such as vaccines and immune checkpoint inhibitors have shown promise in this approach [8–10]. Because breast cancer comprises a heterogeneous collection of diseases, identifying the patient groups which will benefit from particular forms of immunotherapy will be of key importance. Furthermore, identifying the barriers which reduce the efficacy of immunotherapy will be required to more accurately design effective treatment strategies. To this end, the use of blood-based biomarkers rather than tumour tissue biomarkers provides a less invasive approach with the additional advantage of allowing multiple sampling over time.

Interactions between the immune system and cancer can be complex and hard to define, resulting either in tumour suppression, tumour promotion, or both. The immune system is capable of recognising and combating cancer through effector cells such as cytotoxic T cells. This process may be sufficient to result in tumour elimination, but on the other hand it can select tumour cells which resist immune attack [11]. In the latter scenario, the immune system is not only ineffective at providing tumour protection, but may even contribute to disease progression. This is in part due to the ability of tumours to re-programme immune cells so that they suppress anti-tumour immune functions, for example in the case of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs). MDSCs are a heterogeneous group of myeloid cells which have been shown to impair anti-tumour immune responses and which commonly expand in response to pro-inflammatory signals [12]. They are characterised as myeloid cells of granulocytic and monocytic origin, although no unequivocal phenotype that can be used to definitively characterise MDSCs has yet been identified. Much effort is currently being expended in determining approaches to inhibit the suppressive activity of these cells, for example, using certain tyrosine kinase inhibitors [13]. One of the major mechanisms by which MDSCs suppress beneficial immune responses is by impairing T cell function through the release of reactive oxygen species, production of suppressive soluble molecules or through arginine starvation; these mechanisms could also be susceptible to therapeutic blockade to reduce suppressor function [14–17]. This would be desirable because high levels of MDSCs are associated with poor patient prognosis in a range of cancer types [18, 19]. Despite an increasing number of studies on the clinical relevance of MDSCs in human cancers including breast cancer [20–23], surprisingly there are only a few studies examining the clinical role of peripheral MDSCs of monocytic origin in breast cancer to date [23, 24].

In the present study, we assessed circulating populations of myeloid and lymphoid cells in female breast cancer patients, with particular emphasis on cells with monocytic MDSC-like (mMDSC) phenotypes. Due to medical advances, breast cancer is now commonly diagnosed at an early stage. As such we considered it important to additionally determine if alterations in the immune system occur in early disease development (stages 1 and 2 [25]). This may highlight which populations of immune cells could be targeted for effective immunotherapy in particular patient subgroups. To complement this observational approach, circulating myeloid cells were also examined for their ability to suppress T cell activation and proliferation. The aim of this study was to identify disease-associated alterations in breast cancer patients and to uncover suppressive mechanisms used by circulating myeloid cells, which together may provide valuable information for targeted immunotherapy approaches in future.

## Materials and methods

### Samples

Blood samples from 40 breast cancer patients (age range 36–81 years, median age 61) were recruited locally at the Tübingen University Women's Hospital between 2014 and 2016. The cohort included 35 patients with primary tumours and five patients with metastatic disease. Patient tumours were classified according to TNM staging (tumour size (T), nodal status (N) and metastasis (M)). Blood was drawn upon diagnosis, prior to surgery and before receiving any treatment. Apart from the diagnosis of breast cancer, patients did not have any other serious health problems. Detailed characteristics of this patient cohort are summarised in Table 1. Peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-blood using Ficoll–Hypaque gradient centrifugation and stored in a viable state in liquid nitrogen. In addition, we included a control group of 25 age-matched healthy

**Table 1** Clinical characteristics of the breast cancer cohort

| Factor | Patients ($n = 40$) |
|---|---|
| Age | |
| Median (years) | 61 |
| Range | 36–81 |
| Grade | |
| 1 | 2 |
| 2 | 15 |
| 3 | 14 |
| nd | 9 |
| Tumour size | |
| pT1 | 22 |
| pT2 | 12 |
| nd | 1 |
| Nodal status | |
| N0 | 30 |
| N1 | 3 |
| N3 | 1 |
| nd | 1 |
| Metastasis | |
| M0 | 35 |
| M1 | 5 |
| Histological subtype | |
| Ductal | 24 |
| Lobular | 7 |
| Tubular | 1 |
| Papillary | 1 |
| Mucinous + papillary | 1 |
| nd | 1 |
| Hormone receptor and HER2 expression | |
| ER+ | 25 |
| PR+ | 20 |
| HER2+ | 7 |
| HER2 status unknown | 6 |
| Triple negative | 7 |

women (age range 36–84 years, median age 61). PBMCs of this control cohort were obtained from routine blood donations at the Tübingen University Hospital.

All patients gave their written informed consent for the storage and scientific analysis of their biomaterial. The use of the samples was approved by the University of Tübingen Ethics Committee (ethics approval number 626/2016BO2).

## Immunophenotypic analysis of circulating blood myeloid cells and T cells

Flow cytometry was used to phenotype blood myeloid cells (including monocytes, monocytic MDSCs and dendritic cells (DCs)) and lymphoid populations (including differentiation stages of CD4+ and CD8+ T cells) as previously described [26] using the following antibody panels (online resource 1). For the gating of mMDSCs, lineage-negative events were selected before exclusion of CD14-negative cells. This population was then used to gate HLA-DR-positive or HLA-DR-negative events using an HLA-DR-negative internal reference population. To gate DCs, lineage-negative events were first gated, followed by CD14-negative and HLA-DR-positive cells. From this population, myeloid DCs (mDCs) were identified based on CD11c positivity, while plasmacytoid DCs (pDCs) were identified as CD123-positive cells. All gating steps, including those for T cells, are illustrated in online resource 2. For the establishment of antibody panels, fluorescence minus one controls were used. Due to the limited availability of patient material, we could not perform multiple testing of the same sample, but consistency in machine performance was achieved by using cytometer setup and tracking (CST) beads before and after each sample measurement. Furthermore, repeated measurements of the same batch of a biological control donor were used in each run to confirm consistency in measurement conditions.

## T cell/monocyte co-culture suppression assays

The suppressive capacity of CD14+ myeloid cells from breast cancer patients on autologous proliferating T cells was assessed using monocytes isolated from whole PBMC by magnetic cell sorting with human CD14 MicroBeads (Miltenyi Biotech, Teterow, Germany). The isolated CD14+ monocytes were co-cultured with CD14-depleted PBMC at a ratio of 1:1.5 (CD14-depleted PBMC:monocytes) for 5 days in IMDM with GlutaMAX (Life Technologies, Darmstadt, Germany) containing 10% FCS (SERATEC, Göttingen, Germany). CD14-depleted PBMC without the addition of monocytes were included as a positive control. All experiments were performed in 96-well U-bottom plates (Greiner Bio-One, Frickenhausen, Germany) containing a total of $0.25 \times 10^6$ cells per well (i.e. $0.1 \times 10^6$ CD14-depleted PBMC and $0.15 \times 10^6$ monocytes to give a ratio of 1:1.5). In order to assess the degree of T cell proliferation, CD14-depleted PBMCs were stained with CFSE (Invitrogen, San Diego, USA) according to our previous protocol [27] but with the following modifications: cells were incubated with CFSE staining solution for 5 min at room temperature in the dark and then washed with 10 mL PB buffer (5% FCS in PBS) to stop the labelling reaction. T cells were activated with CD3/CD28 T cell activator Dynabeads (Invitrogen, San Diego, USA) (1.5 µL/well). The 1:1.5 ratio (CD14-depleted PBMC:monocytes) was chosen based on a prior study [28] and on preliminary experiments which showed a concentration-dependent relationship between CD14+ myeloid cells and T cell suppression. Following the 5-day culture period, flow cytometry was used to characterise

cell phenotypes and to assess the extent of T cell proliferation. The following antibodies were used: CD3-A700 (BD Pharmingen, Heidelberg, Germany), CD4-APC (Milteny Biotech), CD8-Pacific Blue and CD14-APC-H7 (both from BD Pharmingen). Suppression assays were performed with five patient samples (four with primary early-stage disease, one metastatic) randomly selected from the patient cohort. All experiments were performed using triplicate cultures to ensure consistency in results.

To investigate mechanisms potentially responsible for suppression, co-cultures were treated with inhibitors targeting candidate pathways previously suggested to be involved in mediating immune suppression. These were the reactive oxygen species (ROS) inhibitor superoxide dismutase (200 IU/mL) (Sigma-Aldrich, Steinheim, Germany), anti-TGFβ antibody (10 µg/mL) (R&D Systems, Wiesbaden, Germany) and the STAT-3 (signal transducers and activator of transcription 3) inhibitor AG490 (10 µmol/L) (Sigma-Aldrich).

### Flow cytometry data analysis

Flow cytometry data analysis was performed using FlowJo software version 10.07 (Tree Star, Ashland, USA). Events not part of the main acquisition population were first excluded using a time-versus-side scatter gate. This was followed by removing cell doublets and subsequently the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate. This was followed by gating for specific populations of interest according to the gating strategies shown in online resources 2 and 3. For assessing T cell proliferation in suppression assays, in a first step CD14+ cells were gated out to avoid contamination of the CFSE signal. CD3+ events were gated which was followed by gating both CD4+ and CD8+ populations. An index of CFSE mean fluorescence intensity was created for CD4+ and CD8+ populations for each condition relative to the corresponding positive control in order to determine the relative degree of T cell proliferation between experimental conditions.

### Statistical analysis

Statistical analyses were performed using SPSS version 20 (IBM, Ehningen, Germany) and GraphPad Prism 6 (GraphPad Software, San Diego, USA). To compare two independent groups, non-parametric Mann–Whitney *U* tests were used. To compare changes in the same sample under different experimental conditions, Wilcoxon matched-pair tests were used (these statistical tests included values obtained from biological replicates to consider biological variation and technical replicates to account for measurement error). Correlations were calculated using Spearman correlation analysis. A value of $p < 0.05$ was considered statistically significant. Because this was an exploratory study, we aimed to reduce the chance of obtaining false-negative results. As such statistical analyses were not corrected using the Bonferroni method.

## Results

### T cell phenotypes in peripheral blood are associated with certain clinical features of breast cancer patients

Using flow cytometry, we measured myeloid cell populations and a spectrum of T cell populations from early to late stages of differentiation in the peripheral blood of 40 breast cancer patients. We characterised myeloid cells including monocytes, mDCs and pDCs (16 populations) and lymphoid cells including effector and memory T cells in both the CD4+ and CD8+ compartments (62 populations). These populations were investigated for association with clinical features of the breast cancer cohort; tumour characteristics such as pathological tumour size (pT), tumour grade, HER2 status, oestrogen (ER) and progesterone (PR) receptor expression and patient age were considered. We saw a number of correlations between T cell distribution and breast cancer patient clinical features (selected examples are shown in Fig. 1). For example, patients with larger tumours (pT2 vs. pT1) tended to have higher levels of earlier differentiated CD4+ T cell populations (CD45RA+ CD95− CD27+ CD28+) ($p < 0.01$), while CD4+ phenotypes at later differentiation stages (CD95+) tended to be present at lower levels in these patients ($p < 0.01$) (Fig. 1a). In addition, we observed that a number of later differentiated populations of CD8+ T cells (CD57+ and CD45RA+ CD95+ CD27− CD28−) were negatively associated with hormone receptor expression (Fig. 1b). We furthermore found a number of inverse correlations between patient age and the level of CD8+ T cells including naïve CD8+ T cells (CD8+ CD45RA+ CD95− CD27+ CD28 +) ($p = 0.0001$) and central memory phenotypes (CD8+ CD95+ CD45RA− CD27+ CD28+ and CD8+ CD95+ CD45RA− not gated for CD27 and CD28 expression) ($p = 0.0167$ and $p = 0.0329$) (Fig. 1c). We did not find any relationship between age and tumour characteristics (pT, tumour grade, HER2, ER and PR), suggesting that the associations between them and leukocyte levels are independent of age.

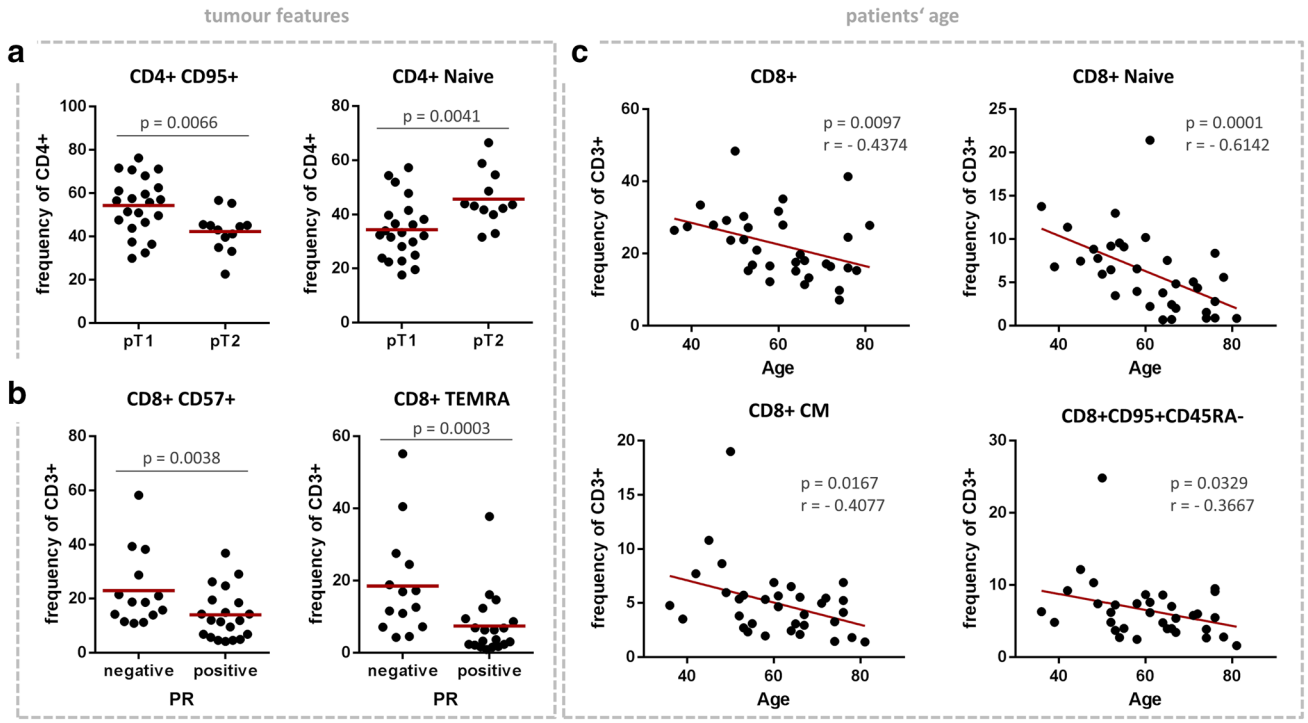



**Fig. 1** Association between peripheral T cells and breast cancer clinical features. Multi-colour flow cytometry was used to analyse immune cell phenotypes in the peripheral blood of breast cancer patients ($n = 40$). We found correlations between circulating immune cells and breast cancer clinical parameters. **a** Association between earlier and later differentiated CD4+ T cells and pathological tumour size (pT). **b** Association between later differentiated CD8+ T cells and progesterone receptor (PR) expression on breast tumours. **c** CD8+ T cell phenotypes and association with patient age. *pT* pathological tumour size, *PR* progesterone receptor, *TEMRA* terminally differentiated effector memory cells re-expressing CD45RA (phenotype: CD45RA+ CD95+ CD27− CD28−), *CM* central memory T cells (phenotype: CD95+ CD45RA− CD27+ CD28+), *Naïve* naïve T cells (phenotype: CD45RA+ CD95− CD27+ CD28+)

## Breast cancer patients have elevated levels of CD14+ HLA-DR−/low MDSC phenotypes in the peripheral blood compared with healthy women

We next examined whether the levels of circulating lymphoid and myeloid cell populations differed between breast cancer patients and controls. We found that the frequencies of cells with the mMDSC phenotype CD14+ HLA-DR−/low was significantly higher in patients when assessing their levels as a percentage of total leukocytes (CD45+), and also relative to CD14+ cells ($p = 0.0084$ and $p = 0.0105$, respectively) (Fig. 2a). Importantly, when only looking at early-stage patients (stages 1 and 2, $n = 33$) in the cohort, we observed the same association ($p = 0.0116$ and $p = 0.0151$), indicating that the differences can already be detected at earlier breast cancer stages (online resource 4). These differences appeared to be specific for cells with a suppressor phenotype; we did not detect differences in the levels of CD14+ monocytes between breast cancer patients and healthy women (Fig. 2a, right panel), nor did we observe differences in mDCs or pDCs (Fig. 2b).

We also observed that populations of lymphoid cells were present at different levels between patients and controls (selected results are shown in Fig. 2). While the frequencies of circulating CD4+ and CD8+ T cells showed no difference between breast cancer patients and healthy women (Fig. 2c), the relative frequencies of several earlier differentiated T cell populations (CD4+ CD45RA+ CD95+ CD27+ CD28+) were elevated in breast cancer patients ($p = 0.0076$), which was again also true when only considering early-stage patients ($p = 0.0026$). In contrast, later differentiated T cells lacking the expression of CD45RA (e.g. central memory cells (CD8+ CD95+ CD45RA− CD27+ CD28+)) tended to be lower in breast cancer patients than healthy women ($p = 0.0466$) (Fig. 2d).

## Circulating CD14+ myeloid cells from breast cancer patients suppress the proliferation of autologous T cells

Our results revealed that within CD14+ monocytes, cells with mMDSC phenotypes (CD14+ HLA-DR−/low) were elevated in breast cancer patients. Thus in order to model

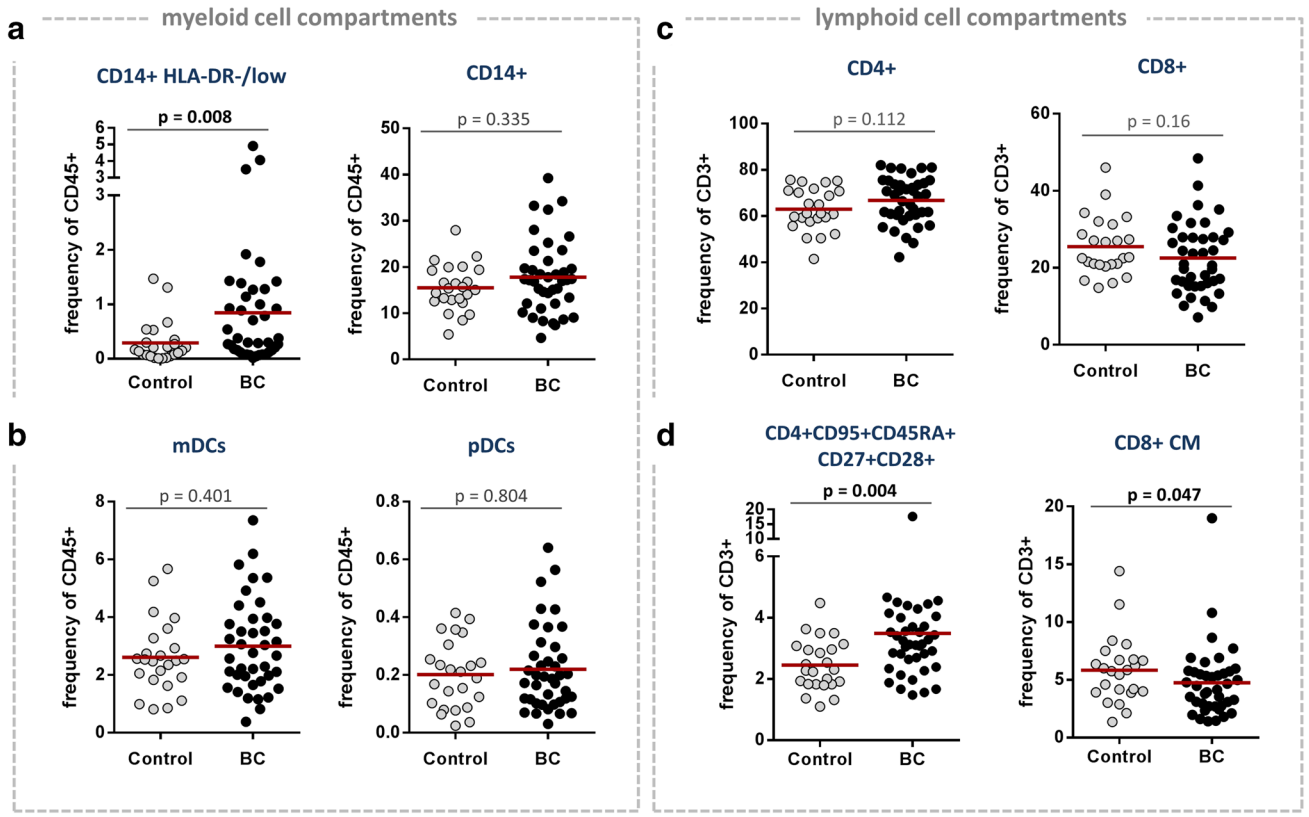



**Fig. 2** Frequencies of immune cell populations in breast cancer patients and healthy women. PBMCs from 40 breast cancer patients and 25 healthy age-matched control women were stained with panels of antibodies for lymphoid and myeloid cells and their levels assessed using multi-colour flow cytometry. **a** Frequencies of cells with the mMDSC phenotype CD14+ HLA-DR−/low (left-hand panel) and of CD14+ cells (right-hand panel) within CD45+ cells. **b** Frequencies of circulating myeloid dendritic cells (mDCs, left-hand panel) and plasmacytoid DCs (pDCs, right-hand panel) within CD45+ cells. **c** Levels of circulating CD4+ and CD8+ T cells as percentages of CD3+ cells. **d** Frequencies of earlier differentiated CD4+ T cells expressing CD45RA within CD3+ cells (left-hand panel); frequencies of later differentiated (CD45RA−) CD8+ CM cells within CD3+ cells (right-hand panel). *BC* breast cancer, *PBMCs* peripheral blood mononuclear cells, *mMDSCs* monocytic MDSCs, *mDCs* myeloid dendritic cells, *pDCs* plasmacytoid dendritic cells, *CM* central memory T cells

immune suppression that these cells may exert in vivo, we compared the suppressive potential of equivalent numbers of isolated CD14+ cells between breast cancer patients and healthy women. Because CD14+ HLA-DR−/low mMDSCs, but not the total levels of CD14+ monocytes, were elevated in breast cancer patients, we asked whether these cells with an mMDSC phenotype from breast cancer patients had suppressive properties.

To determine the suppressive capacity of circulating CD14+ HLA-DR−/low mMDSCs from early-stage breast cancer patients, we co-cultured isolated CD14+ cells with autologous CD14-depleted PBMCs. PBMCs were labelled with CFSE and stimulated with CD3/CD28 beads, with the degree of proliferation by CD4+ and CD8+ T cells measured by flow cytometry after 5 days of culture. We observed potent and consistent suppression of CD4+ and CD8+ T cell proliferation by CD14+ myeloid cells from breast cancer patients ($n = 5$) ($p < 0.0001$) (Fig. 3a). In preliminary experiments, we observed a concentration-dependent association between isolated CD14+ cells and T cell proliferation (data not shown). To investigate whether this suppressive capacity by circulating myeloid cells was specific to breast cancer patients, we tested the suppressive capacity of myeloid cells from healthy age-matched women ($n = 4$). We found that myeloid cells from healthy women could also suppress proliferating T cells, but the suppressive capacity was weaker when compared to breast cancer patients ($p = 0.0037$ for CD8+; trend for CD4+) (Fig. 3a).

## Circulating CD14+ myeloid cells from breast cancer patients suppress immune responses via reactive oxygen species

To investigate the mechanism(s) potentially responsible for the suppressive capacity of breast cancer CD14+ myeloid cells, we treated cells in the model previously used to

investigate immune suppression with inhibitors targeting different myeloid-suppressor pathways, namely TGFβ (inhibitor: neutralising antibody), ROS (inhibitor: superoxide dismutase, SOD) and STAT-3 (inhibitor: AG490). We found that inhibition of ROS via SOD partially restored T cell proliferation ($p < 0.0001$ for CD8+ and $p = 0.0002$ for CD4+), while we also observed weak but statistically significant restoration for anti-TGFβ ($p = 0.0067$ for CD8+; CD4+ not significant) and AG490 ($p = 0.0009$ for CD8+ and $p = 0.0002$ for CD4+) (Fig. 3b). Compared with untreated cultures, those treated with SOD restored CD8+ T cell proliferation by 131% and CD4 73% on average. Treatment with anti-TGFβ and AG490 only led to a 34% (CD8) and 11% (CD4) and 44% (CD8) and 31% (CD4) proliferation increase compared with untreated cultures, respectively (Fig. 3c).

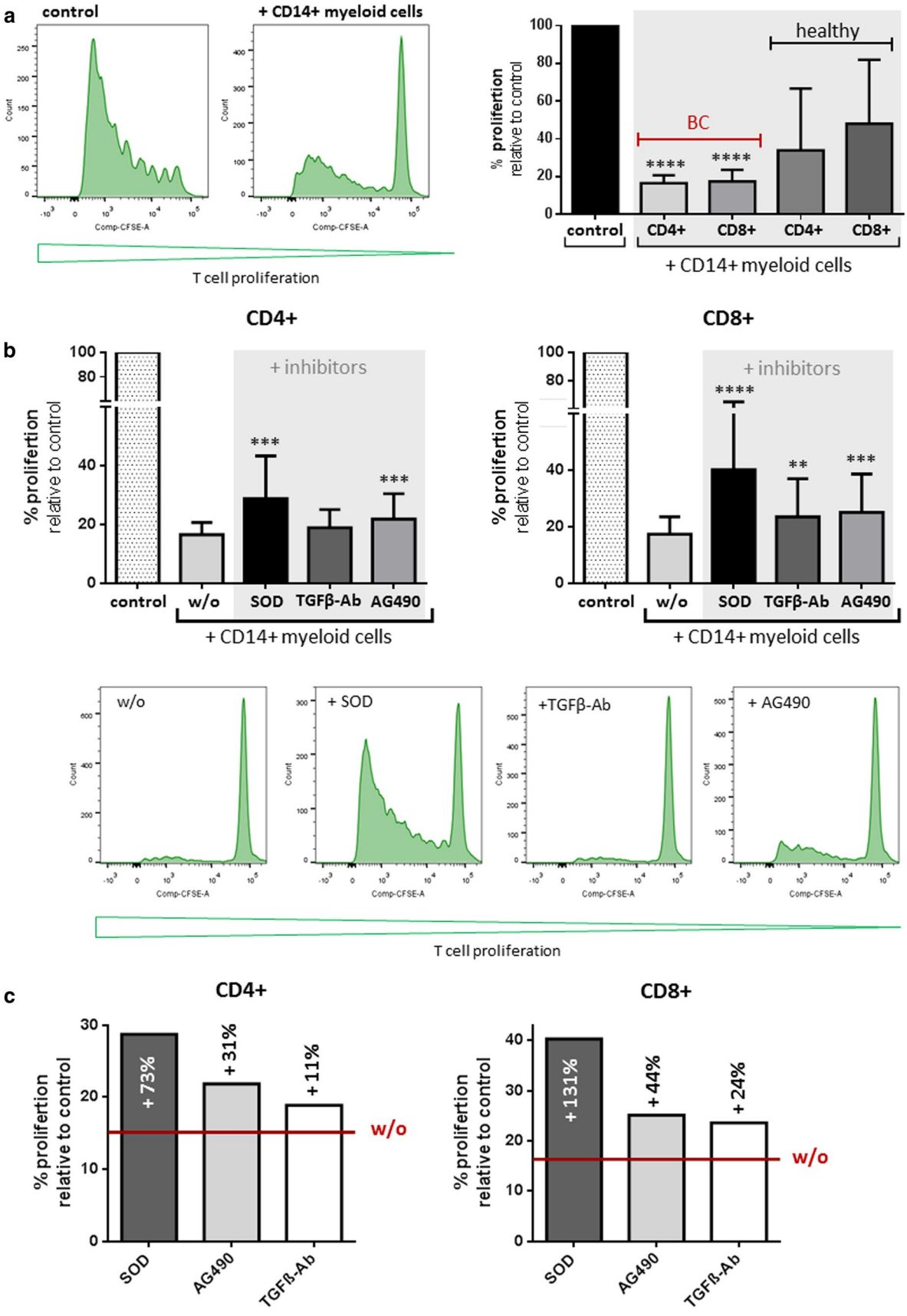
## Discussion

Compared with some other cancer types [2–5], immunotherapy is not yet a routine form of treatment for breast cancer. To gain an indication of which immune cells could be targeted for effective immunotherapy, we considered it an important first step to identify possible alterations in the immune system of breast cancer patients compared with healthy age-matched women. Characterising such "signatures" of immune alteration on both a phenotypical and functional level might provide a means of identifying potential targets for immunotherapy. Due to public health campaigns and medical advances, breast cancer is now typically diagnosed at an early stage of disease. To account for this, we additionally examined if immune perturbations occur early in disease development by comparing the immune signatures between healthy women and early-stage breast cancer patients. To date, studies attempting to identify changes in the immune system in exclusively early-stage breast cancer are rare [29, 30].

Our results reveal that cells with the mMDSC phenotype CD14+ HLA-DR−/low are present at significantly higher frequencies in breast cancer patients than in healthy individuals. Despite the recent expansion in the study of MDSCs in later stage cancer patients [23, 24, 26, 31], there are no studies which have so far assessed whether these cells are clinically relevant in early-stage breast cancer. Thus this study is, to the best of our knowledge, the first to show that CD14+ HLA-DR−/low mMDSCs are elevated early in breast cancer progression (clinical stages 1 and 2). This finding suggests that immune suppression via elevated mMDSCs occurs early in the development of breast cancer, which may help protect tumour cells from immune attack. Interestingly, we found that total monocyte frequencies did not differ between breast cancer and healthy women, indicating the pool of CD14+ myeloid cells in breast cancer to be selectively driven towards MDSC differentiation, thereby leaving other populations of myeloid cells unaffected. Indeed, we did not observe levels of other myeloid cells, such as mDCs, to be altered in breast cancer. Collectively, our findings indicate the importance of mMDSCs (CD14+ HLA-DR−/low), already in earlier stages of breast cancer. Pending clinical follow-up will reveal if these cells can be used to predict patient survival as previously shown in other cancer types [18].

Further to our findings on mMDSCs, we also found elevated levels of early differentiated T cells in breast cancer patients compared with healthy women. Higher levels of these cells indicates potential for the immune system to recognise novel or newly arising tumour antigens present in the tumour and thus mount an immune response against tumour cells. This anti-tumour potential might be counterbalanced by our finding of elevated mMDSCs, which may suppress the activity of beneficial T cells, for example by preventing their differentiation. Indeed, we found elevated levels of both mMDSCs as well as more immature T cells expressing CD45RA (which are not TEMRA cells) in breast cancer patients. This association suggests that mMDSCs may impair the maturation of T cells in cancer patients. This notion is supported in our prior study where patients with tumour antigen-reactive T cells experienced greater clinical benefit if they also had low MDSC levels [26]. In contrast to T cells in the periphery, T cells infiltrating the tumour (TILs) have frequently been shown to be associated with favourable prognoses in breast cancer [7, 29, 32, 33]. The relationship between peripheral and intra-tumoural TILs in breast cancer is not yet known, and thus we cannot judge whether our observation of altered levels of circulating T cells in breast cancer patients relates to the presence of T cells in the tumour. It is conceivable that higher levels of T cells in the blood may act to support the maintenance of intra-tumoural T cells, but such associations remain to be confirmed. It should certainly be considered that immune cells in the periphery may play different roles to those infiltrating the tumour, although there may be more transit in and out of tumours than previously appreciated [34].

Having observed elevated mMDSCs in patients, we then investigated whether CD14+ myeloid cells from breast cancer patients or healthy women are immunosuppressive. To better mimic immune suppression that these cells may exert in vivo, we compared the suppressive potential of equivalent numbers of isolated CD14+ myeloid cells between breast cancer patients and controls. We also aimed to determine potential mechanisms responsible for any immune suppression observed by inhibiting three of the pathways previously shown to be used by MDSCs in other cancer types [35–37], namely TGFβ, STAT-3 and ROS. Our results revealed that CD14+ myeloid cells from breast cancer patients are potent suppressors of autologous T cell proliferation, already in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CD8+
+ 73%
+ 31%
+ 11%
+ 131%
+ 44%
+ 24%
w/o
SOD
AG490
TGFß-Ab

◂**Fig. 3** Suppressive capacity of circulating CD14+ myeloid cells from breast cancer patients. Isolated CD14+ myeloid cells from five breast cancer patients were cultured with autologous CD14-depleted PBMCs labelled with CFSE and stimulated with CD3/CD28 beads. Inhibitors against TGFβ (10 µg/mL), reactive oxygen species (ROS) (SOD; superoxide dismutase (200 IU/mL)) and STAT-3 (AG490, (10 µmol/L)) were added to the cultures to identify the mechanism(s) responsible for the ability of CD14+ myeloid cells to suppress autologous T cells. After 5 days of culture T cell proliferation was measured by flow cytometry. **a** Suppressive capacity of CD14+ myeloid cells from breast cancer patients ($n = 5$) and healthy women ($n = 4$) on autologous CD4+ and CD8+ T cells (****$p < 0.0001$, compared to control without monocytes). **b** Influence of inhibitors targeting TGFβ, STAT-3 and ROS on CD14+ -mediated T cell suppression in breast cancer patients (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$, compared to untreated co-culture (w/o)). **c** Effect on T cell suppression by inhibiting ROS compared with targeting TGFβ or STAT-3. *BC* breast cancer, *Control* no monocytes added, *w/o* without treatment, *ROS* reactive oxygen species, *TGFβ-Ab* neutralising anti-TGFβ-antibody, *STAT-3* signal transducer and activator of transcription 3

patients with early disease. Interestingly, CD14+ myeloid cells from healthy counterparts were also able to suppress autologous T cells, but not as profoundly as myeloid cells from breast cancer patients. This likely reflects normal levels of peripheral tolerance governed by mMDSCs in healthy individuals. The observation that myeloid cells in breast cancer patients more potently suppress immune responses may reflect our finding that cells with the CD14+ HLA-DR−/low mMDSC phenotype are present at higher levels in these patients. The potential association between CD14+ HLA-DR−/low mMDSCs and immune suppression is supported by other studies showing the suppressive features of this particular mMDSC phenotype in a number of other cancer types [28, 38–40]. However, as far as we are aware, this is the first study proposing their suppressive capacity in breast cancer patients. To more conclusively demonstrate the suppressive function of CD14+ HLA-DR−/low mMDSCs, future studies should additionally examine their suppressive function in an isolated system only containing these cells and activated CD4+/CD8+ T cells. This approach would isolate potential effects due to other cells present in circulating blood such as Tregs and other suppressive myeloid populations. However, while establishing the conditions for this study we observed a concentration-dependent association between T cell suppression and isolated CD14+ myeloid cells in the presence of all circulating mononuclear cells, suggesting the effects of other potentially suppressive cell types to be negligible. We chose to use a model including all circulating mononuclear cells to avoid working in a more artificial model with isolated cell types. We further found that inhibiting ROS partially reduced the suppressive effect of CD14+ myeloid cells from breast cancer patients, suggesting ROS as one of the suppressive mechanisms used in breast cancer mMDSC-mediated suppression of T cells, as in other cancer types [28, 41, 42]. However, inhibiting ROS could not completely restore T cell proliferation, suggesting that a combination of other suppressive mechanisms may be simultaneously used by these cells to suppress the immune system, or that ROS inhibition was incomplete. Further functional assays exploring a wider spectrum of candidate suppressive pathways may reveal more information regarding the mechanisms used by these cells to exert immune suppression in breast cancer.

This study shows that systemic immune alterations occur early in the development of breast cancer and that the identified differences between healthy women and breast cancer patients may serve as immunotherapy targets in future. Our results encourage the potential use of strategies targeting CD14+ HLA-DR−/low mMDSCs in breast cancer such as antioxidant treatment strategies. However, developing a more detailed picture of interactions between disease-associated factors and their effect on different immune cell populations (particularly MDSCs) will be crucial for the development of effective immunotherapeutic approaches.

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## Compliance with ethical standards

**Conflicts of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Ethical standards** The experiments comply with the current laws of the country in which they were performed.

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Original Study

# Frequency of Immune Cell Subtypes in Peripheral Blood Correlates With Outcome for Patients With Metastatic Breast Cancer Treated With High-Dose Chemotherapy

Robert M. Lafrenie,[1,2,3] Lisa Speigl,[4] Carly A. Buckner,[1,2] Graham Pawelec,[1,4] Michael S. Conlon,[1] Christopher Shipp[4]

## Abstract

**The frequencies of circulating myeloid and T-cell populations were correlated with clinical outcome for 88 patients with metastatic breast cancer treated with high-dose chemotherapy. The ability to predict outcome depended on the chemotherapy regimen. The frequency of monocytes indicated outcome for patients treated with cyclophosphamide-based chemotherapy, while the frequency of T cells indicated outcome for patients treated with paclitaxel-based chemotherapy.**

**Background:** The frequency of circulating leukocytes has been shown to be a prognostic factor in patients being treated for different types of cancer. In breast cancer, tumor-infiltrating leukocytes may predict patient outcome, but few studies have investigated such associations for circulating leukocytes. **Patients and Methods:** Multiparametric flow cytometry was used to examine the immunophenotypes of circulating peripheral blood mononuclear cells for 88 patients with metastatic breast cancer, which was then correlated to breast cancer–specific survival. Patients had been treated either with high-dose cyclophosphamide-containing regimens (group 1, n = 51 patients) or high-dose paclitaxel-containing regimens (group 2, n = 37 patients). **Results:** The frequency of peripheral blood $CD14^+$ monocytes indicated prognosis for patients in group 1 (but not group 2), while higher levels of $CD11c^+$ dendritic cells indicated a better prognosis for patients in group 2 (but not group 1). The frequency of a number of different $CD4^+$ or $CD8^+$ T cell subtypes also predicted prognosis for patients in group 2. For example, patients in group 2 with a higher frequency of circulating $CD4^+$ or $CD8^+$ naive T cells ($CD45RA^+CD95-CD27^+CD28^+$) showed a poorer prognosis. In contrast, T cells were not associated with prognosis for patients in group 1. **Conclusion:** Circulating leukocytes can predict clinical outcome for patients with breast cancer. Prediction of clinical outcome in this cohort of metastatic breast cancer patients was specific to the type of chemotherapy, and this finding is likely to apply to other therapies.

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**Keywords:** Cancer prognosis, Immunophenotype, Myeloid cell, T cell

## Introduction

The level of immune cell populations both in the tumor and in circulation has been shown to be clinically informative for patients with cancer. For example, the frequency of intratumoral macrophages and $CD4^+$ and/or $CD8^+$ T immune cells has been associated with clinical outcome for patients with cancer, including breast cancer and advanced melanoma.[1-4] The absolute number of circulating $CD14^+$ monocytes[5,6] and the level of inflammatory

R.M.L. and L.S. contributed equally to this work as first authors.

Current address for Christopher Shipp: Natural and Medical Sciences Institute (NMI) at the University of Tübingen, Reutlingen, Germany.

[1]Health Sciences North Research Institute, Sudbury, Ontario, Canada
[2]Laurentian University, Sudbury, Ontario, Canada
[3]Northern Ontario School of Medicine, Sudbury, Ontario, Canada
[4]Department of Internal Medicine II, University Hospital Tübingen, Tübingen, Germany

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Address for correspondence: Robert M. Lafrenie, PhD, Laurentian University, 935 Ramsey Lake Rd, Sudbury, Ontario, P3E 2C6, Canada
E-mail contact: rlafrenie@laurentian.ca

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monocytes[7] or dendritic cells[8] (DCs) correlates with a poorer prognosis in several cancers. However, in patients with breast cancer, a higher frequency of circulating plasmacytoid DCs (pDCs; $CD11c^+CD123^+$) was associated with an improved 5-year survival.[9] The presence of a high level of circulating myeloid-derived suppressor cells (MDSC, $CD14^+CD11b^+$HLA-DR−/low) is associated with grade, stage, and tumor burden and can indicate a relatively poor prognosis for patients with cancer,[10-12] including breast cancer[13,14] or advanced melanoma.[15]

A higher density of T cells in tumor tissue predicts a better clinical outcome for patients with breast cancer or melanoma.[16-18] In patients with melanoma, the presence of circulating $CD4^+$ and $CD8^+$ T cells was also strongly associated with improved prognosis for patients treated with ipilimumab.[19,20] The frequencies of different effector and central memory $CD4^+CD45RA-$[21] or $CD8^+CD45RA-$[22] T cells (designated EM1-EM4 on the basis of the presence or absence of CD27 or CD28 expression) are also linked to prognosis for patients with some cancers.[23,24] The presence of circulating T cells that recognize shared tumor-associated antigens also correlates with clinical outcome. Patients with malignant melanoma that possessed peripheral T cells that can respond to the melanoma-associated antigens NY-ESO-1 and melan-A (but not survivin or MAGE-A3) experienced a better clinical outcome.[25,26] Patients who possessed $CD4^+$ and/or $CD8^+$ cells that respond to NY-ESO-1 or who had only $CD8^+$ cells that respond to melan-A showed improved outcome.[27] Patients with breast cancer that possessed circulating $CD4^+$ and $CD8^+$ T cells that can respond to the HER2 tumor antigen showed an improved clinical outcome and had a lower frequency of monocytes, natural killer cells, DCs, and T-regulatory cells (Tregs).[16,28,29] However, patients with an elevated level of circulating MDSCs and Tregs did not show an improved prognosis associated with the presence of T cells responsive to tumor-associated antigens for breast cancer[28] or advanced melanoma.[13,27]

While the prognostic impact of tumor-infiltrating cells in breast cancer is well established, there are few studies examining the role of blood-based immune cell signatures in relation to clinical outcome. One study showed that a high baseline frequency of late-stage differentiated effector memory $CD8^+$ cells ($CD8^+CD45RA^+CCR7-CD27-CD28-$) was correlated with poorer survival.[30] Patients with breast cancer have a higher frequency of early differentiated T cells and a lower level of more differentiated T cells than healthy individuals without cancer,[20] although the impact of these differences on patient survival remains unknown. As such, the present study is likely the first to perform a detailed characterization of circulating immune cells in association with patient outcome in breast cancer. To achieve this we examined a large number of circulating mononuclear immune cell populations (myeloid and lymphoid) and determined associations with clinical outcome for a group of metastatic breast cancer patients treated with either cyclophosphamide (CTX)- or paclitaxel-based high-dose chemotherapy. CTX and paclitaxel are commonly used as part of standard treatment for patients with metastatic breast cancer.[31] Considering the mounting evidence implicating the functionality of the immune system in patient outcome we sought to determine whether pretreatment immune profiles could be used to improve patient selection for these drugs. This information may be valuable for individualization of patient management.

## Patients and Methods

### *Patients*

All procedures performed in studies involving human participants were in accordance with the ethical standards of Laurentian Hospital, Sudbury, and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in this study.

Cryopreserved peripheral blood mononuclear cells were derived from a group of patients enrolled in clinical trials to test the efficacy of high dose chemotherapy and autologous stem cell transplantation for the treatment of women with metastatic breast cancer between 1991 and 1997 at Sudbury, Ontario, Canada. Patients were enrolled in 5 separate studies; 4 studies included phase 2 clinical trials to study treatment with high dose CTX, mitoxantrone, and vinblastine or carboplatin (patient outcomes for these studies were indistinguishable and are combined into group 1), and one study included a phase 1/2 clinical trial to study treatment with high dose paclitaxel, CTX, and mitoxantrone (group 2).[32,33] Eligible patients were histologically diagnosed with metastatic breast cancer (stage IV), had a Karnofsky performance status of $\geq$ 60%, did not have central nervous system metastases, and had not received chemotherapy for metastatic breast cancer or adjuvant chemotherapy for at least 6 months before enrollment. Patients were hormone receptor-negative, had progressed on hormone therapy, or had rapidly progressing disease where a response to hormone treatment could not be awaited.

Patient characteristics and high dose chemotherapy treatment regimens are listed in Table 1. Informed consent was obtained from all of the patients enrolled in these studies according to the Laurentian Hospital Research Ethics Board. The clinical outcome for these patients has been published previously.[33-35] Blood samples were taken from the patients at enrollment. The peripheral blood mononuclear cells were collected, isolated from fresh blood by Ficoll-Hypaque density gradient centrifugation, and cryopreserved in 5% dimethyl sulfoxide. Vials of cryopreserved cells were removed from liquid nitrogen storage and packaged on dry ice for shipping to Tübingen, Germany. On receipt, they were once again placed in liquid nitrogen until the day of analysis. Profiles were obtained from 88 patients (51 in group 1 and 37 in group 2).

### *Flow Cytometry*

Peripheral blood mononuclear cells were thawed and immediately analyzed by flow cytometry. For phenotyping of blood myeloid cells (including monocytes, MDSCs, and DCs) and lymphoid populations (including the differentiation stages of $CD4^+$ and $CD8^+$ T cells) we used a previously described approach.[13] The antibody panels employed are provided in Appendix A in the online version. To identify DCs, lineage-negative events were gated, followed by CD14−and HLA-$DR^+$ cells. From this population, myeloid DCs were identified on the basis of CD11c expression, while pDCs were identified as CD123-expressing cells. To identify mMDSCs, lineage-negative events were selected before the

exclusion of CD14− cells. This population was then gated for HLA-DR$^+$ or HLA-DR− events using an HLA-DR− internal reference population. The gating steps for both myeloid and T cells are shown in Appendixes B and C in the online version. For the establishment of antibody panels, we employed fluorescence minus one controls. Due to limited availability of patient material, we could not perform multiple testing of the same sample, but consistency in machine performance was verified using cytometer setup and tracking beads before and after each sample measurement. Furthermore, repeated measurements of a biological control donor were used in each measurement to confirm consistency in measurement conditions. An overview of the immune cell phenotypes investigated is provided in Appendix D in the online version.

### *Statistical Analysis*

Breast cancer−specific survival (BCSS) was defined as the time from study enrollment until death from breast cancer. Progression-free survival was defined as the time from study enrollment until documented progression of metastatic disease or censorship. Potential cut points to define positive versus negative marker levels were determined by quantile analysis. Flow cytometry data were sorted by ascending percentage of positive cells, and the patients were divided into groups, each with approximately 10 subjects, and the values for percentage of positive cells that distinguished each group were used as the potential quantile cut points. The quantiles were then compared for differences in BCSS based on pairwise comparisons by log-rank statistics for all patients or those in group 1 or group 2 separately. Only a single cut point was chosen, usually near the median value of positive cells (Appendix E in the online version). The cancer-specific survival likelihood was estimated by the Kaplan-Meier method and compared by log-rank tests. Cox proportional hazards regression models were used to verify single factors. The results of the Cox regression analysis are described by the means, hazard ratios, and $P$ values (Wald test) for each immunophenotype for each group of patients. All reported $P$ values for these analyses were unadjusted. To account for multiple comparisons (for 8 tests per panel), a Bonferroni type I error level of 0.00625 could be used. Correlations between immunophenotypes and clinicopathologic features were examined by Spearman correlation and were subjected to Bonferroni correction to account for multiple testing.

## Results

### *Patient Response to Therapy*

The patients in this study were treated with two different high-dose chemotherapy regimens. Patients in group 1 were treated with high-dose CTX-containing chemotherapy, and patients in group 2 were treated with high-dose paclitaxel-containing chemotherapy. All data were analyzed for the combined set of patients, then separately for those in group 1 or group 2. Clinicopathologic characteristics (Table 1) and clinical outcome (Figure 1) indicate that the patients in groups 1 and 2 were similar with regard to age, hormone receptor status, sites of metastasis, and clinical outcome, thus suggesting that the different therapies had equivalent efficacy.

### *Frequency of Circulating Monocytes and DCs Is Prognostic for Patients With Metastatic Breast Cancer*

Patients with a higher frequency of circulating mature monocytes (CD14$^+$HLA-DR$^+$) showed a significant difference in BCSS. Patients in group 1, with a high frequency of mature monocytes within the CD14$^+$ population (ie, at a cutoff of > 88.8% of gated cells), showed a better BCSS than patients with a low frequency of these cells (14.0 vs. 25.2 months; hazard ratio [HR] = 1.78 (1.01−3.17), $P$ = .049) which was also seen for the whole cohort (16.6 vs. 24.7 months, HR = 1.58 (1.01−2.47), $P$ = .043) but not for patients in group 2 (Figure 2A; Appendix E in the online version). In contrast, patients in group 1 with a high frequency of mature monocytes (CD14$^+$HLA-DR$^+$ cells) within the CD45$^+$leukocyte population (> 38% of gated cells) had a shorter BCSS (21.4 vs. 10.5 months; HR = 0.43 (0.23−0.89), $P$ = .021) (Figure 2B). Further, an increased frequency of cells with the MDSC phenotype (CD14$^+$HLA-DR− cells) was associated with a longer BCSS but only for patients in group 1 (15.0 vs. 26.6 months, HR = 1.83 (1.03−3.26), $P$ = .04) (Figure 2C).

On the other hand, patients in group 2 with a higher frequency of myeloid DCs (CD11c$^+$CD123−/CD16$^+$ cells), had a longer BCSS (13.4 vs. 25.3 months, HR = 4.60 (1.23−17.1), $P$ = .023) as did the entire cohort of patients (17.7 vs. 25.5 months, HR = 1.63 (1.04−3.33), $P$ = .019), but not patients in group 1 (Figure 2D). Similarly, a higher frequency of pDCs (CD11c-CD123$^+$ cells) also indicated a longer BCSS for patients in group 2 (16.3 vs. 25.7 months, HR = 2.09 (1.05−4.16), $P$ = .036) (Figure 2E).

### *Frequency of Circulating CD8$^+$ T Cells Is Prognostic for Patients With Metastatic Breast Cancer*

The frequency of several different cytotoxic CD8+ T-cell phenotypes correlated with prognosis for patients with metastatic breast cancer treated with high-dose paclitaxel-containing chemotherapy (group 2), but not for patients treated with high-dose CTX (group 1). In general, patients with higher frequencies of early differentiated CD8$^+$CD45RA$^+$ cytotoxic T cells were associated with poorer prognosis, while patients with higher frequencies of CD8$^+$CD45RA− later differentiated cytotoxic T cells were associated with a better prognosis. In particular, we found that patients in group 2 with a higher frequency of T cells, including CD8$^+$CD45RA$^+$ (Figure 3A) or CD8$^+$CD45RA$^+$CD95− T cells (Figure 3B) had a shorter BCSS (27.5 vs. 12.6 months, HR = 0.43 (0.21−0.88), $P$ = .021) or (28.7 vs. 12.6 months, HR = 0.42 (0.20−0.87), $P$ = .012), respectively. Similarly, patients in group 2 with a higher frequency of the naive CD8$^+$CD45RA$^+$CD95−CD27$^+$CD28$^+$ phenotype had a shorter BCSS (28.7 vs. 12.6 months, HR = 0.32 (0.15−0.67), $P$ = .0028) (Figure 3C). Patients with a higher frequency of CD8$^+$CD45RA$^+$CD95$^+$CD27$^+$CD28− cells also had a shorter BCSS both in the entire cohort (20.7 vs. 15.0 months, HR = 0.55 (0.30−0.95), $P$ = .033) and in group 2 (8.0 vs. 25.9 months, HR = 6.13 (1.97−19.1), $P$ = .0017) (Figure 3D).

In contrast, patients in group 2 with a higher frequency of later differentiated CD8$^+$ cell types, such as CD8$^+$CD45RA−CD95$^+$CD27−CD28$^+$ cells, experienced a longer BCSS (12.6 vs. 29.4 months, HR = 2.17 (1.05−4.48), $P$ = .037)

## Frequency of Immune Cell Subtypes

**Table 1** Clinicopathologic Characteristics of Patients With Metastatic Breast Cancer

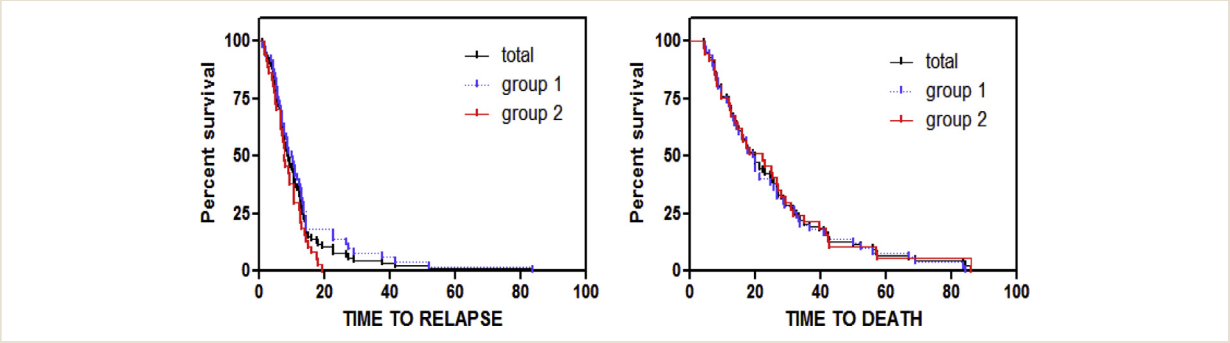
| Clinical Characteristic | Total | Group 1 | Group 2 |
|---|---|---|---|
| Total no. of patients | 88 | 51 | 37 |
| **Age** | | | |
| Mean (standard deviation) (years) | 43.6 (7.7) | 42.7 (7.7) | 44.8 (7.7) |
| < 40 years | 22 | 15 | 7 |
| 40–49 years | 45 | 26 | 19 |
| 50–59 years | 21 | 10 | 11 |
| **Estrogen Receptor (N = 76)** | | | |
| Negative | 29 | 17 | 12 |
| Positive | 47 | 28 | 20 |
| **Progesterone Receptor (N = 75)** | | | |
| Negative | 33 | 16 | 17 |
| Positive | 42 | 28 | 14 |
| **HER2 (N = 76)** | | | |
| Negative | 36 | 20 | 16 |
| Positive | 40 | 24 | 16 |
| **No. of Metastatic Sites** | | | |
| ID, 1 | 50 | 34 | 16 |
| ≥ 2 | 38 | 17 | 21 |
| **Metastatic Sites (No/Yes)** | | | |
| Bone | 43/45 | 16/25 | 17/20 |
| Lung | 60/28 | 38/13 | 22/15 |
| Lymph node | 65/23 | 39/12 | 26/11 |
| Liver | 72/16 | 42/9 | 30/7 |
| Other | 69/19 | 40/11 | 29/8 |
| **Adjuvant Endocrine Therapy** | | | |
| No | 59 | 35 | 24 |
| Yes | 29 | 16 | 13 |
| **High-Dose Chemotherapy Regimen** | | | |
| Mitoxantrone, cyclophosphamide, vinblastine | 29 | 29 | |
| Mitoxantrone, cyclophosphamide, carboplatin | 16 | 16 | |
| Mitoxantrone, cyclophosphamide, paclitaxel | 37 | | 37 |
| Thiotepa, cyclophosphamide, carboplatin | 5 | 5 | |
| Mitoxantrone, cyclophosphamide | 1 | 1 | |

(Figure 3E). Group 2 patients and patients in the entire cohort with a higher frequency of $CD8^+CD45RA^-CD95^+CD27^+CD28^+$ cells showed a longer BCSS (12.3 vs. 25.3 months, HR = 3.16 (1.30–7.66), $P = .011$) and (14.3 vs. 21.4 months, HR = 1.74 (1.03–2.94), $P = .041$), respectively (Figure 3F). A higher frequency of $CD8^+CD95^+$ cells indicated a longer BCSS for patients in group 2 (12.5 vs. 29.4 months, HR = 5.33 (2.24–12.7), $P = .002$) and for the entire cohort (14.0 vs. 25.4 months, HR = 2.03 (1.28–3.21), $P = .0025$) (Figure 3G). A higher frequency of $CD8^+CD45RA^-CD95^+$ cells also indicated a significantly longer BCSS for patients in group 2 (15.1 vs. 31.1 months, HR = 2.61 (1.22–5.57), $P = .013$) (Figure 3H).

### *Frequency of Circulating $CD4^+$ T Cells Is Prognostic for Patients With Metastatic Breast Cancer*

The frequency of several $CD4^+$ T helper cell subtypes also correlated with prognosis for patients with metastatic breast cancer treated with high-dose paclitaxel-containing chemotherapy (group 2). An increase in the frequency of circulating $CD4^+$ cells was associated with an improved prognosis for patients in group 2 (15.5 vs. 25.3 months, HR = 2.35 (1.10–5.02), $P = .028$), but not for the entire cohort or for patients in group 1 (Figure 4A). In addition, a higher frequency of $CD4^+CD95^+$ cells was also associated with a longer BCSS for patients in group 2 (12.5 vs. 31.1 months, HR = 3.98 (1.79–8.83), $P = .007$) (Figure 4B).

**Figure 1** Clinical Outcome of Patients Treated With High-Dose Chemotherapy Including Cyclophosphamide (Group 1) or Paclitaxel (Group 2)




A higher frequency of some early differentiated T helper cell subtypes that express the $CD4^+$ and $CD45RA^+$ markers was associated with a poorer prognosis. For example, there was a shorter BCSS for patients in group 2 with a higher frequency of $CD4^+CD45RA^+CD95-$ cells (28.0 vs. 16.0 months, HR = 0.30 (0.13−0.69), $P = .0046$) (Figure 4C), a higher frequency of $CD4^+CD45RA^+$ cells (25.1 vs. 14.1 months; HR = 0.25 (0.09−0.072); $P = .011$) (Figure 4D), and a higher frequency of $CD4^+CD45RA^+CD95-CD27^+CD28^+$ cells (29.4 vs. 15.1 months, HR = 0.45 (0.22−0.91), $P = .027$) (Figure 4E).

In contrast, there was a longer BCSS for patients in group 2 with a higher frequency of later differentiated T helper cells, $CD4^+CD45RA-CD95^+$ (13.4 vs. 31.1 months, HR = 3.71 (1.68−8.21), $P = .0012$) (Figure 4F); a higher frequency of central memory T helper cells, $CD4^+CD45RA-CD95^+CD27^+CD28^+$ cells (15.1 vs. 25.3 months, HR = 2.33 (1.11−4.87), $P = .025$) (Figure 4G); and a higher frequency of effector T helper cells, $CD4^+CD45RA-CD95^+CD27-CD28^+$ cells (13.4 vs. 31.1 months, HR = 4.53 (2.01−10.21), $P = .0003$) (Figure 4H).

### *Associations Between Clinicopathologic Characteristics and Immune Cell Frequencies in Patients With Metastatic Breast Cancer*

Because previous studies had shown correlations between the frequencies of specific cell populations and hormone receptor status,[3,36,37] we tested for interactions between the frequency of different immunophenotypes and clinicopathologic characteristics. We found no association between patient age and any of the immune cell populations. There were a few associations between estrogen receptor and progesterone receptor status and immunophenotype, but none remained significant after Bonferroni correction for multiple testing. However, there were several $CD4^+$ (but not monocyte, DC, or $CD8^+$) cell immunophenotypes that were associated with differences in HER2 status. $HER2^+$ patients in the entire cohort were associated with a higher frequency of T helper cell types including $CD4^+$ ($P = .009$), $CD4^+CD45RA^+$ ($P = .0018$), $(CD4^+)CD45RA^+CD95^+CD27-CD28^+$ ($P = .0019$), and $(CD3^+)CD4^+CD45RA^+CD95^+$ ($P = .0046$) cells and a lower frequency of effector $(CD4^+)CD45RA-CD95^+CD27-CD28^+$ ($P = .0025$) T cells compared to HER2− patients (data not shown).

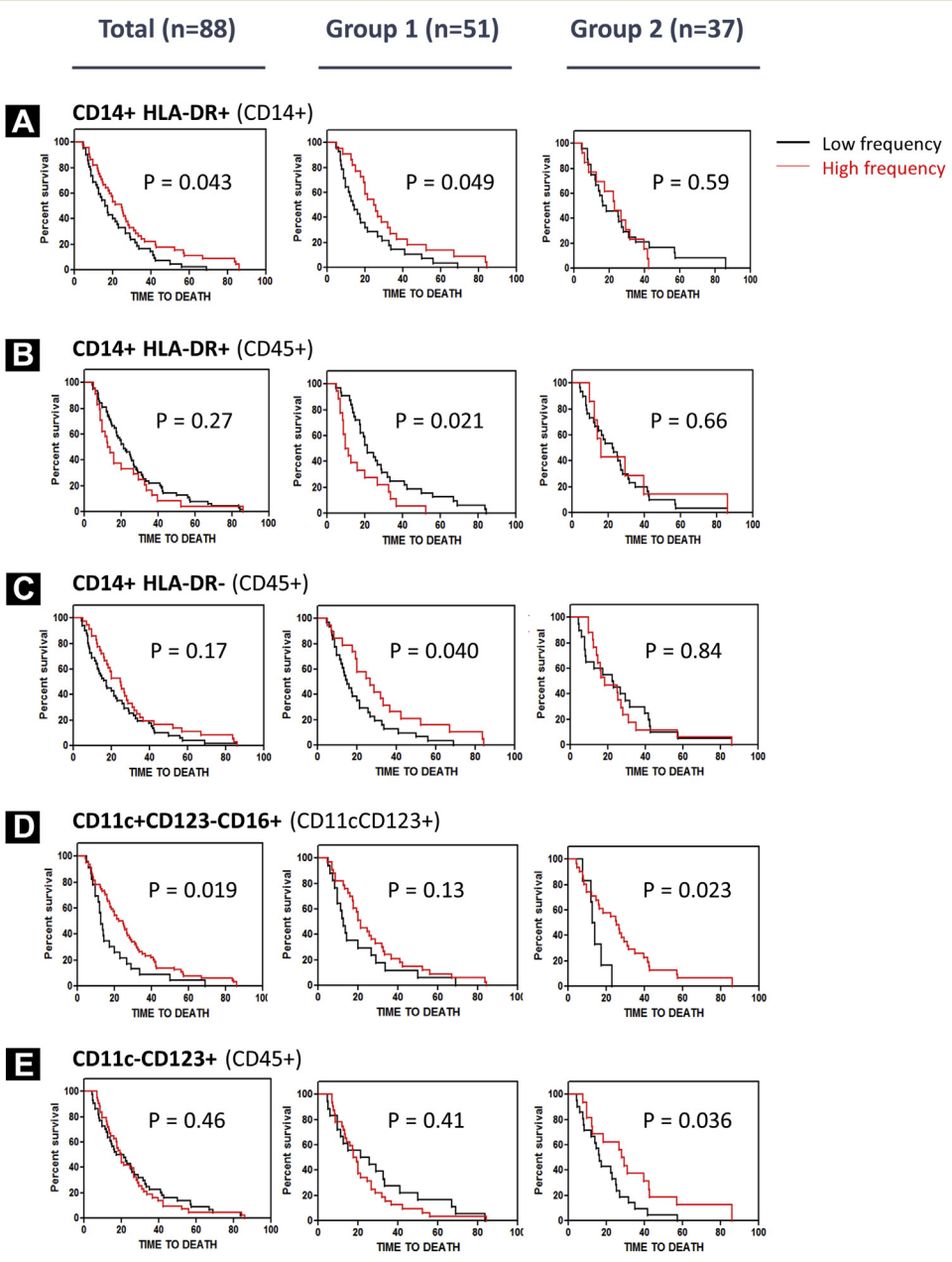
## Discussion

In this study, the relative frequencies of certain monocyte, DC, and/or helper ($CD4^+$) and cytotoxic ($CD8^+$) T-cell subtypes were shown to correlate with BCSS for patients with metastatic breast cancer treated with high-dose chemotherapy. The ability of the frequency of an immune cell type to indicate differences in BCSS was dependent upon the chemotherapy regimen used to treat the patients. Differences in the frequency of some monocyte subtypes could predict prognosis for patients treated with high-dose CTX-based chemotherapy (group 1) while differences in some $CD4^+$ and $CD8^+$ T-cell subtypes could predict prognosis for patients treated with high-dose paclitaxel-based chemotherapy (group 2). Except for their treatment regimens, the patients in group 1 and group 2 were very similar in age, hormone receptor status, and clinical outcome following treatment. In addition, the frequencies of the different immune cell types were similar between the two groups of patients. Since the samples tested for this study were obtained before treatment with chemotherapy the difference in the ability of distinct baseline immune subsets to predict BCSS must be selected by outcome in response to the particular chemotherapy.

Different chemotherapy drugs have differential effects on specific immunophenotypes. For example, treatment with doxorubicin or paclitaxel can eliminate MDSCs in a mouse model[38,39] and treatment of patients with paclitaxel can decrease the number of peripheral MDSC[40] and enhance the immune response.[41] In contrast, treatment with CTX can increase the number of MDSC[42,43] and decrease the function and number of $CD4^+$ and $CD8^+$ Tregs[44,45] and cytotoxic T cells and T helper cells.[46] Therefore, treatment with different chemotherapies can alter immune survival to ablate the effects of the baseline immunophenotype on clinical outcome. For example, paclitaxel treatment eliminates MDSC, which could eliminate their impact after treatment, but has minimal effects on T cells, which allows the effects of baseline T-cell levels to remain prognostic.

Patients treated with CTX-based high-dose chemotherapy (group 1) and a higher frequency of functional monocytes ($CD14^+HLA-DR^+$) within the $CD14^+$ population (frequency range 47%−100%) correlated with a better outcome while patients with a higher frequency of $CD14^+HLA-DR^+$ as a proportion of all $CD45^+$ leukocytes (frequency range 5.4%−62.9%) correlated with a poorer

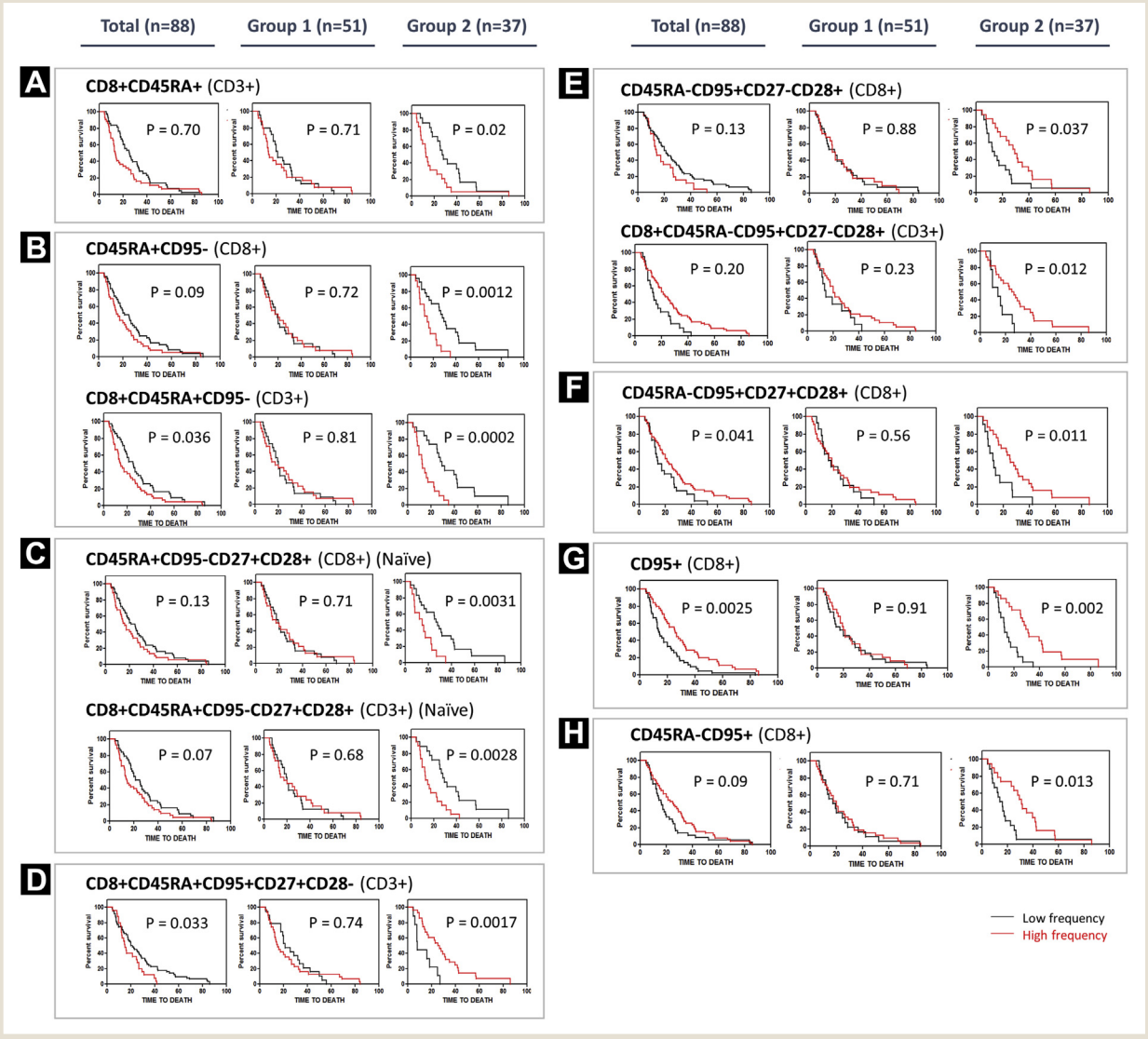
**Figure 2** Impact of Myeloid Immunophenotypes on Breast Cancer–Specific Survival for Patients Treated With Cyclophosphamide-Based (Group 1) or Paclitaxel-Based (Group 2) High-Dose Chemotherapy. Total Cohort (Left, n = 88), Group 1 (Center, n = 51), and Group 2 (Right, n = 37) Were Characterized as High-Frequency (Red) or Low-Frequency (Black) Levels of Indicated Cell Type. (A-C) Indicate Monocytic Populations, Whereas (D and E) Refers to Dendritic Cell Populations. Percentage Survival Was Analyzed Using the Kaplan-Meier Survival Method. Indicated *P* Values Were Determined by Log-Rank Statistics for Comparison. Populations Shown in Brackets After Each Immune Cell Phenotype Indicate the Reference Population Used to Determine the Frequency of Cells in Each Patient




prognosis. Selection of $CD14^+HLA\text{-}DR^+$ cells from the $CD14^+$-gated population identifies the majority of the monocyte population while selection from the $CD45^+$ gated common leukocyte population is likely to include cells in addition to $CD14^+$ monocytes, such as DCs, which could have a significant impact on the relative frequency for each patient.

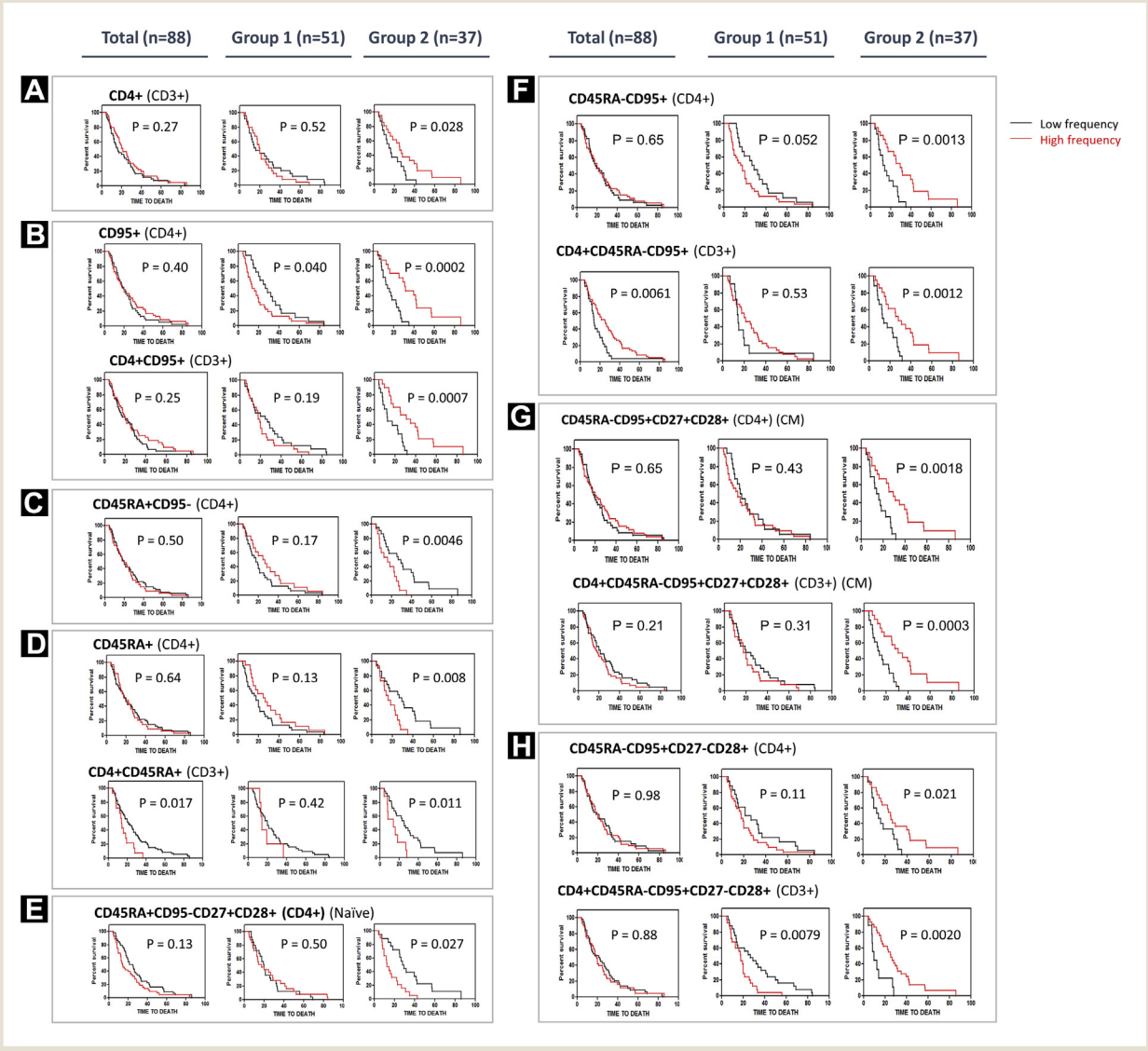
A higher frequency of cells with the MDSC phenotype $CD14^+HLA\text{-}DR-$ within the $CD14^+$ gated population (frequency range 0.01%–24.7%) was unexpectedly associated with a superior survival for patients treated with high-dose CTX. This is different from some previously published results, under other treatment conditions, where higher baseline levels of MDSC (eg, > 1% of

**Figure 3** Impact of CD8+ Cytotoxic T Cell Immunophenotypes on Breast Cancer–Specific Survival for Patients Treated With High-Dose Chemotherapy Including Cyclophosphamide (Group 1) or Paclitaxel (Group 2). Total Cohort (Left, n = 88), Group 1 (Center, n = 51), and Group 2 (Right, n = 37) Were Characterized as High-Frequency (Red) or Low-Frequency (Black) Levels of Indicated Cell Type. (A-D) Refer to CD45RA+ Populations, (E, F and H) Indicate CD45RA− Phenotypes, While (G) Shows a CD95+ Population. Percentage Survival Was Analyzed Using the Kaplan-Meier Survival Method. Indicated *P* Values Were Determined by Log-Rank Statistics for Comparison. Populations Shown in Brackets After Each Immune Cell Phenotype Indicate the Reference Population Used to Determine the Frequency of Cells in Each Patient




total peripheral blood mononuclear cells) in patients with breast cancer are associated with a poorer prognosis.[10,14,47] One reason for such differences is that MDSC have been defined in previous studies using different phenotype markers than those employed here such as CD13, CD15, and CD33 which could impact the ability to compare results across studies.[18,48-50] Some reports that suggest that the levels of MDSC in breast cancer tumors do not correlate with the level of MDSC in the circulation.[51] Further, cryopreservation also depletes granulocytic MDSC which can alter the interpretation of some previous observations.[51] MDSC are thought to interfere with normal activation of adaptive immune responses against the tumor and promote the production of angiogenic growth factors to support tumor growth and spread.[52] The observation that CTX can enhance MDSC levels[42] suggests that it may be able to accelerate the expected inhibitory effect of MDSC cells in patients with lower baseline MDSC frequencies. In this study, the frequency of MDSC did not correlate with outcome for patients treated with high-dose paclitaxel-containing chemotherapy. Treatment with paclitaxel can strongly inhibit MDSC[41] and decrease MDSC number which could ablate the expected inhibitory effect of MDSC in patients with high baseline levels of MDSC.

**Figure 4** Impact of CD4+ Helper T Cell Immunophenotypes on Breast Cancer–Specific Survival for Patients Treated With High-Dose Chemotherapy Including Cyclophosphamide (Group 1) or Paclitaxel (Group 2). Total Cohort (Left, n = 88), Group 1 (Center, n = 51), and Group 2 (Right, n = 37) Were Characterized as High-Frequency (Red) or Low-Frequency (Black) Levels of Indicated Cell Type. (A) Refers to CD4+ T Cells, (B) Indicates CD95+ T Cell Populations, (C-E) Indicate CD45RA+ Phenotypes, (F-H) Show CD45RA− Populations. Percentage Survival Was Analyzed Using the Kaplan-Meier Survival Method. Indicated P Values Were Determined by Log-Rank Statistics for Comparison. Populations Shown in Brackets After Each Immune Cell Phenotype Indicate the Reference Population Used to Determine the Frequency of Cells in Each Patient




Abbreviation: CM, central memory.

Increased levels of DCs, including pDCs ($CD123^+$) and myeloid DCs ($CD11c^+$), were associated with an improved BCSS for patients treated with paclitaxel-based high-dose chemotherapy (group 2) but not for patients treated with CTX-based chemotherapy (group 1). A previous study has shown that an increased frequency of pDCs was associated with a better prognosis for patients with breast cancer[9] which supports the idea that an increased level of antigen-presenting DCs could be associated with an improved antitumor immune response, regardless of the type of therapy given.

We showed that a high frequency of $CD45RA^+$ early differentiated $CD4^+$ and/or $CD8^+$ T cells was associated with a relatively poor prognosis for patients with metastatic breast cancer treated with high-dose paclitaxel-containing chemotherapy. This is consistent with the idea that most $CD45RA^+$ T cells, especially $CD4^+$ T cells, are early stage T cells that have not been previously activated[53] and should not be involved in antitumor activity. In contrast, we showed that a high frequency of CD45RA− later differentiated $CD4^+$ and/or $CD8^+$ T cells was associated with an improved

prognosis which supports the idea that previously activated $CD4^+$ or $CD8^+$ CD45RA− T cells[53] may contribute to antitumor activity. Studies in melanoma also showed that a higher baseline frequency of $CD8^+$ effector memory 1 T cells ($CD8^+CD45RA-CD27^+CD28^+$) was associated with an improved outcome.[20] Our observation that CD45RA− T cells are associated with improved prognosis makes it unlikely that the level of Tregs makes a significant contribution to prognosis in this group of patients. It appears that the level of later differentiated $CD4^+$ subtypes makes the biggest contribution to prognosis for patients in group 2 since the level of $CD4^+$ cells converted to later stages of differentiation is larger than the level of converted $CD8^+$ cells. The median frequency of helper T cells ($CD3^+CD4^+$) in the entire cohort of patients was > 50% (range 14%−78%) and the median frequency of $CD3^+CD4^+CD45RA^+$ T cells was only 13% (range 2%−47%) while the median frequency of cytotoxic T cells ($CD3^+CD8^+$) was 35% (range 14%−57%) and the median frequency of $CD3^+CD8^+CD45RA^+$ T cells was 22% (range 6%−40%).

The increased frequency of cells expressing CD95 in both the $CD4^+$ and $CD8^+$ T cell populations also seems to be associated with an improved prognosis for the patients in group 2, consistent with previous reports.[54,55] Further, the association of $CD95^+$ T cells with an improved prognosis is related to its expression of CD45RA−: T cells that are CD45RA− and $CD95^+$ indicate an improved prognosis while T cells that express CD45RA but not CD95 indicate a poorer prognosis (whereas an increased frequency of $CD45RA^+CD95^+$ cells or CD45RA−CD95− T cells did not indicate a difference in prognosis). In addition, the frequency of $CD45RA^+$ cells that also express $CD95^+$ is different between $CD4^+$ and $CD8^+$ cells. A significantly higher frequency of $CD3^+CD4^+CD45RA^+$ do not express CD95 (median 9.2%) compared to $CD3^+CD4^+CD45RA^+$ cells that are also $CD95^+$ (median 2.7%) while both $CD3^+CD8^+CD45RA^+CD95-$ cells (10%) and $CD3^+CD8^+CD45RA^+CD95^+$ cells (11%) are similar. This is consistent with the observation that a greater number of $CD4^+$ subtypes contribute to prognosis for the patients in group 2. In addition, HER2 status correlates with the frequency of a subgroup of the $CD4^+$ cells (but not $CD8^+$ cells) suggesting the possibility that a group of patients can express anti−HER2−reactive $CD4^+$ cells which may contribute to antitumor activity. However, $HER2^+$ patients with a higher frequency of effector T helper cells that were CD45RA− did not show a significant difference in outcome. Some previous reports have indicated that tumor subtype (or hormone receptor status) can impact the ability of different immune subtypes to predict prognosis in breast cancer patients.[3,36,37] For example, patients with luminal (estrogen receptor positive) breast cancer subtypes had a lower level of T-cell infiltration than patients with nonluminal (estrogen receptor negative) breast cancer and patients with triple-negative breast tumors, which have the poorest prognosis, had higher levels of T-cell infiltration. In this group of patients, the breast cancer type (luminal A/normal, luminal B, HER2 positive, and triple negative) did not correlate with differences in the frequency of the circulating $CD4^+$ or $CD8^+$ immunotypes. This suggests that there is no difference in immunophenotype specific for hormone receptor status in our metastatic breast cancer patients although it is possible that the reported differences in infiltrating T cells are not reflected in circulation.

## Conclusion

Immunophenotypes can be used as biomarkers of prognosis for patients with metastatic breast cancer, although this is highly dependent on the chemotherapy used for treatment. The present study shows that a higher frequency of certain circulating $CD4^+$ immunophenotypes, and to a lesser extent of $CD8^+$ immunophenotypes, in particular those expressing CD95 but not CD45RA, were associated with a better clinical outcome for metastatic breast cancer patients treated with paclitaxel-based high-dose chemotherapy. The frequency of circulating $CD11c^+CD123-$ DCs was also associated with prognosis for this group of patients. In contrast, patients treated with CTX-based high-dose chemotherapy showed that some monocytic cells, but not T cells or DCs, could be correlated with outcome. It will be interesting to see if differences in chemotherapy regimen will identify different prognostic biomarkers in other studies of breast cancer patients.

### *Clinical Practice Points*

- The frequency of circulating myeloid or T cell populations can indicate clinical outcome for some groups of patients with metastatic breast cancer being treated with chemotherapy.
- The utility of using immunophenotype as a biomarker for clinical outcome for patients with metastatic breast cancer depends on the chemotherapy regimen used for treatment.
- Different chemotherapy regimens can differentially affect the ability of immunophenotype to predict outcome in patients with metastatic breast cancer and therefore may also affect the potential success of immune therapies.

## Disclosure

The authors have stated that they have no conflict of interest.

## Supplemental Data

Supplemental appendixes accompanying this article can be found in the online version at https://doi.org/10.1016/j.clbc.2019.05.002.

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# Low levels of intra-tumoural T cells in breast cancer identify clinically frail patients with shorter disease-specific survival

Lisa Speigl [a], Alexandra Grieb [a], Nicole Janssen [a], Sigrid Hatse [b,c], Barbara Brouwers [b], Ann Smeets [d], Giuseppe Floris [e,f], Jithendra Kini Bailur [a], Cindy Kenis [c], Patrick Neven [e], Hans Wildiers [b,c,e], Graham Pawelec [a,g,h,i], Christopher Shipp [a,j,*,1]

[a] Department of Internal Medicine II, University Hospital Tübingen, Germany
[b] Laboratory of Experimental Oncology (LEO), Department of Oncology, KU, Leuven, Belgium
[c] Department of General Medical Oncology, University Hospital Leuven, Leuven Cancer Institute, Leuven, Belgium
[d] Department of Surgical Oncology, University Hospital Leuven, Belgium
[e] Leuven Multidisciplinary Breast Center, University Hospital Leuven, Belgium
[f] Department of Imaging and Pathology, KU Leuven, Leuven, Belgium
[g] John van Geest Cancer Research Centre, Nottingham Trent University, UK
[h] Division of Cancer Studies, King's College London, London, UK
[i] Health Sciences North Research Institute, 41 Ramsey Lake Road, Sudbury, ON, Canada
[j] Natural and Medical Sciences Institute (NMI) at the University of Tübingen, Germany

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ABSTRACT

*Objectives:* The global health status of older patients with cancer influences their clinical course, but little is known regarding the influence of the immune system on the global health of older patients with cancer. The goal of this study was to assess the relationships between patient fitness/frailty status and survival, and the local tumour immune environment of older patients with breast cancer.
*Materials and Methods:* In a cohort of 58 older patients with breast cancer (over 70 years of age), fluorescence microscopy was used to investigate whether levels of intra-tumoural T cells (CD3+) and granulocytic cells (CD15+) could predict patient clinical outcome, and/or whether they correlated with patient physical and mental performance as evaluated by comprehensive geriatric assessment.
*Results:* We observed that patients with higher levels of intra-tumoural T cells were fitter according to a number of clinical health measures including G8 ($p = 0.006$), Karnofsky Index ($p = 0.0372$), and Leuven Oncology Frailty Score (LOFS) ($p = 0.0187$). In contrast, high relative levels of granulocytic cells were found in patients with poorer clinical health (LOFS, $p = 0.0474$). Furthermore, high levels of T cells but not granulocytic cells were associated with longer breast cancer-specific survival ($p = 0.0444$).
*Conclusions:* This is the first study to show that low relative levels of intra-tumoural T cells are associated with inferior patient fitness. In contrast to T cells, we observed that intra-tumoural granulocytic cells displayed an inverse relationship with patient performance. Further research is needed to determine whether boosting the level of intra-tumoural T cells in older non-fit patients can result in improved outcome.


## 1. Introduction

Breast cancer incidence rises dramatically with age; up to a fivefold increase in risk is seen in women above the age of 65 [1]. Consequently, as populations in developed countries continue to increase in life expectancy and to show a greater proportion of older adults, the incidence of older patients developing (breast) cancer will continue to rise [2]. These biological and sociological factors will result in a flood of older patients with breast cancer in the future, placing significant additional strain on health services. However despite these clear trends, older patients are

* Corresponding author at: Department of Internal Medicine II, University Hospital Tübingen, Waldhörnlestr. 22, 72072 Tübingen, Germany.
*E-mail addresses:* alexandra.grieb@student.uni-tuebingen.de, (A. Grieb), sigrid.hatse@kuleuven.be, (S. Hatse), ann.smeets@uzleuven.be, (A. Smeets), giuseppe.floris@uzleuven.be, (G. Floris), cindy.kenis@uzleuven.be, (C. Kenis), patrick.neven@uzleuven.be, (P. Neven), hans.wildiers@uzleuven.be, (H. Wildiers), graham.pawelec@uni-tuebingen.de, (G. Pawelec), mrchristophershipp@gmail.com. (C. Shipp).
[1] Current affiliation: Natural and Medical Sciences Institute (NMI) at the University of Tübingen, Germany.

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nevertheless under-represented in clinical trials, limiting the applicability of many treatments [3]. Age-associated decline impairs the tolerability of standard therapies, and a lack of therapeutic options specifically tailored for this patient group makes their clinical management arguably more difficult [4]. Furthermore, older adults are a highly heterogeneous population in terms of physical health, a factor which by itself can determine patient outcome. For example, frailty is common in the elderly and can influence to what extent a patient tolerates anti-tumour treatment [5], yet there is generally no routine assessment of clinical health in such patients. Chronologic age is a poor indicator of patient health which cannot be used by itself to identify clinically frail patients [6]. Geriatric assessment is one approach to assess the health state of older cancer patients, but its use is currently not standardised. Furthermore, the time consuming nature of geriatric assessment makes it impractical to implement on a routine basis. Therefore, there is a need to easily assess the health state of patients in order to select the most appropriate therapies that will minimise toxicity and maximise therapeutic efficacy [7]. This may allow the identification of older patients who are unable to tolerate conventionally indicated treatment, those at risk for treatment complications and those who can be treated similarly to their younger counterparts [8]. This type of accurate assessment of patient health remains a major clinical challenge to date, but will need to be overcome in future.

Despite steady advances, breast cancer treatment in fact remains suboptimal for patients of all ages, particularly those with late-stage disease [9]. Ineffective treatment is not only due to the inability to apply customised therapy to each patient individually, but it is also the result of an incomplete understanding of disease progression. Recently, an appreciation for the role of the immune system in cancer progression has been gaining momentum in light of studies unequivocally linking the immune system with therapeutic response and patient survival [10–12]. Interactions between the immune system and cancer are complex; they can result either in tumour suppression, tumour promotion, or both [13]. One of the most important anti-tumour factors identified to date are tumour-infiltrating lymphocytes, high levels of which have been shown to correlate with delayed disease progression and favorable clinical outcome in a number of cancer types including breast cancer [14–18]. Despite this, relatively little is known about the differential roles of distinct types of tumour-infiltrating immune cells in cancer progression. For example, whereas patients with breast cancer whose tumours show higher levels of T cells clearly experience a more favorable prognosis [19–21], the role of tumour-infiltrating myeloid cells is much less intensively studied and remains obscure. A better understanding of such aspects may increase the accuracy of predicting patient survival and lead to a more complete understanding of the factors associated with disease progression.

Adapting cancer therapies, particularly emerging immunotherapies, to older patients will become increasingly important in the near future. Compared with younger individuals, distinct changes are found in the immune systems of older individuals [22], making the customisation of cancer (immuno) therapies to this population a unique challenge. The present study so far is one of the few investigations aimed at developing an understanding of the clinical importance of the immune system in elderly breast cancer. We assessed two major immune subsets - T cells and granulocytes - in the tumours of older patients with breast cancer (≥70 years). We tested if the levels or the location of these cells are important for prognosis and/or for patient clinical frailty, evaluated here by geriatric assessment and a suite of other performance status indicators. By combining the measurement of clinical health and immune features, we attempted to identify more accurate methods of assessing patient status. Studies of this type will be important to improve patient management and identify new therapeutic targets for older patients with cancer.

## 2. Materials and Methods

### 2.1. Patient Cohort

We previously performed a prospective, multi-centre study for which 109 patients were recruited in two academic and two regional hospitals in Belgium [23]. Participants were aged 70 years or older with early invasive breast cancer. The first cohort ($n = 57$) consisted of patients who were treated with adjuvant chemotherapy (4 x docetaxel-cyclophosphamide), combined with trastuzumab in HER2-positive cases, and followed by an aromatase inhibitor in ER-positive cases. The second cohort ($n = 52$) consisted of patients where adjuvant chemotherapy was not indicated, and who received an aromatase inhibitor as sole adjuvant systemic treatment. The study was approved by the ethics committees of the participating hospitals and written informed consent was obtained from all patients for the use of their biological material for research purposes. For the present substudy, we included 58 patients for whom tumour biopsies from the surgical specimen were available. All biopsies were taken prior to the initiation of any treatment. A representative tumour block was selected by an expert pathologist. Detailed patient characteristics are summarised in Table 1.

Patients underwent a full geriatric assessment (GA) [24] and quality of life (QoL) evaluation by a trained nurse prior to administration of systemic therapies as previously described [25]. In brief, we assessed the clinical health of this cohort according to the geriatric screening

**Table 1**
Patient characteristics.

| Factor | Patients (n = 58) | |
|---|---|---|
| Age | | |
| Median (years) | 75 | |
| Range | 70–90 | |
| pT | n | % |
| 1 | 11 | 19.0 |
| 2 | 42 | 72.4 |
| 3 | 2 | 3.5 |
| 4 | 3 | 5.2 |
| pN | n | % |
| 0 | 21 | 36.2 |
| 1–3 | 36 | 62.1 |
| x | 1 | 1.7 |
| Histological subtype | n | % |
| Ductal | 48 | 82.8 |
| Lobular | 6 | 10.3 |
| Ductal + lobular | 2 | 3.5 |
| Ductal + other | 2 | 3.5 |
| Molecular subtype surrogate | n | % |
| Basal like | 8 | 13.8 |
| Her2 positive | 2 | 3.5 |
| Luminal A | 19 | 32.8 |
| Luminal B Her2 negative | 24 | 41.4 |
| Luminal B Her2 positive | 5 | 8.6 |
| Hormone receptor expression | n | % |
| ER positive | 49 | 84.5 |
| PR positive | 39 | 64.2 |
| Adjuvant therapy | n | % |
| Chemotherapy | 30 | 51.7 |
| Antihormonal therapy | 48 | 82.8 |
| Trastuzumab | 6 | 10.3 |
| Radiotherapy | 51 | 87.9 |

n: number of patients.
Her2: Human epidermal growth factor receptor 2.
pT: pathological tumour size.
pN: pathological lymph node status.
ER: estrogen receptor.
PR: progesterone receptor.
Molecular subtype: for details see [40].
Chemotherpy: docetaxel-cyclophosphamide.
Antihormonal therapy: Letrozole, Anastrozole, Tamoxifen.

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test G8 and Karnofsky Performance Score (KPS). We also performed a formal geriatric assessment including social data, functional status assessed by Katz's Activities of Daily Living (ADL) and by Lawton's Instrumental Activities of Daily Living (IADL) scales, fall history, self-perceived fatigue assessed by the Mobility–Tiredness test (MOB-T), cognitive status by the Mini Mental State Examination (MMSE), mood by the 15-item Geriatric Depression Scale (GDS-15), nutritional status by the MNA-Short Form (MNA-SF), and comorbidity by the Charlson Comorbidity Index (CCI) [25]. Pain was evaluated in every patient using a Visual Analogue Scale (VAS). Polypharmacy was assessed by the number of different registered drugs (www.bcfi.be) the patient was using during the week preceding study inclusion. Quality of life was assessed with the EORTC QLQ-C30 questionnaire. Geriatric assessment results were also summarised according to the Balducci criteria of frailty, and according to the LOFS (Leuven Oncology Frailty Score) which summarises GA results in a single score, ranging from 10 (very fit) to 0 (very frail) [26].

Tumour characteristics (tumour molecular subtype according St. Gallen criteria, hormone receptor expression and TNM staging), as well as treatment details and toxicity events during therapy were also recorded.

### 2.2. Evaluation of Tumour-infiltrating Leukocytes

Five micrometre thick formalin-fixed paraffin-embedded tissue sections were stained with antibodies against CD3 and CD15 according to the previous protocol [27] but with a modified antigen retrieval method [28]. The following antibodies were used: CD3 rabbit polyclonal (Abcam, Cambridge, UK), CD15 rabbit monoclonal (clone SP159) (Novus Biologicals, Littleton, USA). The secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG (H + L) (Jackson ImmunoResearch Laboratories, West Grove, USA) was used for all experiments. All tissues were stained with secondary antibodies as a negative control. Fluorescently-stained tissue slides were measured with a Zeiss Axiophot fluorescence microscope. Depending on size of tissue piece, an average of 15 images per tumour was captured at 40× magnification so that the number of images captured was roughly proportional to tumour size. Regions were selected in a non-biased manner in the invasive front and tumour centre covering the entire tumour mass (i.e. total tumour region including stromal and intra-epithelial cells). Images were scored by two independent investigators and results compared to identify potential discrepancies. When assessing intra-tumoural CD3+ and CD15+ cells, images from stained slides were compared to images from control tissues (slides stained with the secondary antibody only) for each tumour. The final number of positive cells was normalised so that it was independent of the number of images captured, thus all reported cell counts represent the average number of positive cells per image. The number of positive cells was scored using ImageJ version 1.48 (NIH, Bethesda, USA). This protocol was developed considering the recommendations made by the international working group on evaluating tumour-infiltrating leukocytes (TILs) in breast cancer [29] but could not be followed precisely because this was a novel research investigation that assessed specific populations of tumour-infiltrating cells using a different method (immunofluorescence here as opposed to H&E staining in the working group recommendations).

### 2.3. Statistical Analysis

Statistical analyses were performed using Prism software version 6 (GraphPad, La Jolla, USA). We did not assume that data were normally distributed and therefore 1) Correlations were assessed with non-parametric two-tailed (Spearman) correlation tests and 2) differences between two groups were assessed with two-tailed non-parametric (Mann-Whitney U) tests. For patient survival analysis, the Kaplan-Meier method using the log-rank test was applied. The median value was used as a cut off to divide the cohort into groups of similar sizes. Significant relationships were considered as $p < 0.05$. This was a hypothesis-generating study that did not necessitate correction for multiple testing. As such, all $p$ values are exploratory and require validation in an independent cohort.

## 3. Results

### 3.1. The Tumours of Older Patients With Breast Cancer Are Frequently Infiltrated by CD3+ Cells and CD15+ Cells

We observed the breast tumours of this older patient cohort to be commonly infiltrated by both CD3+ (T-cells) and CD15+ (granulocytic) cells. We found 93% (54 of 58) of breast tissues to contain T cells (mean cell count of 17, range 0–108), while granulocytic cells occurred less frequently (64% of tumours; 35 of 55) and at substantially lower abundance (mean 1, range 0–11) (Fig. 1A). There was no difference in the level of infiltration by CD3+ or CD15+ cells between the tumour centre and invasive front, although we did find that patients with higher levels of CD15+ cells in the tumour centre were younger (median age 72 years) compared with patients who showed less CD15 + infiltration in the tumour centre (median age 76 years) ($p = 0.0102$) (Fig. 1B). Similarly, this group of older patients showed relatively higher levels of granulocytic cells at the invasive front compared with the tumour centre ($p < 0.0001$, data not shown). Apart from this, there were no other associations between chronological age and the levels of tumour-infiltrating CD3+ cells or CD15+ cells. Representative fluorescence images showing tumour infiltration by CD3+ and CD15+ cells appear in Fig. 1C.

### 3.2. Immune Cell Infiltration in Older Breast Tumours is Associated With Tumour Grade and Hormone Receptor Status

Next, we investigated whether the degree of tumour infiltration by CD3+ and CD15+ cells was associated with tumoural features in older patients with breast cancer and found that patients with higher tumour grade had tumours that were more heavily infiltrated by CD3 + cells ($p = 0.0321$). We also observed that patients with higher levels of CD3+ cells in the tumour had lower or absent expression of oestrogen and progesterone receptors ($p = 0.0236$ and 0.0367, respectively) (Fig. 1D). This was also the case when considering CD3 levels in the invasive front and centre separately (ER: $p = 0.0221$ and PR: $p = 0.0237$ for invasive front and ER: $p = 0.0411$ and PR: $p = 0.0143$ for centre). In contrast, tumour infiltration by CD15+ cells showed the opposite relationship for PR expression; tumours with a greater level of CD15+ cells expressed higher levels of the progesterone receptor ($p = 0.0208$, Supplementary Data 1). No other associations between CD3+ and CD15+ cells with patient tumoural features were found.

### 3.3. Clinical Health and Performance Measures of Older Patients With Breast Cancer

We also assessed the global health status of older patients with breast cancer using different performance measures. According to the Balducci method, the majority of patients in this cohort were considered vulnerable (68%) with a mixture of frail (20%) and fit (12%) patients making up the remainder of the population. To obtain a more precise picture of the overall health of the cohort we also applied more detailed methods assessing clinical frailty, such as the (non-geriatric specific) Karnofsky performance scale (KPS) and the Leuven Oncogeriatric Frailty Score (LOFS) [26]. According to KPS, half of the patients from our cohort (51%) were able to perform daily activities required for normal functioning with a score of 100, with the remaining 49% showing some degree of health-related disability (range 90–20). Using the combined assessment method LOFS, we found the median score of this breast

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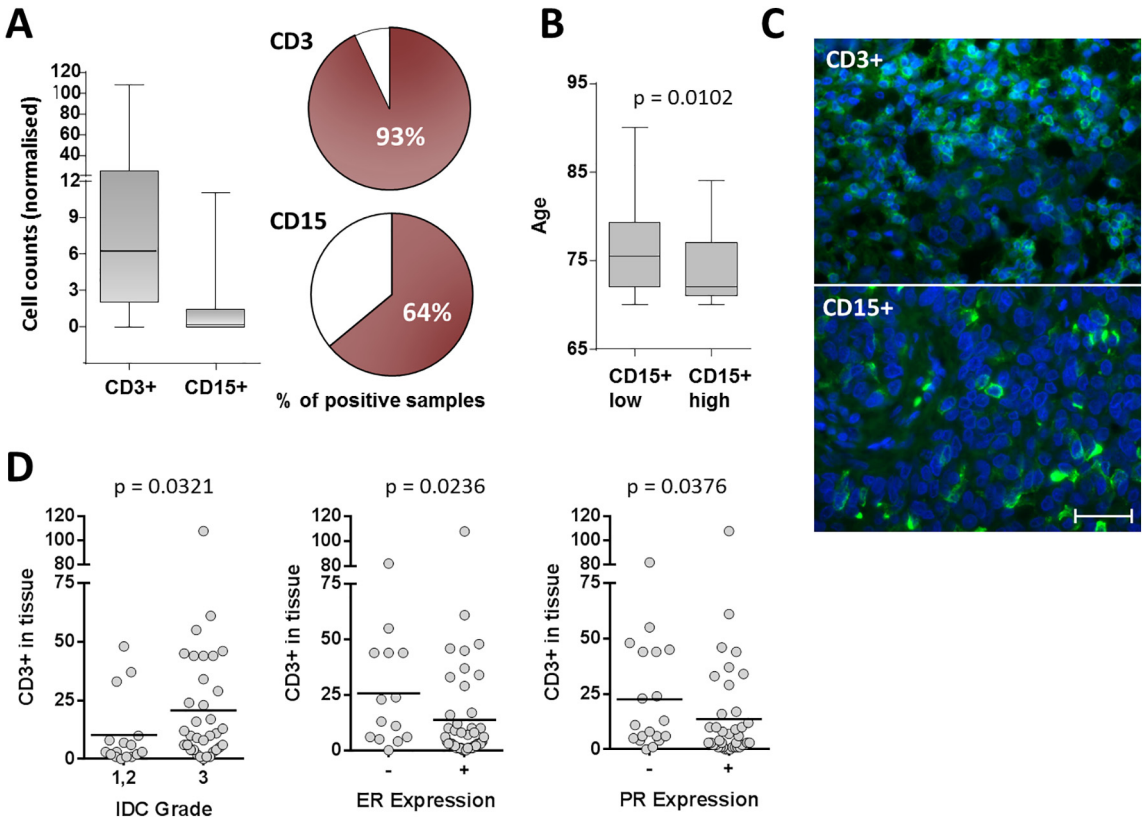


**Fig. 1.** Tumour-infiltrating leukocytes in older patients with breast cancer. Tumour infiltration by CD3+ ($n = 58$) and CD15+ ($n = 55$) cells in the tumour tissues of older patients with breast cancer was determined with fluorescence microscopy and the degree of infiltration investigated for association with patient clinical features. (A) Left panel: Comparison between the degree of tumour infiltration by CD3+ and CD15+ cells; whiskers indicate minimum/maximum values and bars indicate mean values. Right panel: comparison between the proportion of tumours infiltrated by CD3+ (93%) or CD15+ cells (64%) (B) Association between patient age and CD15+ infiltration in the tumour centre. (C) Representative immunofluorescence images of CD3+ and CD15+ cells in breast tumours (40× magnification). Scale bar indicates 50 μm. (D) Tumour infiltration by CD3+ cells and the association with the oestrogen and progesterone receptor expression and tumour grade. In each case the number of infiltrating cells represents the average number of cells in the entire tumour. IDC, invasive ductal carcinoma; ER, oestrogen receptor; PR, progesterone receptor.

cancer cohort to be 8, but it included patients ranging from very frail (LOFS = 1) to fit (LOFS = 10). Selected results showing the clinical health of the cohort are summarised in Fig. 2A.

### *3.4. Levels of Tumour-infiltrating T Cells Correlate with Clinical Frailty and Patient Performance Status*

Our prior study [30] showed that the levels of distinct leukocyte subsets in blood relate to the clinical health of older patients with breast cancer. We further investigated these findings here by examining whether immune features in the tumour tissues of these older patients are also associated with patient health or performance. Examining the levels of tumour-infiltrating CD3+ and CD15+ cells in the context of patient performance measures revealed a number of associations. We found that patients with a higher abundance of tumour-infiltrating CD3+ cells were fitter according to G8 geriatric assessment. This was the case for the level of total CD3+ cells ($p = 0.0060$, Fig. 2C) and those located in the invasive front ($p = 0.0170$, data not shown). In line with this, patients with higher CD3 levels also showed better performance status as measured by KPS ($p = 0.0372$) and superior status according to the LOFS ($p = 0.0187$) (Fig. 2B). These relationships were present when considering total levels of CD3+ cells and those in the invasive front ($p = 0.0028$ for LOFS and $p = 0.0109$ for KPS, data not shown). We observed the opposite trend for infiltration of CD15+ cells: patients with tumours that were more densely infiltrated by total levels of granulocytic cells were less fit according to LOFS ($p = 0.0474$, Supplementary Data 2), in line with our previous work which found higher levels of granulocytic cells in blood to be inversely associated with LOFS [30]. Other correlations between tumour CD15+ infiltration and clinical frailty measures were not identified. Selecting patients who showed high levels of T cells in combination with low levels of granulocytes emphasised these relationships by revealing a relatively homogeneous group of patients with high LOFS scores ($p = 0.0006$, Fig. 2B, far right panel). The full list of associations between intra-tumoural leukocytes and patient performance and including all measured clinical parameters is presented in Supplementary Data 2.

### *3.5. High Levels of Intra-tumoural T Cells Are Present in Longer-Surviving Patients With Breast Cancer*

The present study shows that the clinical health of older patients with breast cancer is reflected by the immune state within the tumour. Considering this, we asked whether the levels of leukocytes in tumour tissue are also informative for patient prognosis. Correlating levels of CD3+ cells and CD15+ cells with patient survival showed higher total levels of intra-tumoural T cells in patients with longer disease-specific survival up to 5 years following diagnosis ($p = 0.0444$) in univariate analysis (Fig. 2C). We observed the same result for CD3+ located at the invasive front ($p = 0.0444$), whereas T cells in the tumour centre were not associated with patient survival (data not shown). In contrast to T cells, granulocytic cells, irrespective of location in the tumour, were not associated with patient clinical course (Fig. 2C). Similarly, overall survival which considers all forms of death was not found to be associated with levels of intra-tumoural leukocytes.

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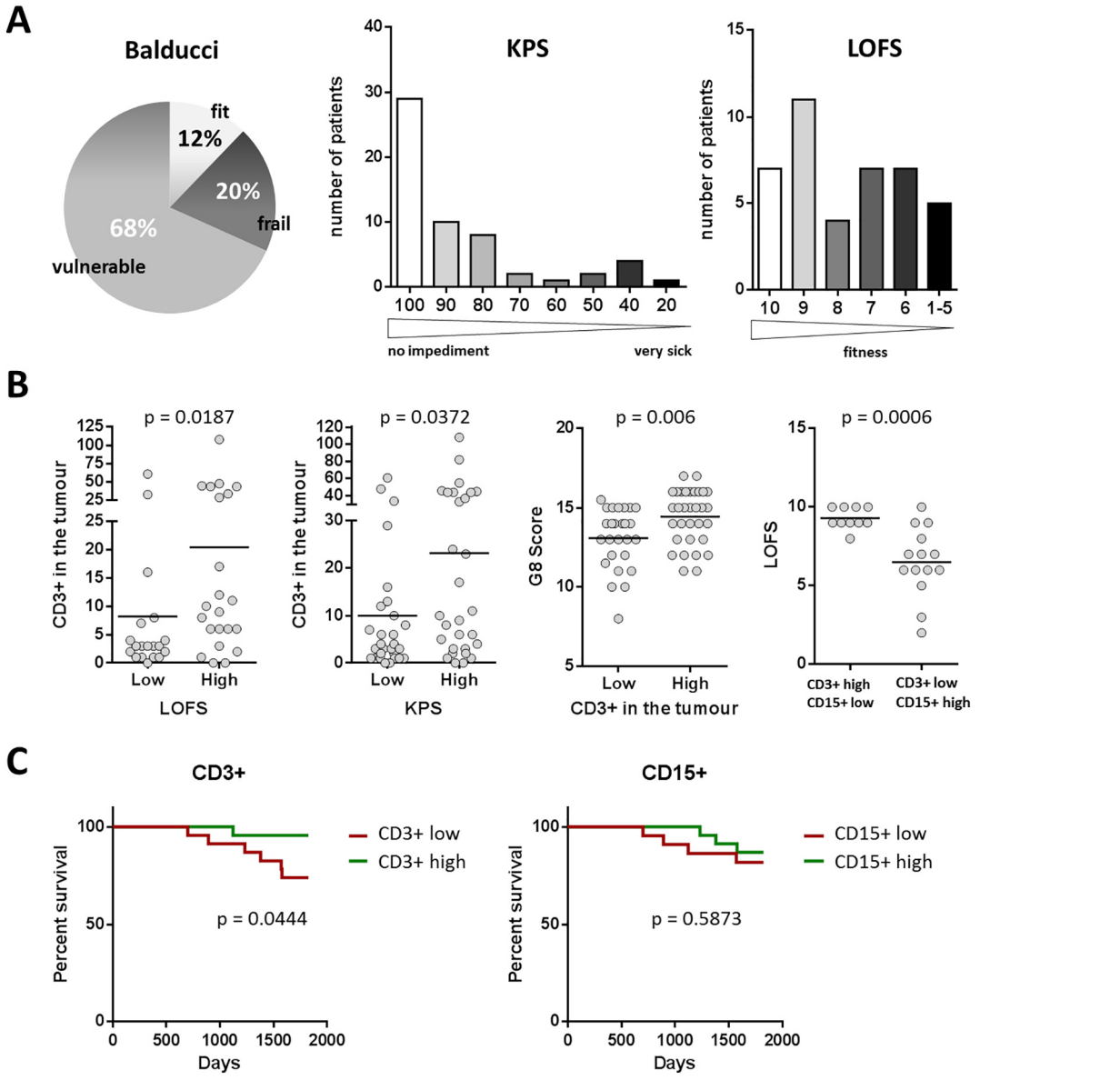


**Fig. 2.** Association between the clinical health of older patients with breast cancer and intra-tumoural leukocytes. The global health of older patients with breast cancer was assessed with G8, KPS (Karnofsky-Index), geriatric assessment (GA), Balducci frailty score, and the Leuven Oncogeriatric Frailty Score (LOFS). These measures were then correlated with tumour infiltration by CD3+ and CD15+ cells. (A) Summarised results for Balducci (left panel), KPS (middle panel) and LOFS (right panel) of the patient cohort (n = 58). (B) Correlations between tumour infiltration by CD3+ (n = 58) and CD15+ cells (n = 55) with the LOFS, KPS and G8 measures of clinical health. (C) Five-year breast cancer-specific survival of intra-tumoural CD3+ and CD15+ cells ($n = 46$). For all groupings of patient parameters (B and C), "low" refers to below median values and "high" indicates values equal to or above the median.

## 4. Discussion

This study examined the tumours of older patients with breast cancer for infiltration by immune cells. Our results showed that both CD3+ and CD15+ cells were commonly found in these breast tumours, suggesting that breast cancer in older patients may be generally immunogenic. In this context, we found that in the oldest patients there were higher relative levels of granulocytic cells at the invasive front compared with the tumour centre. This suggests that with increasing age there is reduced migration of myeloid cells into the tumour core, perhaps reflecting myeloid cell dysfunction that typically occurs with aging [31,32]. Additionally, the immune parameters measured here were found to be clinically relevant; patients with higher tumour grade had greater numbers of intra-tumoural CD3+ cells compared with patients with lower grade tumours. Higher CD3 levels also negatively correlated with the expression of oestrogen and progesterone receptors, in line with prior studies showing that hormone receptor-negative breast tumours are more heavily infiltrated by TILs [33,34].

We also assessed the clinical health of older patients with breast cancer by employing different performance measures. Using the Balducci method, the majority of patients was categorised as vulnerable, with frail and fit patients making up the remainder of the population. However, the Balducci method of frailty assessment is a rather broad approach that places individuals into one of three categorical health states. As such, this method cannot accurately reflect more subtle differences in the fitness of patients which may show a continuum of health states. In order to obtain a more precise picture of the health of this population we applied other methods of assessing clinical health such as KPS and LOFS, which are more nuanced in their categorisation of

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health status. In contrast to the Balducci method, patients with the highest Karnofsky performance scores made up roughly half of the cohort. Similarly, the LOFS which incorporates five fundamental aspects of geriatric assessment (including the ability to autonomously perform activities of daily living, cognitive function, nutritional state and comorbidities), also showed that patients with high fitness scores were common in this cohort. The latter two more refined methods of assessing patient fitness may be more useful for measuring patient status, because we found numerous associations between KPS and LOFS and the intra-tumoural immune features investigated here, whereas no relationships were found with the Balducci classification.

The primary goal of this study was to identify novel markers that can reflect clinical health in older patients with cancer. Although immune cell infiltration in breast tumours is well-recognised, little is known regarding the relationship between the immune system and the fitness of older patients with cancer. The results reported here suggest that the state of the immune system may influence the functioning of older patients with breast cancer, or vice versa. We identified a number of associations between the level of intra-tumoural CD3+ and CD15+ leukocytes and patient clinical health or performance, in turn proposing immune features as potential biomarkers for the clinical health of patients with cancer. Further, patients with higher levels of intra-tumoural CD3+ T cells were fitter and had higher patient performance status according to KPS, LOFS and G8. In contrast, high levels of CD15+ granulocytic cells in the tumour were found in patients with inferior health status according to LOFS. Collectively, these results suggest that superior patient health and functioning is linked with high levels of intra-tumoural T cells, whereas on the other hand granulocytic cells appear to be associated with poorer health in older patients. However, it remains to be clarified whether the intra-tumoural immune status leads to better clinical health, or if superior clinical health results in improved immune status. Increasing age is associated with elevated output of myeloid cells and reduced output of naïve T cells, the latter primarily due to thymic involution [31,35]. Accordingly, we found that patients with tumours which reflected these typical age-associated immune alterations (low intra-tumoural T cells and high myeloid cells) had inferior health. In contrast, patients who showed the opposite trend – i.e. tumours with high levels of T cells or low levels of granulocytic cells, tended to show better health scores. This observation closely links the intra-tumoural immune state with the health of older patients, and consequently proposes the immune system in older non-fit patients as a potential therapeutic target whereby immunostimulatory agents may assist in combatting the tumour or in a higher level of patient fitness. The immune differences between fitter and frail patients observed may be attributed to differing capacities for effective immune surveillance. For example patients with better functioning immune systems may retain more functionally active T cells, reflected here by superior ability to migrate to the tumour site. On the other hand, the characteristic accumulation of myeloid cells associated with age may indicate elevated levels of myeloid-derived suppressor cells (MDSCs), which can suppress beneficial immune responses such as those against tumour cells [36]. An accumulation of granulocytic MDSCs in the tumour may therefore result in suppressed anti-tumour immune responses, in turn relating to a suppressed immune system and impaired health.

In accordance with our findings showing high relative levels of intra-tumoural T cells in patients with superior health, we also found that longer-surviving patients had tumours with a higher abundance of T cells, which is consistent with several previous reports showing this correlation for TILs in general [37–39]. Noteworthy is that we found total T cells and those located in the invasive front to be relevant for patient prognosis, in contrast to T cells in the tumour centre which were not associated with patient survival. This observation may hint that the functional activity of T cells is associated with their location within the tumour. The association between intra-tumoural T cells and patient survival should be considered preliminary until a more complete clinical follow-up period or a larger sample size is available. This would allow a more robust investigation additionally considering other known prognostic factors in a multivariate analysis model also including tumour stage and treatment type which varied within this cohort.

## 5. Conclusion

A number of previous studies has shown that tumour-infiltrating leukocytes are relevant for patient survival, but to the best of our knowledge this is the first study to show that certain TIL populations are associated with patient fitness. These findings closely link the intra-tumoural immune state with patient health and survival, suggesting that the immune system plays important roles in maintaining the overall functioning and survival of older individuals with cancer. This might be achieved by more effective immune surveillance mechanisms in longer surviving patients or those with higher functional status, in which the immune system is better able to control chronic or acute infections in addition to providing more effective protection against cancer.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgo.2018.03.021.

## Disclosures and Conflict of Interest Statements

The sponsors did not play any role in the design or conduct of the study including data collection, management, analysis, interpretation of the data or preparation, review, or approval of the manuscript. The authors have no conflicts of interest to declare.

## Author Contributions

Concept and Design: C. Shipp, G. Pawelec, H. Wildiers, J. K. Bailur
Data Collection: L. Speigl, A. Grieb, N. Janssen, C. Shipp, J. K. Bailur, H. Wildiers, S. Hatse, G. Floris, B. Brouwers, A. Smeets, C. Kenis, P. Neven
Analysis and Interpretation of Data: L. Speigl, C. Shipp, S. Hatse, G. Floris, A. Grieb
Manuscript Writing and Approval: L. Speigl, C. Shipp, G. Pawelec, H. Wildiers, S. Hatse, G. Floris, A. Grieb, N. Janssen, B. Brouwers, A. Smeets, C. Kenis, P. Neven, J. K. Bailur

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# MANUSCRIPT 1

## Putative cancer stem cell markers are frequently expressed by melanoma cells *in vitro* and *in situ* but are also present in benign differentiated cells

**Lisa Speigl[1], Nicole Janssen[1], Benjamin Weide[2], Tobias Sinnberg[2], Graham Pawelec[1,3,4]*, Christopher Shipp[1,5]***

**1** - Department of Internal Medicine II, University Hospital Tübingen, Waldhörnlestr. 22, 72072 Tübingen, Germany

**2** - Department of Dermatology, University Hospital Tübingen, Liebermeisterstr. 24, 72076 Tübingen, Germany

**3** - School of Science and Technology, College of Arts and Science, Nottingham Trent University, Burton St, NG1 4BU Nottingham, United Kingdom

**4** - Department of Haematological Medicine, King's College London, The Rayne Institute, London, United Kingdom

**5** - Current affiliation: The Natural and Medical Sciences Institute at the University of Tübingen, Reutlingen, Germany

***Corresponding authors**

Christopher Shipp
Second Department of Internal Medicine
University Hospital Tübingen
Waldhörnlestr. 22, 72072 Tübingen, GER
Tel.: +49-7071-2983147
Fax: +49-7071-294677
mrchristophershipp@gmail.com

Graham Pawelec
Second Department of Internal Medicine
University Hospital Tübingen
Waldhörnlestr. 22, 72072 Tübingen, GER
Tel.: +49-7071-2982805
Fax: +49-7071-294677
graham.pawelec@uni-tuebingen.de

## Summary

An accurate view of cancer stem cells (CSCs) in solid tumours remains incomplete to date. We studied a panel of putative CSC markers (ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin) in 40 established melanoma cell lines and four early-passage melanoma strains by flow cytometry. We additionally examined 40 formalin-fixed paraffin-embedded melanoma tissues using immunofluorescence microscopy. This was compared with their expression on healthy skin, normal differentiated melanocytes and fibroblasts. Most of the tested putative CSC markers were commonly found in both melanoma cell lines and tissue. When present, these proteins were expressed by the majority of cells in the population in most cases. Differentiated non-malignant cells also expressed CSC markers, indicating that they are not specific markers for CSCs. Culturing cell lines under conditions more characteristic of the tumour microenvironment up-regulated CSC marker expression in a proportion of cell lines, which correlated with improved cell growth and viability.

## Significance

Contrary to the proposition that CSCs are rare, the testing of melanoma cell lines (n=40), early-passage cell strains (n=4) and melanoma tissues (n=40) showed that several putative CSC markers (ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin) are commonly present in a large proportion of melanoma cells *in vitro* and *in situ*. We further show that these putative markers lack specificity for CSCs because they are also expressed on differentiated non-malignant cell types (melanocytes, fibroblasts and skin) which could limit their use as therapeutic targets.

## Key words

melanoma; cancer stem cells; putative; cell lines; tissue

## Abbreviations

CSCs = cancer stem cells; ALDH1A1 = Aldehyde Dehydrogenase 1 family, member A1; ABCG2 = ATP Binding Cassette transporter G2

## Introduction

There has been a paradigm shift in our understanding of cancer over the last several decades. It is now appreciated that instead of consisting entirely of clonally expanded cancer cells, tumours are comprised of different cell types that are heterogeneous in phenotype and function and which interact in complex ways: some non-cancerous cell types in the stroma support tumour growth through multiple mechanisms, and may even represent the majority of cells in the tumour mass. Moreover some cancer cells lie dormant, while others retain the capacity for self-renewal and maintain the heterogeneous lineages of cancer cells which constitute the tumour (Hanahan and Weinberg, 2011). The latter have been dubbed Cancer Stem Cells (CSCs) because they share common features with tissue stem cells, such as self-renewal capacity and the ability to give rise to progeny that can grow and differentiate (Clarke et al., 2006). These cells have therefore been proposed as the driving force behind tumourigenesis, the "seeds" of metastases and a factor associated with the failure of cancer treatment due to their resistance to current therapies (Alix-Panabieres et al., 2007; Balic et al., 2006; Kreso and Dick, 2014; Medema, 2013). However, to date the existence, identification and roles of CSCs remains incompletely understood (Dick, 2008; Lai et al., 2012; Lang et al., 2013; Monzani et al., 2007; Quintana et al., 2010; Redmer et al., 2014; Roesch et al., 2010).

Melanoma is the most aggressive form of skin cancer and one of the deadliest cancers in its metastatic form, but despite a number of recent therapeutic advances most metastatic patients still face a poor long-term prognosis (Flaherty et al., 2012; Hamid et al., 2013; Hodi et al., 2010; Ugurel et al., 2017). An accurate description of CSCs in melanoma may provide a basis for more successful therapies by targeting tumourigenic CSCs. Several studies have attempted to better understand the nature of CSCs in melanoma; however, differences regarding their functional properties and expression patterns were found (Boiko et al., 2010; Civenni et al., 2011; Lai et al., 2012; Monzani et al., 2007; Quintana et al., 2010; Quintana et al., 2008; Redmer et al., 2014; Roesch et al., 2010). Part of the difficulty of comparing the results concerning CSCs across studies is that different studies examined different CSC markers, while the employment of different experimental techniques and sample types are also major contributing factors to these inconsistencies (for example animal or human, *in vitro, in vivo* or *in situ*) (Beretti et al., 2015; Dietrich et al., 1997; Luo et al., 2012; Monzani et al., 2007; Piras et al., 2010).

The present study attempted to perform a comprehensive assessment of commonly-studied CSC markers in melanoma using multiple experimental techniques in samples in a variety of exclusively human sample types. Compared to many studies we have employed a relatively large panel comprising some of the most commonly investigated CSC markers in melanoma,

namely, ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin (for details see supplemental material 1). This panel of markers was studied in a large number of samples relative to the prior studies: 40 established melanoma cell lines, 40 melanoma tissue specimens, four early-passage melanoma cell strains, and three types of differentiated non-malignant cells (melanocytes, fibroblasts and skin sections). Because our understanding of the function and mechanism of CSC markers is primarily derived from *in vivo* studies in animals or human *in vitro* studies, the comparison between cell lines and tissue is an important novelty, while the inclusion of non-malignant samples in this study is a further factor not always addressed in earlier studies.

The diversity of cancer (stem) cells at the genetic and functional level has also been proposed to be governed by environmental factors. To address this, we additionally studied melanoma cell lines in an experimental culture model consisting of low oxygen tension and acidic pH. Hypoxic and acidic conditions are widespread physical features of tumours *in vivo* (Engin et al., 1995; Lartigau et al., 1997) but they are usually not included as part of the *in vitro* modelling of tumours. The aim was to improve the accuracy of *in vitro* culture models and to investigate whether the expression of the CSC markers in melanoma could be influenced by physical features more typical of their environment *in vivo*.

## Results

### Putative CSC markers are commonly expressed by melanoma cells and show variable expression across cell lines

We examined 40 different established melanoma cell lines for their expression of seven putative CSC markers (ALDH1A1, ABCG2, CD44v7/8, CD44v10, CD133, CD271 and Nestin). We first identified how many of the 40 cell lines expressed each of these CSC markers. This was achieved by examining average protein expression on the entire population for each cell line to give a fluorescence index (FI). This approach showed that all 40 cell lines were positive for ALDH1A1, CD271 and Nestin, while around half expressed ABCG2 and only three were positive for CD133. It is worth pointing out that all 40 cell lines expressed the CD44 molecule (Pawelec and Marsh, 2006) yet we could only identify four (10%) which expressed the CD44 splice variant isoforms 7/8 and none which expressed the splice variant isoform 10 (Fig 1 A). In addition, we examined how many cells within each melanoma cell line were positive for each marker by assessing the frequency of expressing cells. The results considering the frequency of positive cells generally agreed with the results obtained when determining average protein

expression on the entire population (FI) described above: ALDH1A1, CD271 and Nestin were found to be expressed on the majority of cells in the population for all 40 cell lines. ABCG2 and CD44v7/8 were found to be present on 0 to 70% and on 0 to 40% of cells in the population, respectively, while CD133 and CD44v10 were found at a lower maximal frequently (Fig. 1B). We observed that when these proteins were expressed, they were usually not present on discrete sub-populations of positive cells, i.e. a single population of expressing cells was observed rather than separate populations of positive and negative cells within a cell line (Fig. 1C). This was the case for both established cell lines and early-passage cell strains. We also noted the absence of discrete positive and negative populations for proteins which showed expression on less than 100% of cells - for example ABCG2 occurred on between 0 and 70% of these melanoma cell line cells, but the majority of cells in the population expressed this protein similarly, and did not show discrete positive and negative populations (Fig. 1C lower panel). However, we also occasionally observed heterogeneity in the expression of these proteins; for example Nestin and CD133 were sometimes found to be expressed at different levels in a fraction of cells in the population (Supplemental Material2). Interestingly, when comparing primary- and metastatic-derived cell lines we observed no marked differences in the expression of the seven CSC markers (Fig. 1D), while we also obtained similar results when comparing early-passage cell strains with established cell lines i.e. common expression of CD271, ALDH1A1, Nestin and ABCG2 (all 4/4), rare expression of CD44v7/8 (1/4) and a lack of expression of CD133 and CD44v10 (data not shown). Finally, we investigated potential relationships between the expression of the seven CSC markers. This analysis revealed correlations between Nestin and ALDH1A1 ($p < 0.0001$, $r = 0.5988$) and ABCG2 ($p = 0.0193$, $r = 0.364$) expression (Fig. 1E).

**Hypoxia and acidity alter the behaviour of melanoma cell lines and change the expression of putative CSC markers**

Because standard *in vitro* cell culture conditions (20% $O_2$ and neutral pH medium) do not accurately reproduce *in vivo* growth conditions, we cultured the established cell lines under conditions designed to better reflect the tumour microenvironment (i.e. 2% $O_2$ and pH 6.7, here designated “experimental conditions”). We were interested in understanding the relationship between the tumour microenvironment and cancer stemness because our previous study had shown that incorporating these features as part of *in vitro* culture can have a dramatic impact on the behavior of melanoma cells (Shipp et al., 2012). Compared with the conventional *in vitro* culture model, experimental culture conditions slowed growth in all melanoma cell lines ($p < 0.0001$) and reduced viability in the majority ($p < 0.01$) (Fig. 2A). We found the same trend towards reduced viability under experimental conditions in a subset of cell lines ($n = 15$) using

a commercial apoptosis kit, which was statistically significant when considering differences in late-apoptotic cells ($p = 0.035$) (data not shown).

We also observed altered expression of the CSC markers by hypoxic and acidic conditions in our experimental culture model. Of the 40 cell lines, most down-regulated ALDH1A1 and ABCG2, while CD271 was up-regulated in the majority, and Nestin up- or down-regulated in roughly equal numbers of cell lines (Fig. 2B). All cells within each cell line showed similar changes in expression under the test conditions. Cell lines negative for any protein under conventional conditions remained negative in the experimental model. The correlations between the expression of Nestin with ALDH1A1 and Nestin with ABCG2 (Fig. 1E) observed under conventional conditions were retained when these cells were cultured in the experimental model ($p = 0.0002$ for ALDH1A1 vs. Nestin and $p = 0.0439$ for ABCG2 vs. Nestin) (data not shown).

**Expression of CD271, Nestin and ALDH1A1 is associated with better melanoma cell line viability and growth under hypoxic and acidic culture conditions**

Changes in the expression of CD271 and Nestin between the conventional and experimental culture models were found to correlate with melanoma cell line viability ($p = 0.0063$ and $p = 0.0258$, respectively) (Fig. 2C). We observed that improved viability was associated with up-regulation of these proteins under experimental culture conditions, as indicated by higher live:dead cell ratios. Despite the majority of cell lines tending to down-regulate ALDH1A1 in the experimental model, we found improved cell growth in those which up-regulated expression of this molecule ($p = 0.0168$) (Fig. 2C). No such associations were found for ABCG2. Because the CD44 variants and CD133 were rarely or not at all expressed by these cell lines, this analysis could not be performed for these proteins.

**Not only melanoma tissues *in situ* but also differentiated non-malignant cell types express putative CSC markers**

To validate the results obtained from melanoma cell lines *in vitro*, we examined the expression of selected CSC markers *in situ* in an equal number of melanoma tissue deposits. Because CD133 and CD44v7/8 were rarely present in melanoma cell lines we considered it important to compare the results for these proteins obtained *in vitro* with those *in situ*. We additionally tested ALDH1A1 and ABCG2 because we found a number of interesting correlations *in vitro* and these proteins have rarely been studied in melanoma tissue, while CD271, Nestin and CD44v10 have been examined *in situ* previously by other investigators (Akiyama et al., 2013; Beretti et al., 2015; Manten-Horst et al., 1995). Consistent with the results in cell lines, all CSC

markers were commonly expressed in the majority of melanoma cells *in situ*, although we did also observe a degree of heterogeneity revealing areas of negative cells or more highly/weakly expressing cells within tumour regions. CD133 in particular was expressed more highly on a proportion of melanoma cells in some tissue samples, although in these cases most cells nevertheless showed positive staining. A common observation was that of lower expression by cells resembling tumour stroma. Notably, an exception to our observation of common CSC marker expression by the majority of cells was ALDH1A1, which we observed to be either commonly expressed by all cells (17.5% of samples) or to be selectively expressed by a subset of cells (45% of samples) *in situ* (Supplemental Material 3).

When comparing tissue with established melanoma cell lines, the four putative CSC markers showed different expression patterns *in situ* (Fig. 3A/B). CD44v7/8 expression was more commonly observed in tissue (37.5% of samples were positive) compared with cell lines (10% of samples). The same was true for ABCG2 which was much more frequently expressed in tissue (77.5%) than in cell lines (46.34%), whereas ALDH1A1 was expressed only in 62.5% of melanoma deposits compared with 100% of cell lines. Interestingly, we found that CD133 was the most commonly expressed putative CSC marker in melanoma tissue with 95% of deposits expressing this protein. This stands in stark contrast to the established cell lines in which only a small proportion (7.5%) of cell lines expressed it according to the FI. Representative immunofluorescence images stained for ALDH1A1, ABCG2, CD44v7/8 and CD133 are shown in Fig. 3C. Supplemental Material 3 shows a single representative stained and control image for all four proteins for two tissue samples (in the case of ALDH1A1 it shows one example demonstrating expression by most cells, with the second example showing expression by a subset of cells) and Supplemental Material 4 shows one example of a complete set of images comprising all fluorescence images captured throughout one tumour sample for all four proteins.

Because we observed that both melanoma tissues and cell lines expressed these putative CSC markers, we then examined their expression on benign differentiated cells, in order to test their specificity as CSC markers and to examine their potential use as therapeutic targets in melanoma. We investigated the expression of these molecules in appropriate control cells – using flow cytometry we tested human dermal fibroblasts and primary human epidermal melanocytes from two different sources. We additionally tested normal human skin sections in the case of CD133 and ABCG2 using immunofluorescence. Our results demonstrate that these seven putative CSC markers are not specific for cancer or normal stem cells, because we detected them in all tested benign differentiated cell types examined here. Interestingly, the benign samples were found to express these proteins at comparable or occasionally even higher levels than in malignant cell types. A comparison between the expression levels of all

proteins tested in benign and malignant cell types by flow cytometry is shown in Fig. 3D. The results for the testing of human skin using immunofluorescence can be found in Supplemental Material 5.

## Discussion

This study was performed to better understand the nature of CSCs in human melanoma, which was achieved by surveying a panel of CSC markers in large numbers of samples *in vitro, in situ* and by including multiple differentiated non-malignant cell types, thereby investigating marker- and sample-dependent differences that may exist. Because mechanistic studies are either performed *in vitro* or *in vivo* with animals, we considered it important to compare melanoma samples *in vitro* with those *in situ* in exclusively human samples. We studied the putative CSC markers CD271, ALDH1A1, Nestin, ABCG2, CD133, CD44v7/8 and CD44v10 in 40 established melanoma cell lines, with four (ABCG2, ALDH1A1, CD44v7/8 and CD133) additionally investigated in an equal number of melanoma tissues. We show that in melanoma cell lines, four of the seven putative CSC markers (CD271, ALDH1A1, Nestin and ABCG2) are commonly expressed, while the remaining three markers were either found very rarely (CD133 and CD44v7/8) or not at all (CD44v10). Except for ALDH1A1, we saw that tissues expressed the CSC markers more frequently than cell lines. Substantial differences were seen for CD44v7/8 and CD133 which were rarely found in cell lines but were more common in tissue, especially in the case of CD133. This infrequent expression of CD133 and CD44v10 in melanoma cell lines is consistent with the results of previous studies (Seiter et al., 1996; Zimmerer et al., 2013). The differences observed between cell lines and tissue may be associated with selection for melanoma tumours or cells which are able to grow *in vitro* as an established cell line. The growth requirements in this artificial environment are likely to differ substantially from those *in vivo*; thus the fraction of melanoma tumours, or individual cells within a tumour, which are able to survive surgical excision, processing and subsequent growth as a monolayer appears to select for melanoma cells or for tumours with a particular profile of CSC marker expression. It is perhaps less likely that these changes occurred during *in vitro* culture, unless they occur very early, because we observed similar results for early-passage and established cell lines.

Collectively, our results suggest that the bulk of melanoma cells express similar levels of CSC markers. It was surprising to us that in the majority of cases, the seven tested markers did not show distinguishable sub-populations of positive and negative cells, which also prevented us from isolating these fractions to investigate if they possess stem-like properties. To strengthen these observations made with established cell lines *in vitro*, we confirmed expression by most

cells *in situ* using excised melanoma tissue and in four early-passage cell strains, the latter less likely to have been altered by *in vitro* culture than established cell lines, and the former not at all. A number of prior studies which have shown CSCs to be expressed in only a small proportion of all melanoma cells utilised freshly resected tumour cells that have undergone enzymatic digestion. This treatment has been shown to reduce the frequency of detected tumour cells expressing CSC markers (Civenni et al., 2011). In contrast, the present study examined formalin-fixed tissue samples, or used cell lines that had undergone brief treatment with a more gentle detachment method than commonly used trypsin, thereby potentially explaining the observation in our study which shows that CSC markers are commonly expressed in melanoma.

Our findings show that the panel of CSC markers investigated here were expressed at a similar level by the majority of melanoma cells. However, these markers are putative and therefore could be non-specific or possibly irrelevant for the identification of CSCs (Zapperi and La Porta, 2012). To address this, we investigated the specificity of these markers for CSCs by testing their expression by benign differentiated cell types of related origin (primary human melanocytes, human dermal fibroblasts and normal human skin). We found that benign differentiated cells as well as cancer cells express these proteins, in line with previous reports for ALDH1, CD44 variants, Nestin and CD133 (Klein et al., 2007; Lugli et al., 2010; Seiter et al., 1996). This finding weakens the proposition that the markers examined here are specific for CSCs and leaves open the possibility that more accurate CSC markers in melanoma may still be discovered. Since we also examined primary- and metastatic-derived cell lines, it is unlikely that the observed results are due to dissemination of phenotypic monoclonal metastatic CSCs from a heterogeneous primary tumour. Our results may have consequences for studies which use these markers to isolate CSCs for functional testing, or for studies attempting to target them therapeutically. Noteworthy differences were seen for ALDH1A1; we observed some melanoma tissues that showed expression by the majority of cells, while other tissues showed the presence of distinct individually positive cells. Perhaps due to genetic heterogeneity, this protein may not represent the same population of cells in every melanoma, i.e. ALDH1A1 may be a marker of CSCs in some melanomas, but not in others.

We additionally cultured established melanoma cell lines in an experimental culture model aimed at more closely mimicking their native environment. We hypothesised that this model may lead to the selection of cells better adapted to it, which may be associated with cells possessing a cancer stem-like phenotype due to the environmental influence on CSCs that is reported to exist. A previous study in melanoma demonstrated that hypoxia can regulate the expression of the CSC marker Oct-4Kumar et al. (2012), thus hinting that features of the

tumour microenvironment are involved in the regulation of CSCs. In line with this, we observed a considerable proportion of cell lines which up-regulated the herein examined CSC markers in response to an experimental culture model of hypoxia and acidity. However we also found that a similar or greater proportion of cell lines down-regulated the markers, highlighting the heterogeneous nature of cancer, even of the same histological origin. The varied results seen in response to our experimental culture model suggest that the role for these proteins may not be the same for all melanomas, whether they represent CSCs or not.

In this study we observed that the expression of CSC markers correlated with certain cellular features, suggesting that there are as yet uncovered roles for these proteins in melanoma. We observed that cells which up-regulated certain CSC markers under hypoxia and acidity showed improved viability or cell growth. The importance of these proteins is underlined by the finding that they were expressed in melanoma cell lines as well as in melanoma tissues, indicating that some of them may be essential for tumour maintenance *in vivo*.

### Conclusions

In the present study we have shown that the expression of CSC markers can differ depending on the nature of the sample type examined and the culture environment employed, which may provide some explanation for the large numbers of conflicting studies previously reported for putative markers of CSCs in melanoma. We found that these proteins are commonly expressed in both melanoma cell lines and tissue, and that they are associated with important features of melanoma cells. Unlike many studies, the inclusion of differentiated non-malignant samples alongside malignant samples in this work allowed us to investigate their specificity for CSCs. This revealed widespread expression of these proteins in non-malignant cells, which questions their definition as CSC markers and may limit their use as therapeutic targets.

## Methods

### Samples

Established melanoma cell lines: Forty cell lines were selected from the European Searchable Tumour Line Database (ESTDAB; http://www.ebi.ac.uk/ipd/estdab) (Pawelec and Marsh, 2006). Thirty eight of the 40 cell lines were metastasis-derived, and two were derived from primary melanomas (EST-66 and EST-83). These cell lines have been certified by DNA fingerprinting and tested for mycoplasma contamination. They are currently also available from the European Collection of Animal Cell Cultures (ECACC, see https://www.phe-culturecollections.org.uk/products/celllines/generalcell/estdab-cell-lines-introduction.aspx).

Early-passage melanoma cell strains: Four early-passage cell strains were derived from metastatic lesions from patients treated at the Tübingen University Hospital according to the following protocol (Lasithiotakis et al., 2008; Mancianti et al., 1988): "TüMel 39" (4$^{th}$ passage), "TüMel 49" (3$^{rd}$ passage), "PDX 25" (P2, P1) and "PDX 35" (P0, P1). The PDX early-passage cell strains were initially passaged in mice before being cultured *in vitro*.

Human epidermal melanocytes: Two sources of primary melanocytes were used: (1) cells in their 3$^{rd}$ passage derived from a circumcision of a healthy individual treated at the Tübingen University Hospital. (2) human adult primary epidermal melanocytes purchased from ATCC (Manassas, Virginia, USA).

Fibroblasts: Neonatal fibroblasts NuFF1 were obtained commercially (Globalstem, Gaithersburg, USA).

Skin sections: Five µm thick sections of formalin-fixed paraffin-embedded normal human skin were sourced commercially (Abcam, Cambridge, UK).

Melanoma tissue samples: Forty formalin-fixed paraffin-embedded metastatic lesions from patients treated at the Tübingen University Hospital Dermatology Department were used. Patients gave their written informed consent for the storage and scientific analysis of tissue samples. The use of these samples was approved by the University of Tübingen Ethics Committee (ethics approval number 017/2016BO2).

**Cell culture**

Established melanoma cell lines were cultured in 40 mL RPMI 1640 medium (Life Technologies, Darmstadt, Germany) supplemented with 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich, Munich, Germany) in either 20% $O_2$ or in 2% $O_2$ using the Concept 1000 Invivo2 hypoxic chamber (Ruskinn Technology (Pencoed, UK)) with media titrated to pH 6.7 with 37% hydrochloric acid (Merck, Darmstadt, Germany). Depending on the generation time of each cell line, between 2 and 4 x $10^6$ cells were seeded per flask. Cell culture and estimation of viability was performed as previously described, but in the current study cells were cultured for seven days and cultures were harvested with Accutase (Sigma-Aldrich) (Shipp et al., 2012). Selected cell lines (n = 2) were repeated to confirm initial results. Early-passage melanoma cell strains were cultured until confluence in medium containing 1% PenStrep (Biochrom, Berlin, Germany). Melanocytes were cultured in DermaLife M melanocyte growth medium (CellSystems, Troisdorf, Germany).

**Flow Cytometry**

Using a BD LSR II, multi-colour flow cytometry was used to measure protein expression as previously described (Shipp et al., 2012) but with the following modifications. Automatic software compensation was performed to minimise spectral overlap between different fluorochromes, and CST beads were run prior to each sample measurement to control for consistency in machine performance. The following antibodies were used: ALDH1A1-PE (Lot: HG09MY1304, Clone: 03) (Sino Biological Inc., North Wales, USA), Nestin-PE (Lot: 2524561, Clone: 10C2) (Merck Millipore, Temecula, USA), ABCG2-PE (Lot: B143287, Clone: 5D3) (Biolegend, San Diego, USA), CD44v7/8-FITC (Lot: 150715, Clone: VFF-17) (Acris Antibodies, San Diego, USA), CD44v10-FITC (Lot: 9E08V1) (Bioss, Woburn, USA), CD133-APC (Lot: 5150611303, Clone: AC133) and CD271-FITC (Lot: 5150609183, Clone: ME20.4-1.H4) (both from Miltenyi Biotec, Teterow, Germany). The DNA-binding dye Ethidium Monoazide Bromide (Biotium, Hayward, USA), was used to exclude dead cells (incubated on ice under bright light for 20 min) before antibody staining. Data were analysed using FlowJo software version 10.0.7. (Tree Star, Ashland, USA). Cell viability was determined with a commercial viability kit according to the manufacturer's instructions (BD Biosciences, Heidelberg, Germany).

**Immunofluorescence**

Melanoma tissue sections (5 µm thick) were prepared and stained with antibodies as previously described (Shipp et al., 2013) but with the following modifications: an EDTA- and SDS-based antigen retrieval solution containing 25 mM Tris–HCl (pH 8.5) (Sigma-Aldrich), 1 mM EDTA and 0.05% SDS (both from SERVA Electrophoresis, Heidelberg, Germany)) was used to unmask antigens. The following antibodies were used: ALDH1A1 rabbit monoclonal (Lot: GR41450-6, Clone: EP1933Y) (Abcam), CD133 rabbit polyclonal (Lot: X13030523) (Fitzgerald, Acton, USA), ABCG2 mouse monoclonal (Lot: D15KF02234, Clone: BXP-21) (Biolegend, San Diego, USA), CD44v7/8 mouse monoclonal (Lot: 051114, Clone: VFF-17) (Bio-Rad, Hercules, USA), Alexa Fluor 488 donkey anti-rabbit IgG (H+L), Cy3 donkey anti-mouse IgG (H+L) (both from Jackson ImmunoResearch Laboratories, West Grove, USA). Fluorescence intensity for each antibody-stained tissue section was compared with fluorescence from a control tissue (secondary antibody only) mounted on the same slide. The software PixelStats recorded mean fluorescence intensity of microscopy images (designed in-house by the University Hospital Tübingen) and was used to create a ratio between the stained and control tissue pieces. A tissue was considered positive if it showed at least a 50% increase in fluorescence over the control tissue. An average of 12 images per tissue (i.e. 12 for stained, 12 for control) covering the entire tumour (including all regions of the tumour centre and at the

invasive front) was captured at 20x magnification. Fluorescently-stained tissue slides were measured with a Zeiss Axiophot fluorescence microscope.

### Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 6 (La Jolla, USA). Changes of less than 10% between different culture conditions for the same cell line were not considered to be different. Cell line tumour-associated antigen and CD44 expression data were obtained from ESTDAB (Pawelec and Marsh, 2006). Correlations were assessed with non-parametric two-tailed (Spearman) correlation tests. Significance between two groups was assessed with two-tailed non-parametric (Mann-Whitney U) tests. Trends across four grouping variables were assessed with two-tailed Fisher's exact contingency tests. Significant relationships were considered as $p < 0.05$.

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## Figures and figure legends

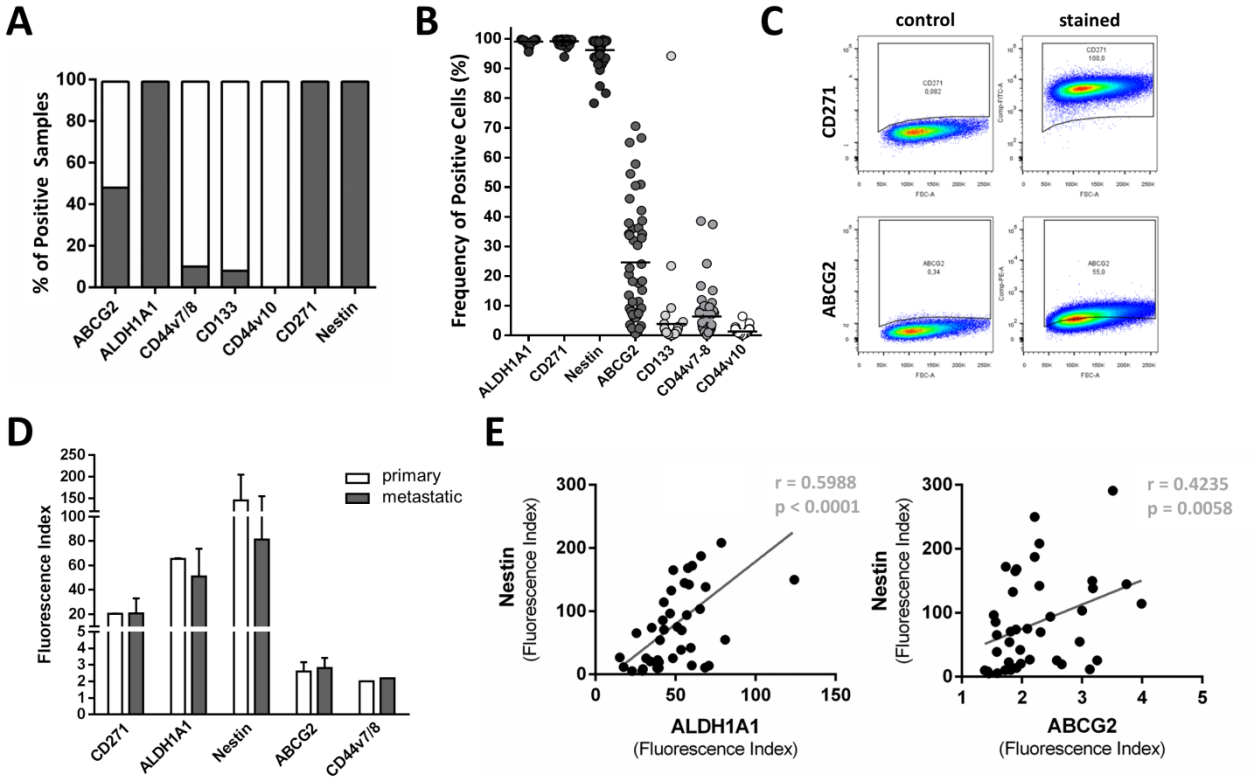



**Figure 1. Expression patterns and correlations of putative CSC markers in established melanoma cell lines.** Forty melanoma cell lines were assessed for their expression of ABCG2, ALDH1A1, CD44v7/8, CD44v10, CD133, CD271 and Nestin using flow cytometry. (A) Average protein expression on the entire population shows Nestin, CD271, ALDH1A1 and ABCG2 to be commonly expressed by established cell lines, while CD133 and CD44 variants were found less commonly. (B) Assessing the frequency of positive cells within each cell line showed Nestin, CD271, ALDH1A1 and ABCG2-positive cells to be common in this panel of 40 established cell lines and in the 4 early-passage cell strains. Lower frequencies of cells were found in the case of CD133 and CD44 variants. (C) Examples of flow cytometry plots showing similar expression by most melanoma cells for CD271 and ABCG2. Although less than 100% of cells are positive for ABCG2, most cells in the population express the protein at a similar level. (D) Expression of CSC markers is similar between primary and metastatic cell lines. (E) Expression levels between ALDH1A1 and Nestin ($r = 0.5988$; $p < 0.0001$) and between ABCG2 and Nestin ($r = 0.4235$; $p = 0.0058$) are correlated.

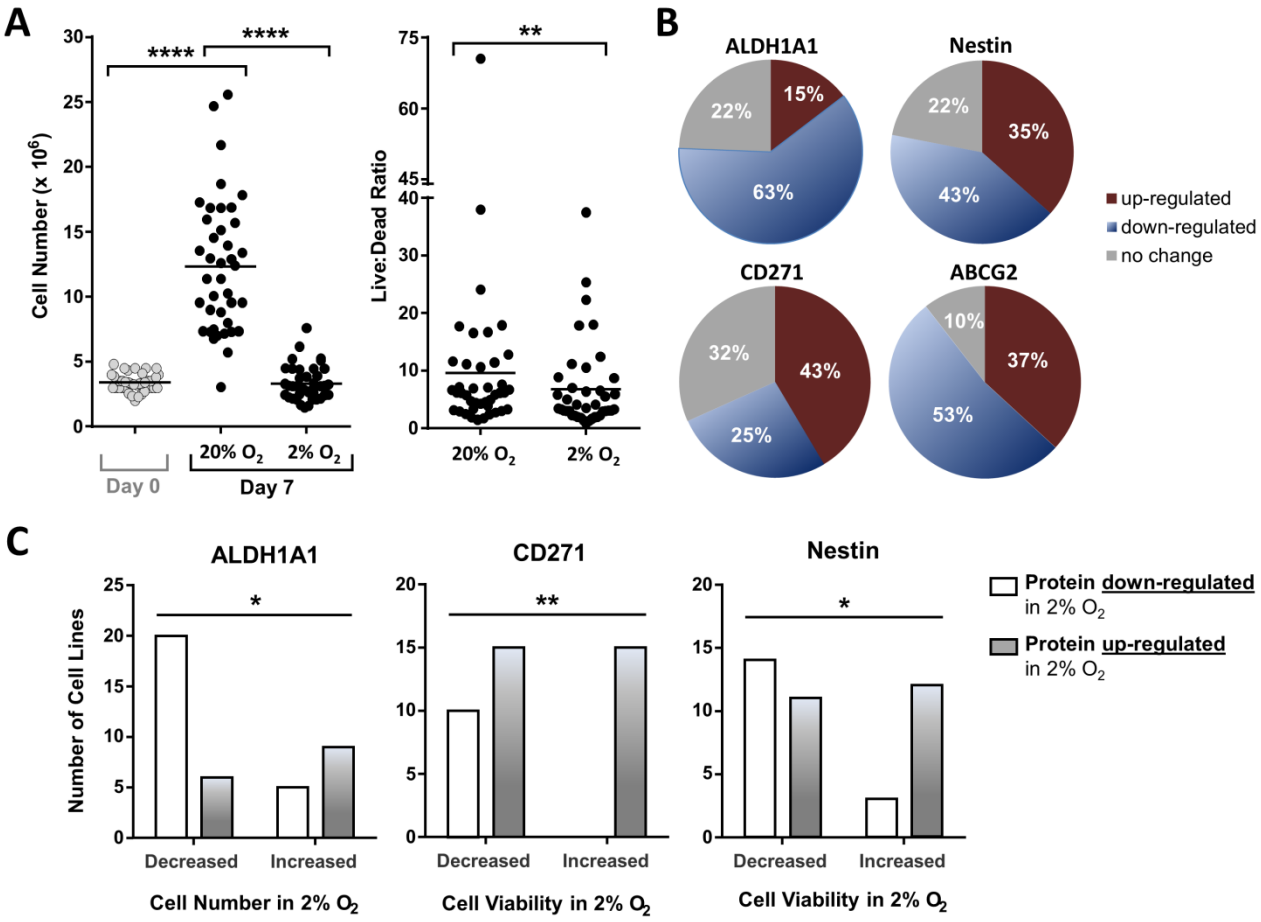



**Figure 2. Effects of hypoxic and acidic culture conditions on melanoma cell lines and the expression of putative CSC markers.** Forty melanoma cell lines were cultured under conventional (20% $O_2$, neutral pH) or experimental conditions (2% $O_2$, pH 6.7) for seven days. Following the culture period, cell lines were harvested and ratios of living to dead cells determined using trypan blue. Changes in the expression of CSC markers under experimental culture conditions were tested for their association with cell line viability and growth. (A) Experimental culture conditions slow the growth of melanoma cell lines and reduce their viability. Cell numbers indicate total numbers of living cells. (B) Experimental culture conditions up- or down-regulate the expression (FI) of putative CSC markers ALDH1A1, Nestin, CD271 and ABCG2. (C) Changes in expression (FI) of Nestin and CD271 are associated with melanoma cell viability under experimental culture conditions, while ALDH1A1 is correlated with cell growth. (* = $p < 0.05$; ** = $p < 0.01$, **** = $p < 0.0001$).

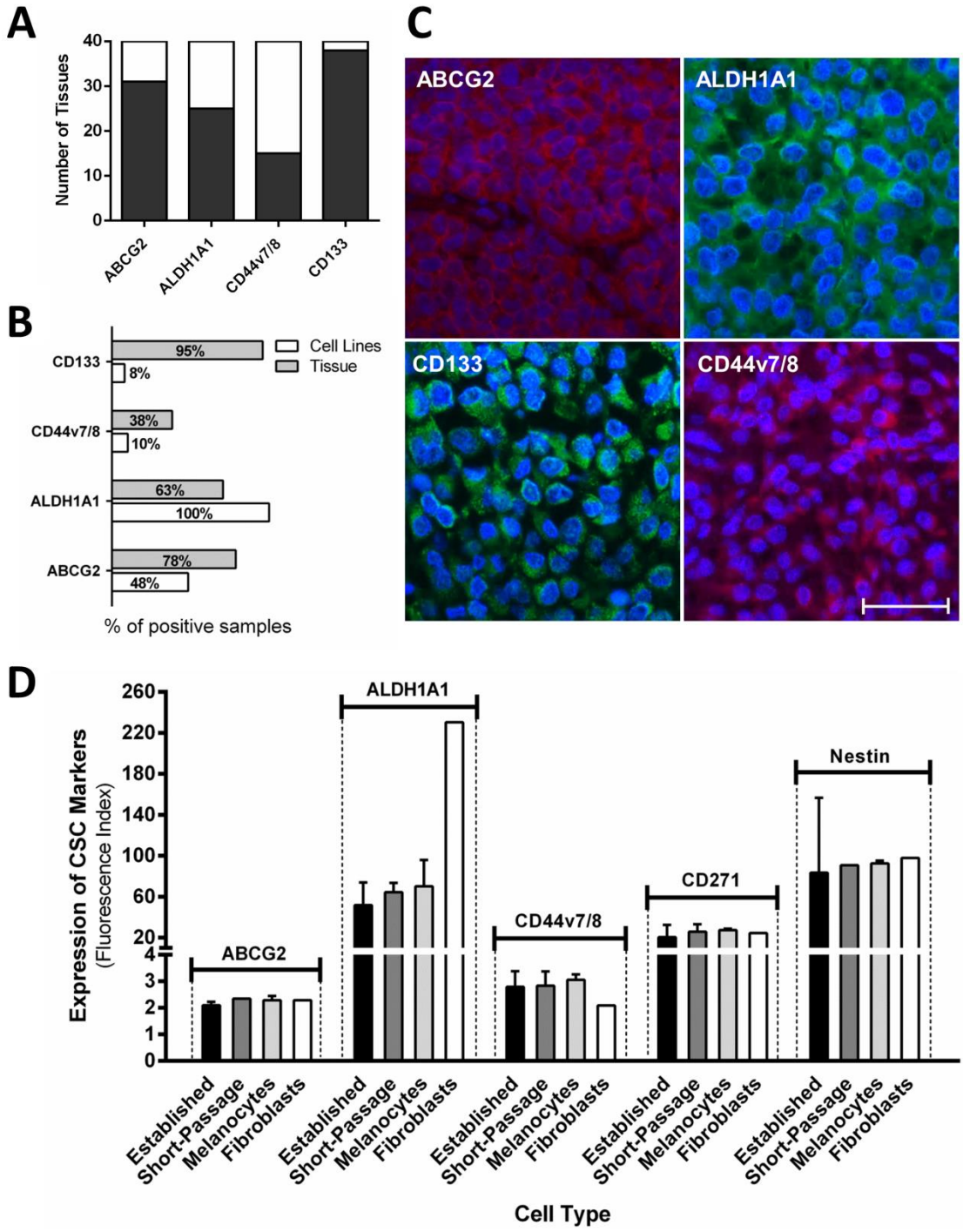



**Figure 3. Expression of putative CSC markers in melanoma tissue, cell lines and differentiated, non-malignant cells.** Immunofluorescence (melanoma tissue) and flow cytometry (cells and cell lines) was used to assess expression levels of putative CSC markers. (A) Almost all 40 melanoma tissues express CD133, while less frequent expression of ABCG2, ALDH1A1 and CD44v7/8 is observed. (B) Forty melanoma cell lines and an equal number of melanoma tissues were compared in their expression of CSC markers; tissues more frequently express CD133, CD44v7/8 and ABCG2, but ALDH1A1 is more common in cell lines. The FI was used to determine positive samples. (C) Representative images of melanoma deposits

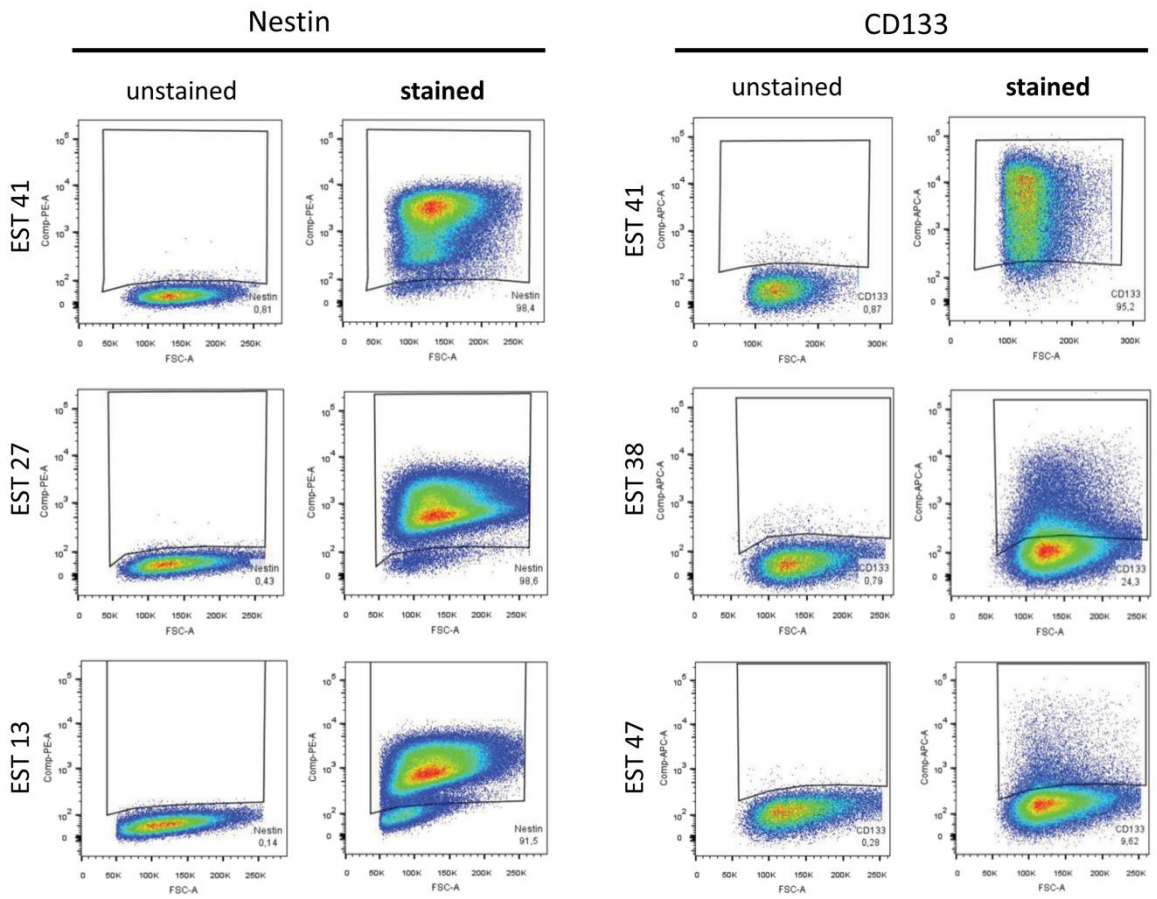
stained for ALDH1A1, ABCG2, CD44v7/8 and CD133. Scale bar indicates 100 µm. (D) Putative CSC markers are expressed at similar levels by established (n = 40) and early-passage (n = 4) melanoma cell lines, primary epidermal melanocytes (n = 2) and dermal fibroblasts (n = 1).

# Supplemental Marerial

## Supplemental Material 1

| **Biological properties of putative CSC markers in this study** | | |
|---|---|---|
| **Putative CSC marker** | **Biological properties** | **References** |
| **CD133** (Prominin-1) | Widely proposed as a CSC marker, it has been found in the CSC fraction of a large variety of human malignancies including melanoma. CD133 can influence cell polarity, migration and interaction of CSCs with surrounding cells, contributing to metastatic potential. | (Rappa et al, 2008, Ding et al, 2012, Fan et al, 2006) |
| **CD271** (Low affinity Nerve Growth Factor Receptor, LNGFR) | Expression has been found on a number of human neural crest derived tissues and in some human cancers including melanomas. It has been shown to drive melanoma initiation and metastasis, thus endowing melanoma cells with stem-like properties. | (Boiko et al, 2010, Chesa et al, 1988, Civenni et al, 2011) |
| **CD44 (v7/8 and v10)** (CD44 isoforms v7/8 and v10) | CD44 is a multifunctional cell-surface glycoprotein with a variety of biological roles, some which are associated with the pathological activities of cancer cells such as migration, proliferation and survival signaling. It has been implicated in the progression and metastasis of several human tumours including melanoma. Due to alternative splicing, a number of CD44 isoforms exist, some which have been associated with tumour growth and metastasis. | (Dietrich et al, 1997, Goodison et al, 1999, Trochon et al, 1996) |
| **ALDH1A1** (ALdehyde DeHydrogenase 1 family, member A1) | ALDH1A1 is a detoxifying enzyme responsible for the oxidation of intracellular aldehydes, thereby mediating self-protection and resistance to alkylating agents used in cancer therapy. This protein has received considerable attention as a marker for cells with enhanced tumourigenic/metastatic potential and elevated therapeutic resistance in several cancers of epithelial origin including melanoma. | (Luo et al, 2012, Sladek, 2003, Ginestier et al, 2007) |
| **Nestin** | Nestin is a type VI intermediate filament protein expressed in organ-specific sites where it serves as a quiescent resource of cells capable of proliferation, differentiation and migration. Nestin has been reported to be present in various neoplasms and was shown to be over-expressed in advanced stages and to correlate with poor prognosis in melanoma patients. | (Neradil and Veselska, 2015, Ehrmann et al, 2005, Ladstein et al, 2014) |
| **ABCG2** (ATP Binding Cassette transporter G2) | Is the second member of the G family of ABC transporters, which transport molecules across extra- and intra-cellular membranes and are involved in drug efflux. It is therefore thought to contribute to multidrug resistance in cancer. ABCG2 expression has been associated with the enhanced tumourigenic potential of cancer cells. | (Monzani et al, 2007, Vlaming et al, 2009, An and Ongkeko, 2009) |

## Supplemental Material 2

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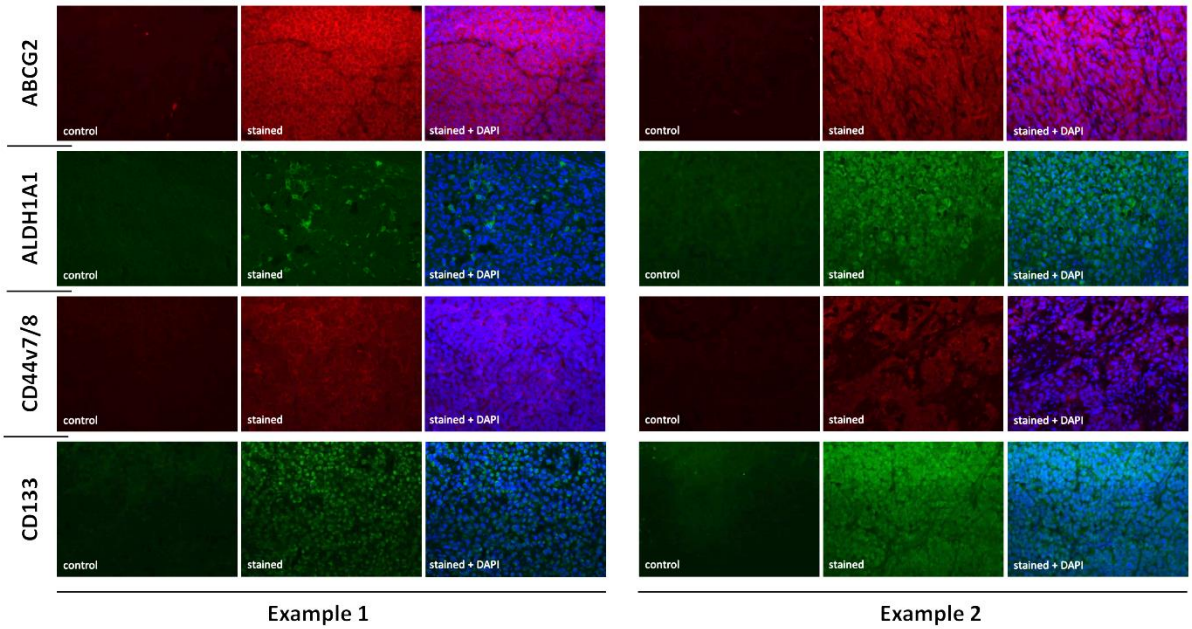

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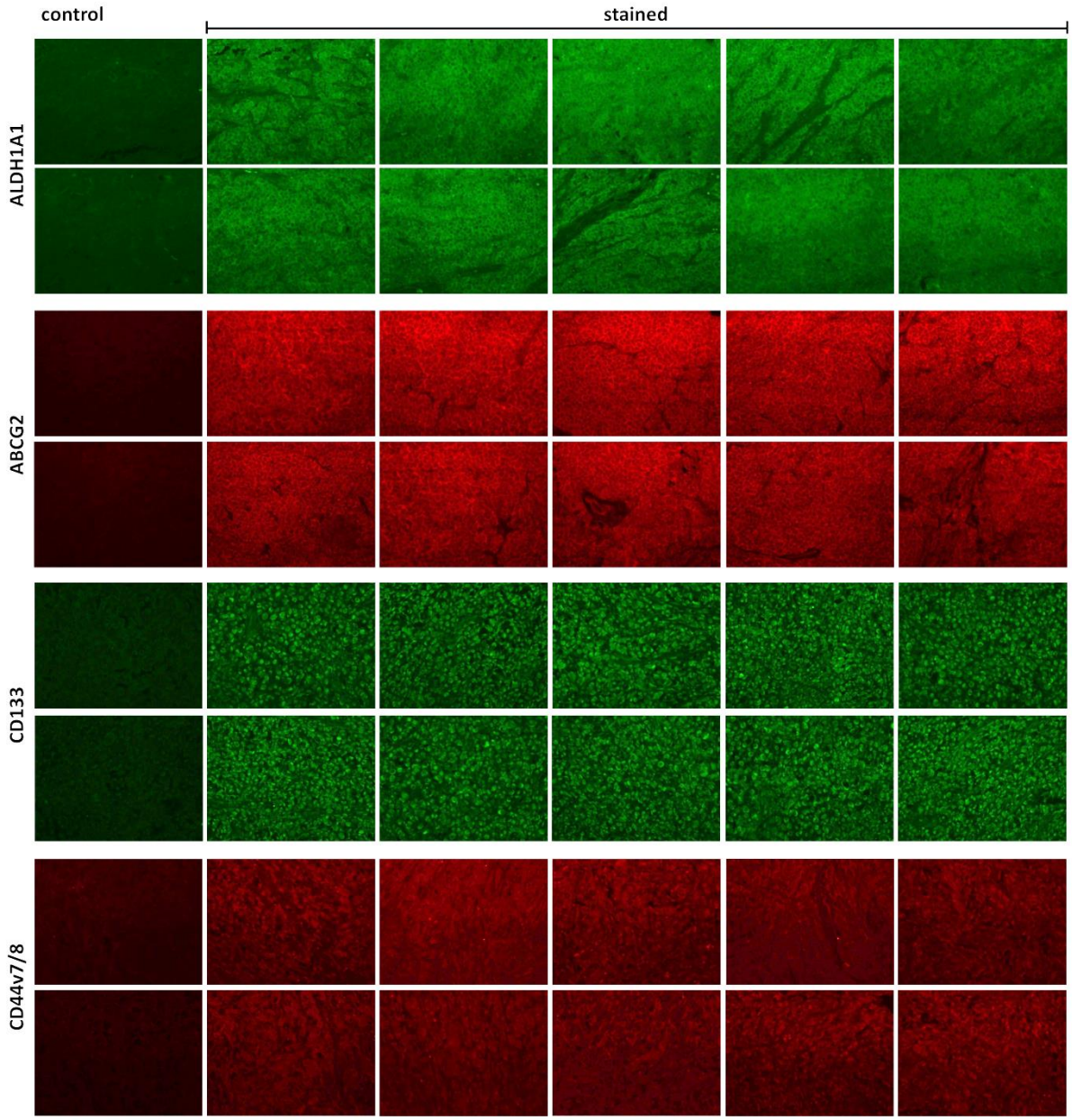

**Supplemental Material 5**