Predicting clinical outcome in melanoma and breast cancer using blood and tumour-based immune biomarkers: a means to monitor anti-cancer immune responses

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Table of contents

1 List of abbreviations

APC	Antigen Presenting Cell
AJCC	American Joint Committee on Cancer
CA	Carbonic anhydrase
CD	Cluster of Differentiation
CFSE	Carboxyfluorescein succinimidyl ester
COX-2	Cyclooxygenase-2
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
ER	Estrogen receptor
ESTDAB	European Searchable Tumour Line Database
FoxP3	Forkhead box P3
GDF-15	Growth/differentiation factor-15
GM-CSF	Granulocyte-Monocyte Colony Stimulation Factor
gp100	Glycoprotein 100
HER2	Human epidermal growth factor receptor-2
HIF-1α	Hypoxia inducible factor-1 α transcription factor
HLA-DR	Human leukocyte antigen-DR
HSP	Heat Shock Protein
ICS	Intracellular cytokine staining
IDO	Indolamin – 2,3 - Dioxygenase
IL-	Interleukin-
IFN-γ	Interferon-gamma
LDH	Lactate dehydrogenase
mDC	Myeloid Dendritic Cell
MDSC	Myeloid-derived suppressor cell
MUC1	Mucin 1
NK cell	Natural Killer cell
NKT cells	Natural Killer T cells
NO	Nitric oxide
NY-ESO-1	New York Esophageal Squamous Cell Carcinoma-1

List of abbreviations

PBMC	Peripheral Blood Mononuclear Cell
pDC	Plasmacytoid Dendritic Cell
PD-1	Programmed Cell Death-1
PD-L1	Programmed Cell Death Ligand-1
PGE2	Prostaglandin E2
PR	Progesterone receptor
ROS	Reactive Oxygen Species
STAT3	Signal transducer and activator of transcription 3
SUR	Survivin
TAA	Tumour-associated antigen
TSCN	Stem memory T cells
TEMRA	Terminally differentiated effector memory cells
TGF-β	Transforming growth factor-beta
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell

2 Abstract

In addition to conventional surgery, chemotherapy and radiotherapy, immunotherapy holds a great deal of promise as an effective form of cancer treatment. This utilises the patient's own immune system to control tumour growth and thus relies on the presence of immune cells which can recognise and subsequently kill or otherwise control cancer. This notion is supported by many studies showing that the presence of T cells recognising tumour-associated antigens (TAAs) is associated with superior survival in a number of cancer types. In contrast, cancer patients with high levels of immune suppressor cells experience worse survival and respond more poorly to therapy. These studies demonstrate that the balance between different immune populations is closely associated with clinical outcome. Therefore, the aim of the work in this thesis was to identify new blood- and tissue-based prognostic markers that more accurately predict patient outcome. Monitoring patients for their possession of TAA-reactive T cells may be important for this, but standardising such biological assays is challenging. Therefore, the first part of this work attempted to identify surrogate markers that may more easily identify patients with beneficial T cell responses. To achieve this, T cells reactive to the shared TAAs MUC1, survivin and HER2 were measured in a cohort of breast cancer patients and were investigated for association with a broad set of immunological parameters. Considering HLA type, serum cytokines, tumour-infiltrating leukocytes and blood leukocyte populations, the latter were found to be the most informative for identifying patients with such antigen-reactive T cells. Moreover, by constructing composite immune profiles, we were able to achieve a sensitivity and specificity of up to 100 % for the identification of patients possessing these antigen-reactive T cells. In addition to predictive immune markers in the periphery, a number of studies has shown that the immune status within the tumour plays a major role in cancer progression. Therefore the second part of this work investigated immune features in the tumour microenvironment which may serve as prognostic markers for patient survival. Unlike in the majority of prior studies which only considered one type of immune feature, for example only

leukocytes or only cytokines, this study assessed a combination of diverse immune parameters in the tumour microenvironment including lymphoid and myeloid cells, secreted cytokines and transcription factors. This investigation showed that patients whose tumours had high relative levels of putatively suppressive CD15+ cells had shorter overall survival. Furthermore, by combining the assessment of CD3+ and CD15+ cells, it was observed that patients with high levels of CD3+ T cells and low levels of CD15+ cells survived the longest. Other studies have yielded similar results regarding the impact of the state of the immune system on clinical outcome. These studies have shown that one of the mechanisms employed by melanoma to escape anti-tumour immunity is the induction of immunosuppressive myeloid-derived suppressor cells (MDSCs), the levels of which correlate with clinical outcome. The mechanisms by which tumour cells induce MDSCs remain unknown, but knowledge thereof may pave the way for new forms of cancer therapy. Therefore, an *in vitro* co-culture model was developed to uncover tumour-immune interactions, with the aim of identifying pathways that allow the induction of such suppressive cells to be prevented. The implementation of a three-way co-culture system employing melanoma cells, activated T cells and monocytes (precursors of MDSCs) allowed the re-creation of MDSC-induced immune suppression *in vitro*. Subsequent targeting of specific molecular pathways in melanoma cells revealed that proteins involved in cellular stress pathways (heat shock proteins) are involved in triggering the differentiation of normal monocytes into MDSCs. These studies show that the levels of immune populations in the periphery and in the tumour can be used to monitor the clinical course of cancer patients. Furthermore, this work identifies new mechanisms of immune suppression by revealing novel pathways used by tumour cells to suppress the immune system.

3 Zusammenfassung

Bei der Behandlung solider Tumore gilt die Immuntherapie zurzeit als einer der vielversprechendsten Therapieansätze. Hierbei macht man sich die Fähigkeit von patienteneigenen Immunzellen zu Nutze, Tumorzellen nach Stimulation zu erkennen und anschließend zu eliminieren. So konnte bereits bei mehreren Entitäten eine längere Überlebenszeit bei denjenigen Patienten festgestellt werden, deren Tumore antigen-reaktive T-Zellen aufweisen. Da es sich bei der Erfassung solcher T-Zell vermittelten Reaktionen jedoch um kosten- und vor allem arbeitsintensive Verfahren handelt, haben diese Untersuchungen noch keinen Einzug in die klinische Routine gehalten. Im Fokus dieser Arbeit stand daher unter anderem die Suche nach Biomarkern, welche die Identifizierung derjenigen Patienten erlaubt, die solch klinisch relevanten Antigen-reaktive T Zellen besitzen. Im ersten Screening wurden HLA Typen, Serumzytokine, Tumor-infiltrierende Leukozyten und Blutleukozyten gemessen, wobei sich Blutleukozyten als am geeignetsten für die Identifizierung von Patienten mit solchen Antigen-reaktiven T Zellen erwiesen. Durch die Kombination verschiedener Parameter konnte darüber hinaus eine 100-prozentige Sensitivität und Spezifität bei der Identifizierung von Patienten mit reaktiven Zellen erreicht werden. Jedoch scheinen nicht nur die Immunzellen der Peripherie allein eine wichtige Rolle für den Tumor zu spielen, zusätzlich scheint die zelluläre Zusammensetzung des Tumorgewebes selbst maßgeblich dessen Wachstum zu beeinflussen. Die Identifizierung von zellulären und löslichen Faktoren innerhalb des Tumors, welche eine prognostische Rolle in Melanom Patienten haben, war daher ein weiterer Schwerpunkt der vorliegenden Arbeit. Hier zeigte sich, dass Patienten mit einer starken Infiltration von potentiell suppressiven CD15+ Zellen, als auch Patienten mit schwacher Infiltration von CD3+ und starker Infiltration von CD15+ Zellen ein geringeres Gesamtüberleben aufwiesen.

Ein weiterer Hinweis für die Relevanz des Immunsystems hinsichtlich des Überlebens zeigten weitere Studien, in denen ein erhöhtes Level an immunsuppressiven MDSCs mit einem verkürzten Überleben der Patienten

assoziiert war. So ist von Melanom Zellen bekannt, dass sie die Fähigkeit besitzen immunsuppressive MDSCs zu induzieren, wobei allerdings wenig über den zugrunde liegenden Mechanismus bekannt ist. Wir untersuchten daher in einem Zellkulturmodel die Wechselwirkungen zwischen Tumor und Immunzellen mit dem Ziel, die Induktion immunsuppressiver Zellen zu blockieren. Hierbei zeigte sich, dass molekulare Signalwege, welche in Stresssituationen relevant sind, für die Differenzierung von MDSCs verantwortlich sind. Durch die Hemmung dieser Signalwege lässt sich die Differenzierung von immunsuppressiven Zellen blockieren, womit sich Möglichkeiten für vielversprechende therapeutische Ansätze ergeben.

4 Publications and manuscripts embedded in this thesis

Publication 1: Peripheral T cell responses to tumour antigens are associated with molecular, immunogenetic and cellular features of breast cancer patients.

Nicole Janssen, Sotirios P Fortis, Lisa Speigl, Christoforos Haritos, Nectaria Sotiriadou, Michael Sofopoulos, Niki Arnogiannaki, Catherine Stavropoulos-Giokas, Amalia Dinou, Sonia A Perez, Graham Pawelec, Constantin Baxevanis, Christopher Shipp

Breast Cancer Res Treat 161 (1), 51-62 2016 Oct 27. doi: 10.1007/s10549-016-4037-z

Publication 2: Inhibiting HSP90 prevents the induction of myeloid-derived suppressor cells by melanoma cells

Nicole Janssen; Lisa Speigl; Graham Pawelec; Heike Niessner; Christopher Shipp

Cellular Immunology 327:68-76 2018 May. doi: 10.1016/j.cellimm.2018.02.012

Manuscript 1: High levels of blood T cells identify breast cancer patients with HER2, MUC1 and SUR-reactive T cells.

Nicole Janssen; Lisa Speigl; Christoforos Haritos; Sotirios P Fortis; Graham Pawelec; Christopher Shipp

Manuscript 2: Intra-tumoural immune features as prognostic markers in metastatic melanoma.

Nicole Janssen; Alexandra Grieb; Benjamin Weide; Graham Pawelec; Christoph Shipp

5 Personal contribution

Publication 1:

I was involved in experiments and evaluation of antigen-reactive T cell data, as well as phenotypic analysis of blood leukocytes. Moreover, I was involved in the analysis and interpretation of the data and in the preparation of the manuscript.

Experimental performance
Collection and assembly of data
Data analysis and interpretation
Manuscript writing

Publication 2:

I was involved in the establishment of a new *in vitro* co-culture model for this study. All experiments to determine the effect of inhibitors tested was performed by myself. I conducted the analysis of the results and was involved in writing the manuscript.

Experimental performance
Collection and assembly of data
Data analysis and interpretation
Manuscript writing

Manuscript 1:

I was involved in experiments and phenotypic anaylsis of blood leukocytes. Moreover, I was involved in the analysis and interpretation of the data and in the preparation of the manuscript.

Experimental performance
Collection and assembly of data
Data analysis and interpretation

Personal contribution

Manuscript writing

Manuscript 2:

I was involved in experiments and evaluation of cell counts and fluorescence intensities of soluble molecules. Additionally data analysis and interpretation was performed by me. I was involved in writing the manuscript.

Experimental performance
Collection and assembly of data
Data analysis and interpretation
Manuscript writing

6 Publications not embedded in this thesis

Differential intratumoral distributions of CD8 and CD163 immune cells as prognostic biomarkers in breast cancer.
Sotirios P Fortis, MSc, PhD student; Michael Sofopoulos, MD; Nectaria N Sotiriadou, PhD; Christoforos Haritos, MD; Christoforos K Vaxevanis, BSc; Eleftheria A Anastasopoulou, PhD; **Nicole Janssen, MSc,** PhD student; Niki Arnogiannaki, MD; Alexandros Ardavanis, MD, PhD; Graham Pawelec, PhD; Sonia A Perez, Ph.D; Constantin N Baxevanis, PhD.
J Immunother Cancer. 2017 Apr 18; 5:39. doi: 10.1186/s40425-017-0240-7.
PMID: 28428887.

CD14+ HLA-DR-/low MDSCs are elevated in the periphery of early-stage breast cancer patients and suppress autologous T cell proliferation.
Speigl L, Burow H, Bailur JK, **Janssen N**, Walter CB, Pawelec G, Shipp C.
Breast Cancer Res Treat. 2018 Apr; 168(2):401-411. doi: 10.1007/s10549-017-4594-9. PMID: 29230664.

Low levels of intra-tumoural T cells in breast cancer identify clinically frail patients with shorter disease-specific survival.
Speigl L, Grieb A, **Janssen N**, Hatse S, Brouwers B, Smeets A, Floris G, Bailur JK, Kenis C, Neven P, Wildiers H, Pawelec G, Shipp C. J Geriatr Oncol. 2018 Nov;9(6):606-612. doi: 10.1016/j.jgo.2018.03.021. PMID: 29685380.

7 Introduction

Cancer is a leading cause of death worldwide where it accounts for approximately 16 % of all deaths. This equates to 8.8 million people who died from cancer in 2015, with 70 % of deaths in low- or middle-income countries [1]. The main characteristic of cancer is the uncontrolled growth of abnormal cells resulting in: repression of normal cells, invasion into adjacent tissue, the spread to distant organs and metastasis being the major cause of death from cancer [2]. Normally, cell proliferation and function is strictly regulated. However, in cancer this regulation is lost due to the accumulation of genetic mutations. However, mutation in a single gene does not usually cause cancer, which requires multiple steps typically consisting of genetic mutations in a number of different genes that include gain of function mutations in proto-oncogenes and loss of function mutations in tumour suppressor and DNA repair genes [3]. Such mutations can be caused by a diverse range of factors, including physical or chemical carcinogens such as irradiation or components of tobacco smoke but also by biological factors such as infections with certain viruses [1]. With the accumulation of such mutations a normal cell transforms into a precancerous cell which eventually leads to cancer. During this process, cancer cells acquire traits including sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, reprogramming energy metabolism, evading immune destruction, inducing angiogenesis and activating invasion and metastasis [4,5].

7.1 Breast cancer

All forms of cancer resulted in 14.1 million cases being diagnosed in 2012, with the majority being cancer of the lung, breast and colon. Breast cancer represents the second most common form, account for 12 % of all cancers. In women, it is the most common cancer, representing with 25 % of all cancer types [6]. Over the past few decades the incidence of breast cancer increased, while the death rate has significantly decreased. This is attributed to earlier discovery and subsequent

improvements in therapy [7]. Despite this, it is still the most leading cause of death in women, with approximately 522 000 deaths in 2012 [6].

Breast cancer is heterogeneous genetically and clinically, with several histological and molecular subtypes being identified. The subtype classification is of great importance for prognosis and prediction of response to chemotherapy and endocrine therapy [8]. It can be broadly categorised into *in situ* carcinoma and invasive carcinoma. The *in situ* breast carcinoma most commonly begins in the lining of the milk ducts or in the lobules of the breast and are therefore referred to as ductual or lobular carcinoma. If the cancer cells then spread into surrounding tissue of either breast ducts or lobules, it is then referred to as invasive ductal carcinoma or invasive lobular carcinoma [9]. In addition to traditional clinicopathological variables, including tumour size, tumour grade and nodal involvement, immunohistochemistry markers such as the hormone receptors, estrogen (ER) and progesterone (PR) as well as the expression of human epidermal growth factor receptor-2 (HER2) are analysed in order to further characterise breast cancer. Based on this, breast tumours can be further categorised into at least three major subtypes: luminal, HER2+ and basal like [10,11]. This is of great importance, as each subtype has different risk factors for incidence, response to treatment, risk of disease progression and different preferential organ sites of metastasis. Based on this knowledge, treatment can be customised in order to improve disease-specific survival [12]. Thus, luminal tumours positive for the hormone receptors ER and PR consequently often respond well to hormone therapies. HER2+ tumours have an amplification of the ERBB2 oncogene and overexpress HER2. This subtype can be treated with anti-HER2 therapies. In contrast, basal-like tumours neither express hormone receptors nor do they express HER2 and are therefore known as triple-negative breast cancer. These properties result in triple negative tumours being insensitive to some of the most effective therapies available for breast cancer including anti-HER2 and endocrine therapies. Although only a minority of breast cancer is categorised as triple-negative, it is an aggressive form whereby only 20 % of patients respond to chemotherapy [13]. Therefore, there is a need to develop better therapies and to

identify biomarkers that can be used to select which patients should receive which treatment in order to improve disease outcome.

7.2 Malignant melanoma

In contrast to breast cancer, melanoma is relatively rare with 232 000 newly diagnosed cases throughout the world in 2012, only representing 1.7 % of all cancer types. However, according to the US National Cancer Institute, the incidence rate of melanoma has more than doubled since 1975 – with 7.5 new cases per 100 000 in 1975 versus 25 per 100 000 in 2014 [14]. This is a more rapid increase in the incidence rate compared to any other solid tumour type [14,15]. The 5-year survival rate for primary melanoma of the skin is about 92 %. However, looking at late stage melanoma, the 5-year survival rate drops dramatically to 15 % - 20 % [1]. As with many other cancers, patient outcome depends on the tumour stage at diagnosis. According to the American Joint Committee on Cancer (AJCC), melanoma can be classified into stages I – IV by characteristics of the primary tumour (T) (thickness, ulceration, mitotic rate), involvement of locoregional lymph nodes and soft tissue metastases (N) (microscopic vs macroscopic lesions) and the presence of distant metastases (M) (serum concentration of lactate dehydrogenase and localization of metastases) [16]. In stage I melanoma, the tumour is restricted to the dermal layer with a 5-year survival rate of 90 – 95 %. With progressing disease, tumour size increases, loco-regional metastases develop and finally the tumour spreads into distant organs, typically accompanied by high serum LDH levels. The latter is defined as stage IV melanoma with a 5-year survival probability of 15 – 20 % [16–18]. However, with the introduction of new treatment approaches such as immunotherapies or targeted therapies, 5-year survival of metastatic melanoma patients may rise to as high as 35 % [19].

7.3 Immune system and Cancer

The role of the immune system in cancer has been discussed for more than a century, starting with Paul Ehrlich who first proposed the idea that the immune system could repress carcinomas, which would otherwise occur at higher

frequencies. Burnet and Thomas then further postulated a protective role of the immune system according to the theory of cancer immune surveillance, suggesting that lymphocytes can recognise and eliminate continuously arising transformed cells [21,22]. However, despite the notion that the host immune system can provide protection from cancer, it has been shown that it may also be involved in tumour progression. Therefore, the hypothesis of cancer immune surveillance was modified into the concept of immunoediting, dividing the process from immune surveillance to tumour escape into three phases (Elimination, Equilibrium and Escape) [20]. Immune surveillance occurs during the elimination process. In the equilibrium phase, the immune system prevents tumour growth as well as promotes tumour cell variants with reduced immunogenicity. These cells are then able to develop mechanisms to escape immune control and enter the last "escape" phase in which cancer cells grow in an uncontrolled manner [20].

7.4 Immunosuppression

Immunosuppression by cancer cells can be mediated by multiple mechanisms in order to avoid being recognised or attacked by the immune system. Blocking these mechanisms of immunosuppression and understanding components and the mechanisms responsible for suppression of anti-tumour immune response are of great interest in order to increase the efficacy of immunotherapies.

One mechanism used by tumours to avoid T cell recognition is changing the antigenic profile. This occurs during immune selection against tumour cells expressing immunologically relevant epitopes, therefore leaving those behind which are not recognised by T cells of the adaptive immune system [23]. This leads to a selection of tumour cells highly resistant to T cell responses, thus supporting tumour growth. In addition, tumours can down-regulate and mutate their antigen processing machinery resulting in impaired antigen presentation and thus immune evasion from T-cell responses [24]. Furthermore, immunosuppressive cytokines, chemokines and growth factors are either synthesised by tumour or stromal cells. For example, TGF-β is a cytokine capable of inhibiting T-cell activation, proliferation and differentiation [25] and high serum levels are associated with poor clinical outcome in several types of cancer [26–29]. Several other cytokines have been

identified that impair anti-tumour immune response by directly acting on T cells or by recruiting or expanding regulatory cells like regulatory T cells (Tregs) or myeloid derived suppressor cells (MDSCs). Although Tregs were originally described as regulators for preventing organ-specific autoimmune disease, several studies suggest an important role in anti-tumour immune responses and their involvement in tumour growth and progression. In multiple malignancies, including breast and skin cancer, an accumulation of Tregs has been shown in either the circulation or the tumour itself [30–33], the levels of which are associated with poor overall survival [34]. Supporting the role of Tregs in preventing anti-tumour immune responses, studies targeting these cells have been shown to improve the efficacy of immunotherapy [35–37]. More recently, another regulatory cell type, the MDSC, has gained great attention due to its pro-tumoural and immunosuppressive activity [38,39].

7.5 Myeloid derived suppressor cells (MDSCs)

Myeloid cells may be terminally differentiated cells including dendritic cells, macrophages and granulocytes, however, in the case of cancer a typical high inflammatory environment induces a higher than normal level of immature myeloid cells which have immunosuppressive properties. Due to their suppressive nature and myeloid origin, these cells are called myeloid-derived suppressor cells [40,41]. Regarding their characterisation, in humans no definite phenotype has been identified so far, although a combination of different markers has been reported to mark these cells. They are negative for the lineage markers of T cells (CD3), B cells (CD19) and natural killer cells (CD56) and always positive for myeloid markers including CD11b and CD33 with low expression of human leukocyte antigen-DR (HLA-DR) [42,43]. In addition to these markers, MDSCs can be further distinguished based on the expression of CD14 and CD15 - granulocytic MDSCs express CD15 and lack CD14 while monocytic MDSCs can be defined by their CD14 expression.

Recently, MDSCs gained a great deal of attention for their role in cancer. Due to their suppressive activity, MDSCs represent a major barrier in effective cancer therapy. In support of this, melanoma patients with high relative levels of MDSCs experience shorter overall survival and respond more poorly to immunotherapy

with the anti-CTLA-4 antibody ipilimumab [44,45]. Mechanisms of MDSC-mediated immunosuppression include (1) depletion of nutrients required for T cell activation, (2) generation of oxidative stress (3) cleavage of L-selectin on naïve T cells required for homing and activation of T cells and (4) induction of Tregs. These mechanisms of immunosuppression might represent potential targets for therapeutic intervention. Studies targeting pathways associated with MDSC-mediated immunosuppression (including arginase, ROS, COX-2 and STAT3) may restore T cell proliferation in co-culture experiments. In addition to blocking the suppressive activity of MDSCs, it might also be possible to prevent the differentiation of myeloid cells into MDSCs or to induce the differentiation of MDSCs into non-suppressive cells. Recently, Mao *et al*. have shown that co-culturing CD14+ monocytes from healthy individuals with melanoma cells results in the induction of monocytes with an MDSC-like phenotype possessing suppressive activity [46]. These data suggest that either cell-cell contact or soluble factors produced by melanoma cells are able to induce the differentiation of MDSCs. Several soluble factors have already been identified to be involved in the differentiation of monocytes into suppressive MDSCs, including GM-CSF, IL-6 and PGE2 [47–53]. In addition, upstream signalling pathways like COX2 and STAT3 have also been identified to be involved in the differentiation and suppressive function of MDSCs [46–48]. Targeting these pathways resulted in restored T cell proliferation after treating melanoma-educated monocytes or patient-derived MDSCs with different inhibitors. These data suggest that combining agents that block the differentiation or suppressive function of MDSCs with current immunotherapeutic strategies may be a useful approach that could result in more efficacious treatment.

7.6 Tumour microenvironment

The tumour microenvironment consists of tumour cells, fibroblasts, myofibroblasts, blood and lymph vessels as well as tumour-infiltrating immune cells, chemokines and cytokines, with each component contributing to either tumour suppression or tumour development and progression [55]. Among non-immune components, tumour-associated fibroblasts are involved in the formation and remodelling of the

extracellular matrix as well as in the supply of factors which promote the growth of cancer cells [54]. With increasing tumour mass, the formation of new blood vessels is required to supply sufficient oxygen and nutrition, and this also provides routes for local invasion and distant metastases. Especially the role of immune factors in tumour progression and control has gained more and more attention recently. Analysis of the tumour microenvironment of several cancer types revealed that many or most tumours are infiltrated by T cells. In colorectal cancer, intra- and peritumoural infiltration with CD8+ T cells has been shown to have greater prognostic power than the standard clinical staging system for predicting post-operative survival [56–58]. Such associations between the level of tumour-infiltrating lymphocytes and clinical outcome have been also shown in several other cancer types [59–62]. Although high infiltration with T lymphocytes is generally associated with good prognosis, tumour progression is still frequently seen despite the presence of high levels of such intra-tumoural immune cells. This suggests that factors such as suppressive cells within the tumour microenvironment impair the function of these beneficial immune cells. Indeed, studies have shown that infiltration by MDSCs or regulatory T cells promotes tumour progression via multiple suppressive mechanisms. These cells dampen beneficial anti-tumour immune responses by both innate and adaptive immune cells. In addition to cellular components of the tumour microenvironment, soluble factors may also alter anti-tumour immune responses. In support of this, studies have shown that soluble immune molecules in the tumour microenvironment such as PGE2 or IL-6, correlate with patient survival [63,64] and influence the migration, differentiation and functional state of infiltrating immune cells [67]. As well as providing prognostic information relating to patient survival, the assessment of the intra-tumoural immune context can also assist in predicting which patients will respond to certain forms of therapy, something of great importance in clinical patient management. Consistent with this, analysis of the tumour microenvironment revealed an association between the density of tumour-infiltrating T cells and clinical response to PD-1 antibodies [65].

7.7 Immunotherapy

Based on the current knowledge of the immune system and its role in tumour defence, an approach in cancer therapy, referred to as immunotherapy, was has been established. This form of therapy aims to boost the host's immune system, re-enabling it to effectively target and destroy malignant cells. Numerous studies have been conducted to develop treatment approaches with different mechanisms of action including monoclonal antibodies, adoptive immunotherapies or cancer vaccines targeting different tumour-associated antigens. Encouraging results were observed in breast cancer when HER2-positive patients were treated with the monoclonal antibody trastuzumab targeting HER2. Until then, HER2-positive breast cancer patients had the worst overall survival and recurrence-free survival compared to all other types including triple-negative breast cancer [66]. With the introduction of trastuzumub the median survival of HER2-positive breast cancer patients was increased up to 25.1 months compared to 20.3 months in the group of patients receiving chemotherapy alone [68]. However, as a passive immunotherapy, therapeutic effect will be achieved only during the administration of the antibody. In contrast active immunotherapy strategies potentially induce long-term immune activation even after treatment has been completed. Breakthrough results were achieved in metastatic melanoma patients treated with a monoclonal antibody called ipilimumab, which targets cytotoxic T lymphocyte antigen-4 (CTLA-4) expressed on T cells. In patients with unresectable metastatic melanoma vaccinated with the tumour-associated antigen glycoprotein 100, an improved overall survival of 10.1 months was achieved with ipilimumab, compared to 6.4 months in the group of patients treated with vaccine alone [69]. Later studies targeting another "checkpoint" molecule, programmed death-1 (PD-1) or programmed death-ligand 1 (PD-L1) have demonstrated superior clinical responses compared with ipilimumab in melanoma. There are also promising results in other cancer types [70–75]. Similarly, cancer vaccines also aim to prime or boost immune responses against tumour cells, and both approaches have been shown to stimulate T cell responses resulting in clinical tumour regression or prolonged patient survival [76,77].

Despite these encouraging advances in cancer therapy, not every patient is equally likely to benefit from a certain form of therapy. The effectiveness of such treatments is presumably based on T cell responses against tumour-associated antigens (TAAs) expressed by cancer cells, allowing these T cells to kill or otherwise inhibit tumour cells and induce tumour regression.

7.8 Antigen-reactive T cells

The immune system can distinguish non-transformed cells from cancer cells by differential recognition of certain proteins. However, these targets for the immune system are not necessarily cancer cell-specific and as such can also be found on normal tissue. Therefore, molecules that are more commonly found on cancer cells give rise to so-called tumour-associated antigens (TAA). These proteins can be products of (1) aberrantly expressed genes such as HER2, which is also expressed in non-transformed cells but at lower levels (2) genes encoding viral products like the human papillomavirus proteins (3) mutated cellular genes such as p53, (4) molecules that are expressed during certain stages of development like melanocyte antigen (Melan-A) or (5) molecules that are expressed in germ cells of the testis and ovary but are silent in non-transformed somatic cells, like NY-ESO-1.
One of the most extensively studied TAAs in breast cancer is HER2. This protein is overexpressed in approximately 20 – 30 % of breast carcinomas and is correlated with greater tumour aggressiveness and poor prognosis [78]. Since HER2 is a cell surface molecule, it represents a good target for cancer therapy with antibodies. Indeed, as noted above, treatment with anti-HER2 monoclonal antibody was associated with longer time to progression, higher response rate, longer response duration and improved overall survival [68]. Because antibody-based therapies rely on the expression of the targeted molecule at a certain expression level on the relevant cell type, vaccination that induces or stimulates pre-existing anti-HER2 immune responses might result in superior therapeutic response. In support of this, Bailur *et al.* observed that breast cancer patients who possessed HER2-reactive CD8+ T cells experienced improved overall survival [79]. In Phase I/II clinical trials aiming to elicit T cell responses to HER2 vaccines in breast cancer patients, partial clinical responses were observed [80,81].

8 Thesis aims

The primary aims of this thesis were 1) to identify new blood or tissue-based prognostic markers in melanoma and breast cancer, and 2) to develop a better understanding of interactions between tumour cells and cells of the immune system, with the aim of understanding mechanisms of tumour-induced immune suppression and identifying potential therapeutic targets in melanoma. In breast cancer patients, the presence of HER2-reactive CD8+ T cells had previously been shown to be associated with superior overall survival. Therefore, the current project investigated whether T cells which recognise other tumour-associated antigens may also act as prognostic markers. This project increased the number of candidate antigens tested to include the tumour-associated antigens MUC1 and Survivin, in addition to attempting to validate the previous results for HER2. Because the measurement of antigen-reactive T cells is costly, time intensive and technically challenging they are not used as a part of routine clinical monitoring. Therefore, this study sought to identify surrogate markers which may more easily be used to indicate the presence of these cells. The identification of surrogate markers may also provide a better understanding of the immune states favourable to the presence of these cells, thus improving our understanding of breast cancer immunobiology. While this project in breast cancer was especially focused on the immune profile in peripheral blood, the project in melanoma was focused on identifying immune features in the tumour microenvironment which may serve as prognostic markers. Here, diverse immune parameters covering both soluble and cellular features in the tumours of melanoma patients were assessed. Using immunofluorescence, protocols for the detection of T cells, myeloid cells and a panel of soluble immune factors were established. These factors were then quantified in tumour tissue and investigated for relationship with patient clinical features.

It is being ever more appreciated that immune suppressive cells are of great clinical importance in melanoma, for example they have been shown to correlate with patient survival and to impair the efficacy of immunotherapy. Melanoma cells

have been shown to induce such myeloid cells with immune suppressive activity in vitro, but little is known regarding how melanoma cells convert healthy cells into immune suppressive cells. Therefore, unlike in the prior observational studies, a subsequent project in melanoma involved the development of an *in vitro* model to investigate interactions between melanoma and immune cells, with the aim of identifying potential new therapeutic targets that can prevent tumour-induced suppression of the immune system, which may have the additional benefit of enhancing the efficacy of immunotherapy.

9 Results and Discussion

9.1 Publication 1

Peripheral T cell responses to tumour antigens are associated with molecular, immunogenetic and cellular features of breast cancer patients

Previously, our group demonstrated an association of peripheral HER2-reactive T cells with superior survival in breast cancer patients [79]. The present study analysed patient features associated with the presence of such T cell responses to tumour antigens, with the goal of identifying biomarkers that may be routinely used to select breast cancer patients with responses to tumour antigens. Surrogate markers for antigen-reactive T cells may be more easily implemented into routine clinical practice compared to the complex and expensive protocol required to measure them directly. The introduction of routine detection of such tumour-antigen reactive T cells may improve therapeutic management of breast cancer patients. This study included analysis of (1) the type of reactive T cell (CD4+ or CD8+) and (2) the produced cytokine pattern (interferon-γ (IFN-γ), TNF-α, IL-2, IL-4, IL-10 and IL-17 was simultaneously measured in the same cell) of T cells reactive to HER2, MUC1 and Survivin antigens using intracellular cytokine staining (ICS) (3) immunogenetic factors (HLA type), (4) soluble signalling molecules in serum using Luminex-based technology, as well as immune cells (5) in the peripheral blood using immunophenotyping (monocytes, MDSCs, pDCs, mDCs, T cells and NK cells) and (6) within the tumour using immunohistochemistry (CD4, CD8, FoxP3 and CD163).

Cryopreserved PBMCs from 50 patients with non-metastatic invasive carcinoma were collected at the St. Savas Cancer Hospital in Athens between February 2014 and May 2015, one day prior to surgery. The study included women with an age range of 27–78 (median age 56 years) with early stage non-metastatic invasive ductal carcinoma. Patients with AJCC stages 1, 2A, 2B, 3A and 3C were included,

while patients with tumours extending to the chest wall as well as AJCC stages 3B and 4 were excluded.

Although only 30 % of patient tumours overexpressed HER2, almost every patient possessed T cells responding to HER2 (96 %) (Fig. 1a). These results are in line with studies indicating the benefit of HER2 vaccines in HER2-"negative" patients [82–84] . In terms of MUC1 and SUR, data on tumour expression are not available. Here, were observed antigen-reactive T cells in 80 % and 72 % of patients (Fig. 1a). Consistent with previous studies of our group, we found that CD4+ T cells responses were more common compared to CD8+, and also produced a larger number of different cytokines (Fig. 1C) [79]. Furthermore these two different compartments were associated with different clinical characteristics. According to Mann-Whitney U testing, patients with more advanced disease (higher tumour grade) had weaker CD4+ T cell responses to MUC1 ($p = 0.045$) (Fig. 2a) while those patients with oestrogen receptor (ER)-positive tumours had stronger CD8+ T cell responses to HER2 ($p = 0.031$) (Fig. 2b). In contrast, patients with SUR-reactive T cells showed no associations with any clinical parameters including T, N or AJCC stage, total lymph node involvement as well as expression of Ki67, HER2 or progesterone receptor in the tumour.

In order to identify immune parameters associated with the presence or absence of antigen-reactive T cells, immune features including serum cytokines, HLA type and tumour leukocyte infiltration were analysed for correlations with T cells responding to HER2, MUC1 and SUR. However, these analyses revealed only a few correlations with T cell responses. Here, the presence of HLA-A*01 was associated with a lack of T cell response to MUC1 ($p = 0.045$), while HLA-A*02+ patients were more likely to possess MUC1-reactive T cells ($p = 0.0001$) (Fig. 3a). In terms of serum cytokines, high levels of IL-10 were associated with HER2-reactive (Fig. 3b) and SUR-reactive CD8+ T cells. Interestingly, no association between tumour leukocyte infiltration and *in vitro* T cell responses were observed for HER2-reactive T cells (Fig. 4a), but high infiltration of CD163+, CD4+ T cells (Fig. 4b) as well as combined cell counts for both CD4+ and CD8+ T cells was associated with the presence of T cells responding to the MUC1 antigen. In contrast, patients with low numbers of tumour-infiltrating CD8+ T cells had more frequent CD8+ T cells

responding to the SUR antigen (Fig. 4c). Interestingly, no correlations for these particular cell types in tissue were found for the same cell type in blood. These differences between blood and tissue and their association with TAA-reactive T cells suggest that cellular location and phenotype are both relevant for their impact on T cell responses to tumour-associated antigens in blood. Therefore, unless location-specific markers are found, the identification of marker proteins which accurately reflect cellular function remains difficult.

Analysing peripheral blood leukocytes for their association with TAA-reactive T cells, only a few correlations were observed between T cell responses and potential suppressive cells like Tregs and MDSCs, although the presence of such suppressive cells is one mechanism dampening beneficial antigen-reactive T cell responses. This suggests that (a) either these potential suppressive cells are not suppressive in breast cancer patients or (b) that these cells are not able to suppress antigen-reactive T cells in blood or (c) circulating suppressive cells are not relevant for the activity of antigen-reactive T cells. Notably, several inverse relationships between TAA reactive T cells and blood leukocytes were observed. For example, high levels of blood monocytes was associated with absent or weaker CD4+ and CD8+ T cells responsive to HER2 and MUC1. Comparable inverse relationships were observed for the HLA-DR-negative fraction within monocytes, NK cells, mDCs and CD4+ and CD8+ T cells expressing markers of proliferation (Fig. 5), while several other populations were positively associated with antigen-reactive T cells (Table 2). These data suggest that blood leukocytes are able to suppress T cell responses to tumour antigens, but remain imperfectly defined on a phenotypic level.

Notably, combining different immune parameters resulted in increased specificity and sensitivity in identifying patients with TAA-reactive T cells as well as in identifying patients with a strong or weak response (Fig. 6a). Thus, patients with below median levels of monocytes and NK cells identified a group in which all patients possessed HER2-reactive T cells with an average stimulation index of 14.5, compared to patients with above median levels of these cells who had an average of stimulation index of 2.3 (Fig. 6b, left panel). In addition, combining multiple immunological parameters like tumour-infiltrating cells and blood

leukocytes resulted in superior prediction of patients responding to TAAs. For example, high levels of T cell infiltration and low levels of blood CD16+ mDCs were associated with an improved accuracy in identifying patients with MUC1-reactive T cells as well as identifying those with a stronger response (Fig. 6b, middle panel).

Recent studies have shown an association between the clinical benefit of immunomodulatory antibodies and the presence of anti-tumour T cells. These findings suggest that patients with pre-existing TAA-reactive T cells may be more likely to respond to such therapies [85,86]. Therefore, identifying biomarkers associated with T cells responding to TAAs may provide the basis for selecting breast cancer patients more likely to benefit from such treatments. Furthermore, patients lacking TAA-reactive T cells but who exhibit an immune profile associated with T cells responding to TAAs may also benefit from treatment with immunomodulatory drugs due to these patients having an "immune environment" that is favourable for facilitating such anti-tumour responses. The same principle may be applied for the use of cancer vaccines; patients with an immune profile associated with the presence of anti-tumour immune responses may be more likely to generate an immune response to the vaccine and benefit clinically [76,77,91–93]. Furthermore, previous studies highlighted that not only the presence of TAA-reactive T cells per se is relevant for patient survival, but also the production of certain cytokines. These results allow the selection of patients with TAA-reactive T cells that produce specific cytokines, and as such, may allow better prediction of patients most likely to respond to therapy or have a favourable prognosis according to the immune profiles identified here.

In order to validate whether the composite set of biomarkers identified here will allow superior survival prediction, patient clinical follow-up will need to be analysed. Furthermore, selecting patients using these biomarker combinations may allow more personalised treatment management and prevent unnecessary treatment for patients with a favourable prognosis or the administration of more aggressive therapies for patients with a poorer prognosis.

9.2 Publication 2

Inhibiting HSP90 prevents the induction of myeloid-derived suppressor cells by melanoma cells

Recent findings by Mao *et al*. showed that melanoma cells induce the differentiation of monocytes into immune suppressive myeloid-derived suppressor cells (MDSCs) which may resemble a mechanism of immune suppression that could also occur *in vivo* in melanoma patients [46]. Based on these observations, we aimed to investigate pathways in melanoma cells responsible for the induction of MDSCs. We ultimately aimed to prevent the induction of MDSC-mediated immune suppression by targeting specific molecular pathways in tumour cells. Therefore, established melanoma cell lines were pre-treated with a panel of different inhibitors tested for their potential to alleviate T cell suppression by preventing MDSC differentiation. In brief, melanoma cells were co-cultured with isolated CD14+ monocytes and autologous CD3+ T cells. Suppressive activity of MDSCs was assessed by measuring CD4+ and CD8+ T cell proliferation using CFSE dilution. In addition, monocytes were analysed phenotypically for CD14 and HLA-DR expression in order to investigate their differentiation into MDSC-like cells. Before co-culturing melanoma cells with isolated immune cells, they were pre-treated with inhibitors targeting known pathways involved in the differentiation and suppressive function of MDSCs, such as geldanamycin (inhibition of Heat Shock Protein (HSP) 90), methylene blue (an HSP70/NO Synthase inhibitor), U-104 (Carbonic anhydrase (CA) inhibitor), acetylsalicylic acid (cyclooxygenase (COX) inhibitor) and AG490 (a JAK/STAT inhibitor).

To understand tumour-immune cell interactions we first cultured monocytes and T cells together with a melanoma cell line and observed changes to the monocyte phenotype and T cell proliferation over time. To this end we co-cultured the established tumour cell line EST-200 with monocytes and T cells for 24, 48, 72, 96 or 120 hrs *in vitro*. This revealed that after 72 hrs T cell proliferation started to decrease when T cells were co-cultured with monocytes and melanoma cells compared to the control cultures consisting of T cells and monocytes alone. We

found that CD4+ T cell suppression continued to increase until 96 hrs, while suppression of CD8+ T cells continued to increase up until the final measurement point of 120 hrs (Fig. 2A). T cell proliferation was not inhibited when T cells were co-cultured with melanoma cells alone, suggesting T cell suppression in the co-culture experiment (together with monocytes and melanoma cells) is due to interactions between monocytes and melanoma cells. As low levels of HLA-DR have been used as a marker for suppressive myeloid cells and MDSCs, we next investigated whether T cell suppression was associated with changes in monocyte phenotype, which may indicate their differentiation towards immunosuppressive MDSCs. We observed a decrease in HLA-DR expression on monocytes cultured with melanoma cells and T cells compared to the control monocytes cultured just with T cells (Fig. 2B). This decrease in HLA-DR indicates that the cells have differentiated towards suppressive MDSCs, which is in line with observations of greater T cell suppression over time. Thus, this *in vitro* model has shown that melanoma cells are able to induce differentiation of monocytes into cells that phenotypically and functionally resemble myeloid-derived suppressor cells.

Similar results were observed when performing these experiments with additional donors and more melanoma cell lines. Six healthy donors were tested with a greater number of established cell lines (EST-41, EST-83, EST-145, EST-152 and EST-200); all cell lines resulted in suppression of T cell proliferation when cultured with monocytes compared to control cultures consisting of monocytes cultured with T cells but without melanoma cells. Comparing the degree of suppression, we observed that EST-145, EST-152 and EST-200 induced the greatest degree of suppression with between 90 – 70 % of T cell suppression compared to control cultures without melanoma cells (Fig. 3, top panel). This was accompanied by phenotypic changes on monocytes that are in line with changes previously reported to be associated with differentiation towards MDSCs, namely down-regulation of HLA-DR and up-regulation of CD14 expression (Fig. 3, bottom panel). Interestingly, we observed different degrees of T cell suppression comparing co-cultures with melanoma cell lines tested here, suggesting that different melanoma

cell lines vary in their capacity to induce immunosuppression via MDSCs, and which thus may employ different mechanisms of immune suppression.

Several soluble factors have been identified as being involved in the differentiation of monocytes into suppressive MDSCs, including GM-CSF, IL-6 and COX-2/PGE2 [51] (53). Neutralizing GM-CSF, IL-6 and IL-1beta in tumour cell line- PBMC co-cultures abolished the induction of CD33+ suppressor cells, and reduced their suppressive activity towards T cells to a degree comparable to controls [53] (55). Despite these advances in the understanding of MDSC biology, the molecular pathways in tumour cells responsible for inducing the differentiation of MDSCs remain to be elucidated. In order to identify pathways in melanoma cells responsible for the induction of suppressive MDSCs, we pre-treated melanoma cells for 18 hrs with a panel of different inhibitors including those against heat shock proteins (hsps) which act as molecular chaperones (geldanamycin: HSP90; methylene blue: HSP70), pH regulatory proteins (U-104: Carbonic anhydrase inhibitor) and inhibitors of COX (acetylsalicylic acid) and JAK/STAT signaling (AG490).

Assessing this panel of inhibitors for their ability to prevent immune suppression in this *in vitro* model showed no significant effect on restoring T cell proliferation for acetylsalicylic acid or AG490. In contrast, geldanamycin was the most effective at restoring T cell proliferation, followed by methylene blue. Occasionally we also observed an effect for U-104, however this was less consistent. Interestingly, the effect of the inhibitors differed according to the cell line tested. Geldanamycin alleviated T cell suppression in 3 of 4 tested melanoma cell lines (EST-145, EST-152, EST-200), which was followed by methylene blue and U-104 in terms of strength of effect (Fig. 4B – D). However, for EST-41 we observed a different trend. Here, methylene blue was the most effective in alleviation of T cell suppression, followed by geldanamycin and U-104 (Fig. 4A). In accordance with this functional analysis assessing T cell proliferation, reduced T cell suppression was associated with an increase in HLA-DR expression on monocytes compared with monocytes in cultures without inhibitor (Fig. 4, bottom panel). Interestingly, EST-41 was an exception also in terms of phenotypic analysis; no association between HLA-DR

expression on melanoma-educated monocytes and degree of T cell suppression was observed with this cell line.

In order to exclude a direct effect of the drugs on T cell proliferation, T cells were directly cultured with pre-treated melanoma cells. This showed T cell proliferation to be comparable to the culture where melanoma cells had not been pretreated with an inhibitor. This suggests that due to the thorough washing protocol employed, no drugs remained, or that the levels were too low to have an effect on T cell proliferation. Because the inhibitor might affect the viability of melanoma cells and therefore may result in impaired induction of T cell suppression, we performed the experiment with double the number of melanoma cells to compensate for any potential effect on cell viability. Because we observed no differences in T cell suppression with twice the number of melanoma cells, this suggests that alleviation of T cell suppression is not related to the number of melanoma cells and thus occurs through blocking molecular pathways in melanoma cells.

Collectively, these results show that targeting HSP70 and HSP90 prevents the differentiation of monocytes into suppressive cells on both a functional and phenotypic level [87–90]. Both molecules belong to the family of molecular chaperones and play essential roles in maintaining the integrity of intracellular proteins [99]. With the help of co-chaperones, they are responsible for the correct folding, function and degradation of a large number of cellular proteins. Particularly in the case of cancer, the harsh tumour microenvironment results in the up-regulation of such molecules in order to sustain the function of cancer cells. This is partly achieved through chaperones maintaining the function of key signaling molecules required for aberrant cell division [94]. Therefore, results showing restored T cell proliferation upon blocking HSP70 or HSP90 suggest that there are overlapping networks between pathways involved in cancer maintenance and those involved in immune suppression. This idea is supported the action of chemotherapeutic drugs which have been shown to exert their beneficial effect partly through immunomodulatory mechanisms [95].

As geldanamycin was shown to be the most effective in alleviating T cell suppression, we next investigated the time-dependent effect of this drug on preventing the induction of suppressive cells. For this, we pre-treated established

melanoma cell line EST-200 with geldanamycin and co-cultured these cells with monocytes and T cells for 24, 48, 72, 96 or 120 hrs. This revealed a decrease in T cell suppression over time compared to co-cultures with melanoma cells that had not been pretreated (Fig. 5). In accordance with functional analysis, we observed higher HLA-DR expression on melanoma-educated monocytes over time compared to control cultures not pretreated with geldanamycin. It was interesting that although melanoma cells were treated only for a duration of 18 hrs, alleviation of T cell suppression could be observed even 5 days post-exposure, suggesting that a single dose of this drug is sufficient for a long-lasting effect. In addition, we analysed GM-CSF production in a co-culture experiment consisting of melanoma cells and monocytes, as GM-CSF has been shown to be involved in the differentiation of suppressive MDSCs. Here, we observed decreased GM-CSF expression when melanoma cells were pre-treated with geldanamycin, suggesting HSP90 to be involved in the production of GM-CSF. Collectively, these results suggest that blocking HSP90 by pre-treating melanoma cells with geldanamycin prevents the conversion of healthy monocytes into immune suppressive cells.

The tumour mass has distinctive features when compared to the surrounding tissue, including lowered oxygen tension and acidic pH, which contributes to the up-regulation of heat-shock proteins like HSP70 and HSP90, or the induction of the transcription factor hypoxia inducible factor 1 (HIF-1a) [96]. Although chemotherapeutic drugs are typically less effective under these conditions [95,97], the inhibitors tested here target molecules which are up-regulated in response to cellular stress such as hypoxic tolerance and may not suffer from reduced efficacy under these conditions. On the contrary, they may even be more effective under conditions of the tumour microenvironment [98]. Therefore we repeated the previous experiments under conventional (20 % O2) conditions and compared these results obtained under hypoxia (2 % O_2). Pre-treating melanoma cells with geldanamycin resulted in a greater alleviation of T cell suppression under hypoxia compared to the conventional condition (20 %). A similar trend was observed for methylene blue being equally effective under hypoxia and hyperoxic conditions (Fig. 6). These results suggest that the efficacy of drugs targeting HSP90 is not diminished in a hypoxic environment *in vitro*. Furthermore an even greater effect was observed

when inhibiting HSP90, suggesting that melanoma cells are even more dependent on these molecules under hypoxic conditions.

This study identified novel mechanisms in tumour cells responsible for the conversion of healthy monocytes into immune suppressive cells. Especially interesting for potential clinical application is that a single sub-lethal dose of these drugs was sufficient to alleviate T cell suppression. Furthermore, in contrast to common chemotherapeutic agents, the activity of these drugs was not diminished under hypoxic conditions, which may better represent the tumour microenvironment. Further investigations are necessary to validate the clinical role of hsps.

9.3 Manuscript 1

High levels of blood T cells identify breast cancer patients with HER2, MUC1 and SUR-reactive T cells. (manuscript under revision in biomarkers)

Recent findings from our group, showed that (1) the presence of peripheral HER2-reactive T cells is associated with patient survival in breast cancer and (2) compared with tumour-infiltrating leukocytes, HLA type and serum cytokines, blood leukocytes are the most informative immune parameter for the identification of patients possessing TAA-reactive T cells. We therefore greatly expanded the panel tested focusing mainly on blood leukocytes, with the aim of identifying markers that can more accurately select patients who possess T cells responding to tumour-associated antigens. These markers may then be used as part of routine patient monitoring, in turn avoiding the complex and extended duration of directly testing for these antigen-reactive T cells.

Using intracellular cytokine staining we assessed (1) T cells (CD4 or CD8) reactive to HER2, MUC1 and Survivin and their produced cytokine pattern (measuring interferon-γ (IFN-γ), TNF-α, IL-2, IL-4, IL-10 and IL-17 simultaneously in the same cell) (3) cellular components in the blood including T cells, NK cells and B cells using extracellular staining in the blood of breast cancer patients. For this study, cryopreserved PBMCs from 50 patients with non-metastatic invasive ductal carcinoma were collected at the St. Savas Cancer Hospital in Athens between

February 2014 and May 2015, one day prior to surgery. The study included women with an age range of 27–78 (median age 56 years). Patients of AJCC stages 1, 2A, 2B, 3A and 3C were included, while patients with tumours extending to the chest wall as well as AJCC stages 3B and 4 were excluded.

In this study we observed that all leukocyte types, but particularly T cells, were associated with clinical parameters including tumour grade, AJCC stage, Ki67 tumour expression and lymph node infiltration. Here, higher AJCC stage and higher tumour grade was related to low levels of CD4+ T cells (AJCC stage: $p = 0.02$, tumour grade: $p = 0.03$) (Fig. 2a) and high levels of CD3hiCD56dim cells that may resemble NKT cells (AJCC stage: $p = 0.06$, tumour grade: $p = 0.003$,) (Fig. 2B). Interestingly, no such associations were observed for Tregs, suggesting that reduced levels of CD4+ T cells are due to a reduction in helper CD4+ T cells with disease progression. However, in terms of the number of tumour-infiltrated lymph nodes the same trend was observed for both CD4+ T cells and regulatory T cells; patients with a greater number of infiltrated lymph nodes had lower levels of both CD4+ T cells ($p = 0.02$) (Fig. 2C) and CD4+ regulatory T cells ($p = 0.04$). Furthermore, low levels of NK cells were related to higher stage and higher percentage of tumour cells expressing Ki67 (AJCC stage: $p = 0.01$, Ki67: $p = 0.04$), while the opposite trend was observed for B cells; high levels of B cells were associated with higher tumour grade and Ki67 tumour expression (tumour grade: $p = 0.02$; Ki67: $p = 0.06$) (Fig. 2D). Several other leukocyte populations were also observed to correlate with clinical parameters. Next, we examined the relationships between the major types of leukocyte populations. Here we observed only one significant correlation between B cells and T cells; this showed that high frequencies of CD20+CD40+ B cells were associated with high levels of CD4+ regulatory T cells (CD4+CD25+FoxP3+) ($p = 0.02$) (Additional File 8).

Although previous studies have shown that tumour antigen-reactive T cells are predictive of patient prognosis, the generation and detection of such cells requires time- and resource-intensive culture protocols and thus limits their use as part of routine clinical monitoring. Hence, identifying immune parameters that select patients possessing TAA-reactive T cells may improve patient monitoring and clinical patient management. Having shown that blood leukocytes, compared to

serum cytokines, HLA type or tumour-infiltrating lymphocytes, are the most informative immune parameters for identifying patients possessing antigen-reactive T cells, in this study we expanded the panel of blood leukocytes tested to identify biomarkers that can more accurately select patients with response to tumour antigen. Thus, these blood-based biomarkers can be more easily implemented into clinical routine compared to the complex and expensive protocols required for detecting antigen-reactive T cells.

Accordingly, we have increased the number of populations tested to include 302 different blood leukocyte populations: 213 for T cells, 19 in the case of B cells, and 70 NK cell populations (Additional File 9). We observed NK cells to be associated with T cell responses to MUC1 and SUR but not with HER2 (full set of correlations in Additional File 10). Here, both, low or high levels of different NK cell populations were shown to correlate with MUC1- and SUR- reactive T cells. For example, presence of CD4+ T cells responding to the MUC1 antigen was associated with relative high levels of CD27+ NK cells ($p = 0.04$) (Fig. 3A), while another population of NK cells (CD56dimCD16-NKG2D+) was found to be lower in patients with MUC1-reactive T cells ($p = 0.04$) (Fig. 3A). In contrast to NK cells, B cells were found to be associated only with T cell responses to HER2. Here, only one correlation between CD8+ HER2-reactive T cells and B cells was found; patients possessing CD8+ T cells responding to HER2 had higher levels of CD19+CD38-CD27+CD20- B cells ($p = 0.04$) (Fig. 3B). Although we were able to select patients with TAA-reactive T cells using different B and NK cell populations, we could only identify selected T cell responses to certain antigens. In contrast, blood T cells were found to be associated with both CD4+ and CD8+ T cells responding to all three antigens tested. Here, compared to NK cells and B cells, T cells were found to be most informative for selecting patients with T cell responses to HER2, MUC1 or SUR. Interestingly, the T cell populations found to be associated with TAA-reactive T cells did not share any common features and covered various differentiation stages, including naïve as well as late-differentiated T cells. For example, patients with T cells responding to the SUR antigen had higher levels of CD8+ CD27- T cells ($p = 0.0007$) (Fig. 3C), while patients with MUC1-reactive T cells had higher levels of CD8+ TEMRA T cells ($p = 0.01$) (Fig. 3C). Noteworthy

was that the majority of patients possessing TAA-reactive T cells had higher levels of blood T cells, suggesting that these phenotypes may directly contribute to the generation of antigen-reactive T cells. This is further supported by the observation that, unlike B and NK cells, T cells were relevant for every type of response and accounted for the vast majority of all correlations with TAA-reactive T cells. Underlining these findings and their clinical importance, T cells were found to be the most informative for patient clinical parameters. These data suggest that altering the immune system of patients lacking TAA-reactive T cells so that it more closely mirrors the immune system of patients with TAA-reactive T cells might result in improved anti-tumour immune responses and thus superior clinical outcome.

Our previous study revealed that combining multiple immune parameters can more accurately identify patients with TAA-reactive T cells. For example, combining parameters such as tumour-infiltrating leukocytes and blood leukocytes, as well as combining different blood leukocyte populations resulted in improved specificity and sensitivity in the identification of patients with TAA-reactive T cells (84). Based on these results, the present study aimed to identify markers that can predict patients possessing TAA-reactive T cells even more accurately by testing a markedly increased number of leukocyte populations. Indeed, in this study we were able to achieve specificity and sensitivity of up to 100 % in selecting patients with TAA-reactive T cells by combining multiple blood leukocyte populations. For example, by stratifying patients according to median levels of stem memory T cells (TSCM) and CD3-CD56- cells, we were able to identify a group of patients who all possessed MUC1-reactive CD4+ T cells (Fig. 4). Similarly, a group of patients with above median levels of NK cells (CD3-CD56hiCD16-CD27+) and NKT cells (NKG2D+ CD3+CD56dim) all possessed CD8+ T cells responding to the Survivin antigen (Fig. 4).

This study identified populations of blood leukocytes that allow the accurate identification of breast cancer patients with functional TAA-reactive T cells. The identification of such markers could replace the direct measurement of antigen-reactive T cells, which requires a time consuming, expensive and labour intensive *in vitro* protocol, as well as additional expertise in order to analyse and interpret the

results. Furthermore, difficulties in standardising culture protocols, due to user- or manufacturer-dependent differences, plus the limited availability of material from cancer patients limits the routine implementation of this approach into the clinical setting. Thus, identifying blood-based surrogate markers that can more easily be measured but still select patients with TAA-reactive T cells may represent an alternative to allow patient clinical monitoring and thus allow more personalised treatment. Especially in light of recent advances in immunotherapy, which presumably relies on the generation and amplification of already existing antigen-reactive T cells, identifying patients possessing such TAA-reactive T cells, or identifying patients with an immune profile favourable to generating such an immune response, may improve the selection of patients more likely to respond the immunotherapy. Furthermore, identifying immune profiles of patients lacking TAA-reactive T cells might represent therapeutic targets, whereby immune parameters may be altered in order to better resemble the immune profile of patients with TAA-reactive T cells, potentially creating a more favourable environment to generate anti-tumour immune responses.

These findings are intended as the first step towards replacing the time-consuming and costly procedure of detecting TAA-reactive T cells. By using a directly and more easily measured parameter such as blood leukocytes, this approach may more easily be integrated into routine monitoring in order to select patients with antigen-reactive T cells and thus identify patients with a more or less favourable prognosis. Ongoing clinical follow-up of these prospectively recruited patients will reveal which of the biomarkers identified here are relevant to patient survival compared with the assessment of antigen-reactive T cells.

9.4 Manuscript 2

Intra-tumoural immune features as prognostic markers in metastatic melanoma. (in preparation)

Recently, investigation on the topic of the tumour microenvironment has revealed that the state of the immune system within the tumour plays a major role in cancer progression, patient clinical outcome and response to therapy. To date, prior

studies have been largely focused on investigating lymphocytes, typically T cells, in the tumour mass. In contrast, other populations of leukocytes, transcription factors and soluble mediators of immune response (such as cytokines) have been relatively neglected. Therefore, this study was designed to identify immune parameters including soluble factors and cellular components in the tumour microenvironment in order to assess which of these are most relevant for patient outcome.

This study assessed T cells (CD3+) and myeloid cells (CD15+) in addition to a panel of soluble immune factors (IL-6, GM-CSF, TNF, IL-1Ra, IL-2, IL-10, GDF15, PGE2) as well as the transcription factor STAT3 in metastatic melanoma tissues of 76 patients using fluorescence microscopy. Slide-mounted formalin-fixed paraffin-embedded tissue sections (5 µm thick) from metastatic melanoma patients were collected at the Dermatology Department of Tübingen University Hospital. The study population consisted of 44 men and 32 women with a median age of 63 (range 35 – 89 years). Patients of AJCC stages 3, 4A, 4B and 4C were included in this study.

The analysis of soluble factors as well as immune cells within the tumour microenvironment revealed that the majority of melanoma tissues were positive for most molecules tested (Fig. 1A). However, the levels of expression as well as the number of infiltrating cells varied across melanoma patients, with IL-6, GM-CSF and IL-1Ra showing the greatest range (Fig. 1B). Overall, CD15+ granulocytic cells were less commonly found compared to CD3+ T cells (Fig. 1C and 1D), although no significant difference were observed comparing the relative distribution of T and granulocytic cells between the center and margin of the tumour (Fig. 1E). In addition to T cells and granulocytic cells, we also attempted to analyse CD14+ cell infiltration. Although we tested two commercially-available antibodies (goat polyclonal anti-CD14 from Novus Biologicals and mouse monoclonal anti-CD14 from Santa Cruz (clone 5A3B11B5)) and a number of different staining protocols, unfortunately we were not able to establish a staining protocol which enabled us to quantify CD14+ cells.

Soluble molecules often act in concert and may generate an environment that either enhances or suppress infiltration of immune cells into the tumour. Therefore,

we examined whether the presence of certain soluble molecules is associated with higher infiltration of immune cells into tumour tissue. Indeed, we found that high CD15+ infiltration is associated with low relative expression of PGE2 ($p = 0.02$) (Fig. 2A), but no other association between immune cell infiltration and soluble molecules was observed. Furthermore, analyzing the relationship between different soluble molecules revealed several correlations between their expression levels. For example, expression of IL-6 and GM-CSF ($p < 0.0001$, $r = 0.7$), TNF and STAT3 ($p < 0.0001$, $r = 0.7$), IL-2 and IL-10 ($p < 0.0001$, $r = 0.5$) (Fig. 2B), plus other combinations of these cytokines were found to be positively correlated (Supplementary Data 1).

Several studies have shown that the state of the immune system plays a major role in patient outcome. However, the majority of those studies mainly focus on tumour-infiltrating cells and miss analysis of soluble factors, although they have been shown to influence anti-tumour immune responses and thus patient survival. Therefore we investigated whether immune parameters tested here are associated with clinical features including disease stage, progression time (stage III to stage IV), age and gender as well as patient survival. In terms of clinical parameters, only stage and age were found to correlate with the immune features tested here. We observed that patients with stage IV disease had higher levels of TNF compared to patients with less advanced stage III disease ($p = 0.02$) (Fig. 3A). Furthermore, we observed age to be associated with CD15+ cell infiltration; older patients were found to have lower numbers of CD15+ cells in the margin of the tumour ($p = 0.046$) (Fig. 3B). In order to investigate the role of immune parameters tested here on patient survival, we first stratified patients according to the conventional method of using the median values to split the cohort into relative high and low values for a particular parameter. Using this approach, Kaplan-Meier survival analysis revealed that none of the immune parameters tested here (neither soluble nor cellular factors) was associated with patient survival (data not shown). These results are in contrast to several other studies showing superior overall survival in patients with high CD3+ infiltration in several types of cancer [56–58,100–106]. However, in melanoma, the role of tumour-infiltrating T cells remains to be elucidated due to inconsistent results from different investigators. While Hillen *et al*. reported a

negative association with the level of intra-tumoural T cells on overall survival [67], other studies report a positive relationship - patients with high levels of intra-tumoural T cells, or one study considering peri-tumoural T cell activation markers, were found to have longer survival [107,108]. Another study, in accordance with the results presented here, reported no significant association between the level of intra- or peri-tumoural CD3+ cells and overall survival [109]. Such discrepancies between these studies might be explained by the great diversity of cells that are detected when using only CD3 to identify T cells; sub-populations of CD3+ cells may vary considerably in functional properties or activities. For example, CD3+ T cells include helper cells, cytotoxic cells but also regulatory T cells or exhausted and anergic cells. Therefore, combining multiple markers to more accurately characterise their phenotypes, as well as including markers representing their functional state, may increase the accuracy of predicting patient prognosis. In support of this, one study highlighted the prognostic difference with different T cell populations in a cohort of melanoma patients. In that study, high T cell infiltration per se was associated with poorer survival, but prolonged survival was found when the degree of cells expressing the T cell activation marker CD69 was considered [67].

However, in contrast to the results showing no association between the intra-tumoural immune parameters investigated here and patient survival, we observed that several immune parameters were associated with patient survival when using a different method to analyse the experimental data. In this approach, we grouped patients according to relative high and low values using different cut-off values to determine the threshold with the minimum p-value and thus the greatest difference in survival. Here, we observed that patients who had low relative levels of GM-CSF or IL-10 ($p=0.035$ and $p=0.043$ respectively), or high relative levels of PGE2 showed longer survival ($p=0.03$) (Fig. 4A and B). In addition, higher levels of infiltrating T cells or low levels of infiltrating granulocytic cells were associated with better patient survival ($p = 0.028$ and $p = 0.0006$, respectively) (Fig. 4C). Because this approach requires an additional number of statistical tests, we adjusted the significance threshold accordingly. Considering this, only the association between low relative levels of intra-tumoural CD15+ cells and improved patient survival

remained significant (p = 0.0006, adjusted significance threshold p = 0.0009) (Fig. 4C).

Recent studies have shown that high numbers of tumour-infiltrating immune cells are associated with superior survival in many types of cancer [56,57]. However, despite the presence of such tumour-infiltrating immune cells, tumour progression is still frequently seen, suggesting that there are factors in the tumour microenvironment inhibiting an effective anti-tumour response by these cells. Indeed, several mechanisms have been identified that induce T cell dysfunction by either directly inhibiting T cell function, for example the expression of inhibitory ligands like PD-L1 on tumour cells or APCs, or indirectly via the induction of regulatory cells such as Tregs or MDSCs [24,110]. High levels of IDO and PGE2 have been demonstrated to inhibit T cell function while IL-6 and several other factors have been shown to convert monocytes into immunosuppressive MDSC. These mechanisms have the potential to impair T cell function within the tumour microenvironment and as such may limit the prognostic value of tumour-infiltrating leukocytes due to changing the immunological context which influences the functional state of these cells. To better consider the immunological context of the immune parameters investigated here, we assessed combinations of these features as potential prognostic markers. Thus, by combining immune features we could demonstrated that patients with high levels of infiltrating CD3+ T cells and low levels of CD15+ cell infiltration had better survival compared to patients without these combinations (p = 0.03) (Fig. 5A). Furthermore, we observed a trend for a survival advantage in patients with high relative levels of CD3 infiltration in combination with low relative levels of the potentially suppressive cytokine IL-10 (p = 0.1), as well as for patients with low relative levels of infiltrating CD15+ cells and low IL-10 expression (p = 0.08) (Fig. 5B). These results suggest that the interaction between different intra-tumoural leukocytes as well as between leukocytes and soluble factors in the tumour microenvironment are important for their functional state. This hypothesis is supported by a study showing that high levels of tumour-infiltrating CD8+ T cells are associated with the recruitment of regulatory T cells into the tumour microenvironment, which might represent a negative feedback

mechanism following infiltration by cytotoxic T cells. Therefore, assessing cytotoxic T cells only might limit their prognostic accuracy [111].

These results, especially in light of the increasing clinical use of immunotherapies, indicate the importance of a more comprehensive analysis of the tumour microenvironment. Supporting this notion, immune features of the tumour microenvironment have been shown to be associated with response to immunotherapies [57,112–119]. For example, Tumeh *et al*. observed an association between the density of tumour-infiltrating T cells and clinical response to PD-1 antibodies [65]. Thus, there is an urgent need to understand the constitution of the tumour microenvironment in order to optimize personalized treatment management or for the identification of novel therapeutic targets.

Collectively, these preliminary results show that intra-tumoural immune parameters either individually or in combination may act as potential prognostic markers in metastatic melanoma. Unlike in many other cancer types, tumour infiltration by T cells was not associated with patient survival, but granulocytic cells may be a more promising marker for predicting patient survival. These results require validation in an independent cohort of patients and should be considered preliminary until then.

10 References

1. Society, A. C. 2017. Cancer Facts & Figures 2017. (2017). Retrieved from https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html (accessed 17.12.2018)
2. Bray, F. & Møller, B. Predicting the future burden of cancer. *Nature reviews. Cancer* **6,** 63–74 (2006).
3. Gerger, A. *et al.* A multigenic approach to predict breast cancer risk. *Breast cancer research and treatment* **104,** 159–164 (2007).
4. Hanahan, D. & Coussens, L. M. Accessories to the Crime. Functions of Cells Recruited to the Tumor Microenvironment. *Cancer cell* **21,** 309–322 (2012).
5. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer. The next generation. *Cell* **144,** 646–674 (2011).
6. Ferlay, J. *et al.* Cancer incidence and mortality worldwide. Sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer* **136,** E359-86 (2015).
7. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2016. *CA: a cancer journal for clinicians* **66,** 7–30 (2016).
8. Stingl, J. & Caldas, C. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. *Nature reviews. Cancer* **7,** 791–799 (2007).
9. Sims, A. H., Howell, A., Howell, S. J. & Clarke, R. B. Origins of breast cancer subtypes and therapeutic implications. *Nature clinical practice. Oncology* **4,** 516–525 (2007).
10. Perou, C. M. *et al.* Molecular portraits of human breast tumours. *Nature* **406,** 747–752 (2000).
11. Sørlie, T. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 10869–10874 (2001).

References

12. Perez, E. A. 2011. Breast cancer management. Opportunities and barriers to an individualized approach. The oncologist 16 Suppl 1: 20-22. (2011).
13. Dai, X. *et al.* Breast cancer intrinsic subtype classification, clinical use and future trends. *American journal of cancer research* **5,** 2929–2943 (2015).
14. Noone AM, Howlader N, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA. SEER Cancer Statistics Review, 1975-2015, National Cancer Institute. Retrieved from https://seer.cancer.gov/statfacts/html/melan.html, based on November 2017 SEER data submission (accessed 17.12.2018).
15. Eggermont, A. M. M., Spatz, A. & Robert, C. Cutaneous melanoma. *The Lancet* **383,** 816–827 (2014).
16. Balch, C. M. *et al.* Final version of 2009 AJCC melanoma staging and classification. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **27,** 6199–6206 (2009).
17. Neuman, H. B. *et al.* A single-institution validation of the AJCC staging system for stage IV melanoma. *Annals of surgical oncology* **15,** 2034–2041 (2008).
18. Cummins, D. L. *et al.* Cutaneous malignant melanoma. *Mayo Clinic proceedings* **81,** 500–507 (2006).
19. Hodi, F. S. *et al.* Abstract CT001. Durable, long-term survival in previously treated patients with advanced melanoma (MEL) who received nivolumab (NIVO) monotherapy in a phase I trial. *Cancer research* **76,** CT001-CT001 (2016).
20. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting. From immunosurveillance to tumor escape. *Nat Immunol* **3,** 991–998 (2002).
21. BURNET, M. in *Immunological Surveillance* (Elsevier1970), pp. 161–185.
22. Burnet, M. Cancer--A Biological Approach. III. Viruses Associated with Neoplastic Conditions. IV. Practical Applications. *BMJ* **1,** 841–847 (1957).
23. Khong, H. T., Wang, Q. J. & Rosenberg, S. A. Identification of multiple antigens recognized by tumor-infiltrating lymphocytes from a single patient.

Tumor escape by antigen loss and loss of MHC expression. *Journal of immunotherapy (Hagerstown, Md. : 1997)* **27,** 184–190 (2004).

24. Marincola, F. M., Jaffee, E. M., Hicklin, D. J. & Ferrone, S. Escape of human solid tumors from T-cell recognition. Molecular mechanisms and functional significance. *Advances in immunology* **74,** 181–273 (2000).
25. Li, M. O., Wan, Y. Y., Sanjabi, S., Robertson, A.-K. L. & Flavell, R. A. Transforming growth factor-beta regulation of immune responses. *Annual review of immunology* **24,** 99–146 (2006).
26. Zhao, J. *et al.* Clinical and prognostic significance of serum transforming growth factor-beta1 levels in patients with pancreatic ductal adenocarcinoma. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas* **49** (2016).
27. Tas, F., Yasasever, C. T., Karabulut, S., Tastekin, D. & Duranyildiz, D. Serum transforming growth factor-beta1 levels may have predictive and prognostic roles in patients with gastric cancer. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **36,** 2097–2103 (2015).
28. Lin, Y., Kikuchi, S., Obata, Y. & Yagyu, K. Serum levels of transforming growth factor beta1 are significantly correlated with venous invasion in patients with gastric cancer. *Journal of gastroenterology and hepatology* **21,** 432–437 (2006).
29. Tas, F., Karabulut, S., Yasasever, C. T. & Duranyildiz, D. Serum transforming growth factor-beta 1 (TGF-β1) levels have diagnostic, predictive, and possible prognostic roles in patients with melanoma. *Tumor Biol.* **35,** 7233–7237 (2014).
30. Sakaguchi, S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annual review of immunology* **22,** 531–562 (2004).
31. Viguier, M. *et al.* Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *Journal of immunology (Baltimore, Md. : 1950)* **173,** 1444–1453 (2004).

References

32. Sharma, S. *et al.* Tumor cyclooxygenase-2/prostaglandin E2-dependent promotion of FOXP3 expression and CD4+ CD25+ T regulatory cell activities in lung cancer. *Cancer research* **65,** 5211–5220 (2005).
33. Bates, G. J. *et al.* Quantification of regulatory T cells enables the identification of high-risk breast cancer patients and those at risk of late relapse. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **24,** 5373–5380 (2006).
34. Takenaka, M. *et al.* FOXP3 expression in tumor cells and tumor-infiltrating lymphocytes is associated with breast cancer prognosis. *Molecular and clinical oncology* **1,** 625–632 (2013).
35. Morse, M. A. *et al.* Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood* **112,** 610–618 (2008).
36. Ruter, J. *et al.* Altering regulatory T cell function in cancer immunotherapy. A novel means to boost the efficacy of cancer vaccines. *Frontiers in bioscience (Landmark edition)* **14,** 1761–1770 (2009).
37. Juang, C.-M. *et al.* Regulatory T Cells. Potential Target in Anticancer Immunotherapy. *Taiwanese Journal of Obstetrics and Gynecology* **46,** 215–221 (2007).
38. Gros, A. *et al.* Myeloid Cells Obtained from the Blood but Not from the Tumor Can Suppress T-cell Proliferation in Patients with Melanoma. *Clinical Cancer Research* **18,** 5212–5223 (2012).
39. Schouppe, E., van Overmeire, E., Laoui, D., Keirsse, J. & van Ginderachter, J. A. Modulation of CD8(+) T-cell activation events by monocytic and granulocytic myeloid-derived suppressor cells. *Immunobiology* **218,** 1385–1391 (2013).
40. Gabrilovich, D. I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. *Nature reviews. Immunology* **12,** 253–268 (2012).
41. Mantovani, A. The growing diversity and spectrum of action of myeloid-derived suppressor cells. *European journal of immunology* **40,** 3317–3320 (2010).

References

42. Poschke, I. & Kiessling, R. On the armament and appearances of human myeloid-derived suppressor cells. *Clinical Immunology* **144,** 250–268 (2012).
43. Talmadge, J. E. & Gabrilovich, D. I. History of myeloid-derived suppressor cells. *Nature reviews. Cancer* **13,** 739–752 (2013).
44. Weide, B. *et al.* Myeloid-derived suppressor cells predict survival of patients with advanced melanoma. Comparison with regulatory T cells and NY-ESO-1- or melan-A-specific T cells. *Clinical cancer research : an official journal of the American Association for Cancer Research* **20,** 1601–1609 (2014).
45. Martens, A. *et al.* Baseline Peripheral Blood Biomarkers Associated with Clinical Outcome of Advanced Melanoma Patients Treated with Ipilimumab. *Clinical cancer research : an official journal of the American Association for Cancer Research* **22,** 2908–2918 (2016).
46. Mao, Y. *et al.* Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms. *Cancer research* **73,** 3877–3887 (2013).
47. Mao, Y. *et al.* Inhibition of tumor-derived prostaglandin-e2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity. *Clinical cancer research : an official journal of the American Association for Cancer Research* **20,** 4096–4106 (2014).
48. Obermajer, N., Muthuswamy, R., Lesnock, J., Edwards, R. P. & Kalinski, P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood* **118,** 5498–5505 (2011).
49. Filipazzi, P. *et al.* Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **25,** 2546–2553 (2007).
50. Bayne, L. J. *et al.* Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. *Cancer cell* **21,** 822–835 (2012).

References

51. Lechner, M. G., Liebertz, D. J. & Epstein, A. L. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. *Journal of immunology (Baltimore, Md. : 1950)* **185,** 2273–2284 (2010).
52. Solito, S. *et al.* A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells. *Blood* **118,** 2254–2265 (2011).
53. Lechner, M. G. *et al.* Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines. *Journal of translational medicine* **9,** 90 (2011).
54. Bhowmick, N. A., Neilson, E. G. & Moses, H. L. Stromal fibroblasts in cancer initiation and progression. *Nature* **432,** 332–337 (2004).
55. Weber, C. E. & Kuo, P. C. The tumor microenvironment. *Surgical oncology* **21,** 172–177 (2012).
56. Angell, H. & Galon, J. From the immune contexture to the Immunoscore. The role of prognostic and predictive immune markers in cancer. *Current Opinion in Immunology* **25,** 261–267 (2013).
57. Fridman, W. H., Pagès, F., Sautès-Fridman, C. & Galon, J. The immune contexture in human tumours. Impact on clinical outcome. *Nature reviews. Cancer* **12,** 298–306 (2012).
58. Pagès, F. *et al.* Effector Memory T Cells, Early Metastasis, and Survival in Colorectal Cancer. *N Engl J Med* **353,** 2654–2666 (2005).
59. Zhang, L. *et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *The New England journal of medicine* **348,** 203–213 (2003).
60. Al-Shibli, K. I. *et al.* Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **14,** 5220–5227 (2008).
61. DeNardo, D. G. *et al.* Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer discovery* **1,** 54–67 (2011).

References

62. Galon, J. *et al.* Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science (New York, N.Y.)* **313,** 1960–1964 (2006).
63. Johansson, C. C. *et al.* Prognostic significance of tumor iNOS and COX-2 in *immunotherapy : CII* **58,** 1085–1094 (2009).
64. Wouters, M. *et al.* Interleukin-6 receptor and its ligand interleukin-6 are opposite markers for survival and infiltration with mature myeloid cells in ovarian cancer. *Oncoimmunology* **3,** e962397 (2014).
65. Tumeh, P. C. *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515,** 568–571 (2014).
66. Slamon, D. *et al.* Human breast cancer. Correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235,** 177–182 (1987).
67. Hillen, F. *et al.* Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. *Cancer immunology, immunotherapy : CII* **57,** 97–106 (2008).
68. Slamon, D. J. *et al.* Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *The New England journal of medicine* **344,** 783–792 (2001).
69. Hodi, F. S. *et al.* Improved survival with ipilimumab in patients with metastatic melanoma. *The New England journal of medicine* **363,** 711–723 (2010).
70. Brahmer, J. R. *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *The New England journal of medicine* **366,** 2455–2465 (2012).
71. Weber, J. S. *et al.* Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037). A randomised, controlled, open-label, phase 3 trial. *The Lancet. Oncology* **16,** 375–384 (2015).
72. Robert, C. *et al.* Nivolumab in Previously Untreated Melanoma without BRAF Mutation. *N Engl J Med* **372,** 320–330 (2015).

References

73. Combined Nivolumab and Ipilimumab or Monotherapy in Untreated Melanoma. *N Engl J Med* **373,** 1270–1271 (2015).
74. Brahmer, J. *et al.* Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *The New England journal of medicine* **373,** 123–135 (2015).
75. Motzer, R. J. *et al.* Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. *The New England journal of medicine* **373,** 1803–1813 (2015).
76. Belli, F. *et al.* Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes. Clinical and immunologic findings. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **20,** 4169–4180 (2002).
77. Coulie, P. G. *et al.* A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 10290–10295 (2001).
78. Schmidt, M. *et al.* Long-term prognostic significance of HER-2/neu in untreated node-negative breast cancer depends on the method of testing. *Breast cancer research : BCR* **7,** R256-66 (2005).
79. Bailur, J. K., Gueckel, B., Derhovanessian, E. & Pawelec, G. Presence of circulating Her2-reactive CD8 + T-cells is associated with lower frequencies of myeloid-derived suppressor cells and regulatory T cells, and better survival in older breast cancer patients. *Breast cancer research : BCR* **17,** 34 (2015).
80. Ladjemi, M. Z., Jacot, W., Chardès, T., Pèlegrin, A. & Navarro-Teulon, I. Anti-HER2 vaccines. New prospects for breast cancer therapy. *Cancer Immunol Immunother* **59,** 1295–1312 (2010).
81. Disis, M. L. *et al.* HER-2/neu vaccine-primed autologous T-cell infusions for the treatment of advanced stage HER-2/neu expressing cancers. *Cancer immunology, immunotherapy : CII* **63,** 101–109 (2014).
82. Mittendorf, E. A. *et al.* Clinical trial results of the HER-2/neu (E75) vaccine to prevent breast cancer recurrence in high-risk patients. From US Military

Cancer Institute Clinical Trials Group Study I-01 and I-02. *Cancer* **118,** 2594–2602 (2012).

83. Schneble, E. J. *et al.* The HER2 peptide nelipepimut-S (E75) vaccine (NeuVax™) in breast cancer patients at risk for recurrence. Correlation of immunologic data with clinical response. *Immunotherapy* **6,** 519–531 (2014).
84. Benavides, L. C. *et al.* The impact of HER2/neu expression level on response to the E75 vaccine. From U.S. Military Cancer Institute Clinical Trials Group Study I-01 and I-02. *Clinical Cancer Research* **15,** 2895–2904 (2009).
85. Yuan, J. *et al.* Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proceedings of the National Academy of Sciences of the United States of America* **108,** 16723–16728 (2011).
86. McGranahan, N. *et al.* Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science (New York, N.Y.)* **351,** 1463–1469 (2016).
87. Almand, B. *et al.* Increased Production of Immature Myeloid Cells in Cancer Patients. A Mechanism of Immunosuppression in Cancer. *The Journal of Immunology* **166,** 678–689 (2001).
88. Zea, A. H. *et al.* Arginase-producing myeloid suppressor cells in renal cell carcinoma patients. A mechanism of tumor evasion. *Cancer research* **65,** 3044–3048 (2005).
89. Diaz-Montero, C. M. *et al.* Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer immunology, immunotherapy : CII* **58,** 49–59 (2009).
90. Nagaraj, S. & Gabrilovich, D. I. Myeloid-derived suppressor cells in human cancer. *Cancer journal (Sudbury, Mass.)* **16,** 348–353 (2010).
91. Peper, J. K. & Stevanović, S. A combined approach of human leukocyte antigen ligandomics and immunogenicity analysis to improve peptide-based cancer immunotherapy. *Cancer immunology, immunotherapy : CII* **64,** 1295–1303 (2015).

References

92. Karanikas, V. *et al.* Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus. *The Journal of Immunology* **171,** 4898–4904 (2003).
93. Lonchay, C. *et al.* Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. *Proceedings of the National Academy of Sciences of the United States of America* **101 Suppl 2,** 14631–14638 (2004).
94. Whitesell, L. & Lindquist, S. L. HSP90 and the chaperoning of cancer. *Nat Rev Cancer* **5,** 761–772 (2005).
95. Brown, J. M. Tumor hypoxia in cancer therapy. *Methods in enzymology* **435,** 297–321 (2007).
96. Guillemin, K. & Krasnow, M. A. The hypoxic response. Huffing and HIFing. *Cell* **89,** 9–12 (1997).
97. Weinmann, M., Belka, C. & Plasswilm, L. Tumour hypoxia. Impact on biology, prognosis and treatment of solid malignant tumours. *Onkologie* **27,** 83–90 (2004).
98. Shipp, C., Derhovanessian, E. & Pawelec, G. Effect of culture at low oxygen tension on the expression of heat shock proteins in a panel of melanoma cell lines. *PloS one* **7,** e37475 (2012).
99. Nollen, E. A. A. & Morimoto, R. I. Chaperoning signaling pathways. Molecular chaperones as stress-sensing 'heat shock' proteins. *Journal of cell science* **115,** 2809–2816 (2002).
100. Al-Attar, A. et al. T cell density and location can influence the prognosis of ovarian cancer. Pathology oncology research : POR 16, 361–370 (2010).
101. Cai, X.-Y. *et al.* Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. *Journal of cancer research and clinical oncology* **132,** 293–301 (2006).
102. Nakano, O. *et al.* Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma. Clinicopathologic demonstration of antitumor immunity. *Cancer research* **61,** 5132–5136 (2001).

References

103. Ropponen, K. M., Eskelinen, M. J., Lipponen, P. K., Alhava, E. & Kosma, V. M. Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *The Journal of pathology* **182,** 318–324 (1997).
104. Setälä, L. P. *et al.* Prognostic factors in gastric cancer. The value of vascular invasion, mitotic rate and lymphoplasmacytic infiltration. *Br J Cancer* **74,** 766–772 (1996).
105. Simpson, J. A. D. *et al.* Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. *Gut* **59,** 926–933 (2010).
106. Vesalainen, S., Lipponen, P., Talja, M. & Syrjänen, K. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. *European journal of cancer (Oxford, England : 1990)* **30A,** 1797–1803 (1994).
107. Bogunovic, D. *et al.* Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proceedings of the National Academy of Sciences of the United States of America* **106,** 20429–20434 (2009).
108. Ladányi, A. *et al.* T-cell activation marker expression on tumor-infiltrating lymphocytes as prognostic factor in cutaneous malignant melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **10,** 521–530 (2004).
109. Weiss, S. A. *et al.* Immunologic heterogeneity of tumor-infiltrating lymphocyte composition in primary melanoma. *Human pathology* **57,** 116–125 (2016).
110. Gajewski, T. F. Failure at the Effector Phase. Immune Barriers at the Level of the Melanoma Tumor Microenvironment. *Clinical Cancer Research* **13,** 5256–5261 (2007).
111. Spranger, S. *et al.* Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Science translational medicine* **5,** 200ra116 (2013).
112. Romano, E. *et al.* Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients.

Proceedings of the National Academy of Sciences of the United States of America **112,** 6140–6145 (2015).

113. Andre, F., Berrada, N. & Desmedt, C. Implication of tumor microenvironment in the resistance to chemotherapy in breast cancer patients. *Current opinion in oncology* **22,** 547–551 (2010).
114. Denkert, C. *et al.* Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28,** 105–113 (2010).
115. Gajewski, T. F., Louahed, J. & Brichard, V. G. Gene signature in melanoma associated with clinical activity. A potential clue to unlock cancer immunotherapy. *Cancer journal (Sudbury, Mass.)* **16,** 399–403 (2010).
116. Halama, N. *et al.* Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer research* **71,** 5670–5677 (2011).
117. Hornychova, H. *et al.* Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. *Cancer investigation* **26,** 1024–1031 (2008).
118. Iwamoto, T. *et al.* Gene pathways associated with prognosis and chemotherapy sensitivity in molecular subtypes of breast cancer. *Journal of the National Cancer Institute* **103,** 264–272 (2011).
119. Wu, T. & Dai, Y. Tumor microenvironment and therapeutic response. *Cancer letters* **387,** 61–68 (2017).

11 Acknowledgements

“Every failure is a step to success. Every detection of what is false directs us towards what is true: every trial exhausts some tempting from error.” – William Whewell

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12 Curriculum vitae

13 Appendix

<u>Publication 1:</u> Peripheral T cell responses to tumour antigens are associated with molecular, immunogenetic and cellular features of breast cancer patients.

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Breast Cancer Res Treat. 2017 Jan;161(1):51-62. doi: 10.1007/s10549-016-4037-z. Epub 2016 Oct 27.

<u>Publication 2:</u> Inhibiting HSP90 prevents the induction of myeloid-derived suppressor cells by melanoma cells

Nicole Janssen[a*], Lisa Speigl[a], Graham Pawelec[a,b,c,d], Heike Niessner[e], Christopher Shipp[a,1]

Cell Immunol. 2018 May;327:68-76. doi: 10.1016/j.cellimm.2018.02.012. Epub 2018 Feb 21.

<u>Manuscript 1:</u> High levels of blood T cells identify breast cancer patients with HER2, MUC1 and SUR-reactive T cells.

Nicole Janssen[1], Lisa Speigl[1], Christoforos Haritos[2], Sotirios P. Fortis[2], Graham Pawelec[1,3,4,#] and Christopher Shipp[5,#] (under revision in Biomarkers)

<u>Manuscript 2:</u> Intra-tumoural immune features as prognostic markers in metastatic melanoma

Nicole Janssen[1,2], Alexandra Grieb[1], Benjamin Weide[3], Graham Pawelec,[1,4,5,6] and Christopher Shipp[1],[7,#] (in preparation)

Breast Cancer Res Treat
DOI 10.1007/s10549-016-4037-z

CLINICAL TRIAL

Peripheral T cell responses to tumour antigens are associated with molecular, immunogenetic and cellular features of breast cancer patients

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Abstract
Purpose Breast cancer is a leading cause of cancer deaths in women, but despite steady improvements in therapies, treatment is still suboptimal. Immunotherapy holds promise as a more effective therapy for breast cancer; supporting this, our prior study showed that patients possessing HER2-reactive CD8+ T cells in blood experience survival superior to patients without these cells. Here, we define a composite set of biomarkers that identify patients with T cell responses to tumour antigens.
Methods We assessed T cell responses following in vitro stimulation with the HER2, MUC1 and SUR tumour-associated antigens (TAA) by flow cytometry and intracellular cytokine staining in 50 breast cancer patients. We also measured HLA type, serum cytokines, tumour-infiltrating leukocytes and blood leukocyte populations.
Results We found few correlations between TAA-reactive T cells and HLA type, serum cytokines and tumour-infiltrating leukocytes, whereas blood leukocyte phenotypes broadly correlated with TAA responses. This showed monocytes, natural killer cells, dendritic cells and T cells to be inversely associated with both CD4+ and CD8+ T cells reactive to tumour antigens. Moreover, combining multiple parameters improved the accuracy in predicting patients with TAA-responsive T cells.
Conclusion This study therefore defines composite immune profiles that identify patients responding to TAAs which may allow better personalisation of cancer therapies.

Keywords Tumour-associated antigen · Blood leukocytes · Breast cancer · HER2 · Survivin · MUC1

Abbreviations

AJCC	American Joint Committee on Cancer
ER	Oestrogen receptor
FoxP3	Forkhead box P3
HER2	Human epidermal growth factor receptor 2
HLA	Human leukocyte antigen

Nicole Janssen and Sotirios P. Fortis have contributed equally to this work.

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IFNγ	Interferon gamma
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
mDCs	Myeloid dendritic cells
MDSCs	Myeloid-derived suppressor cells
MUC1	Mucin 1
N	N stage
NK	Natural killer
NR	Non-responder
PBMCs	Peripheral blood mononuclear cells
pDCs	Plasmacytoid dendritic cells
PR	Progesterone receptor
R	Responder
SI	Stimulation index
SUR	Survivin
T	Tumour stage
TAA	Tumour-associated antigens
TGF-β	Transforming growth factor-β
TNF	Tumour necrosis factor
Tregs	Regulatory T cells

Introduction

Breast cancer is the most common cancer in women (http://globocan.iarc.fr), but mainstay treatments are still suboptimal for most patients. To address this, there is a growing interest in using immunotherapy to treat breast cancer. Immunotherapy can take many forms including monoclonal antibodies targeting tumour antigens expressed on cancer cells, so-called checkpoint inhibitors, cancer vaccines or other treatments that aim to boost the immune system. Of these, immunomodulatory antibodies that target T cell suppressor ligands or receptors have shown highly encouraging results in several types of cancer [1–3]. The effectiveness of such treatments is presumably based on T cell immune responses against tumour-associated antigens (TAAs) expressed by cancer cells, allowing these cells to kill or otherwise inhibit tumour cells and induce tumour regression. Similarly, cancer vaccines also aim to prime or boost immune responses against tumour cells, and it has been shown that both of these approaches result in stimulated T cell responses and clinical tumour regression or prolonged patient survival [4–6].

One of the most widely investigated TAAs in breast cancer is the human epidermal growth factor receptor 2 (HER2). Our previous study showed that breast cancer patients whose peripheral blood mononuclear cells (PBMCs) mounted in vitro CD8+ T cell responses to HER2 peptides experienced prolonged survival relative to patients without such responses [7]. Consistent with this, HER2 vaccination has also resulted in clinical therapeutic responses [8, 9] and tumour regression [10]. These studies closely link T cells specific for TAAs with improvements in patient clinical status. As with most types of immune responses, TAA-reactive T cells are governed by immune regulatory factors such as regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs), which can dampen anti-tumour immune responses including those against TAAs [11–19]. Tregs and MDSCs induce immune suppression through a range of processes including cytolysis via granzyme B and perforin, production of inhibitory signalling molecules such as IL-10, transforming growth factor-β (TGF-β) or prostaglandin 2, or induction of T cell dysfunction through arginine depletion [20, 21]. This may offer the possibility of targeting these cells to enhance anti-tumour immunity [22]. Because many of these effector molecules are soluble, the activity of such regulatory cells may be reflected by systemic levels of suppressive factors that they produce. Indeed, serum cytokines have been associated with patient outcome [23, 24], supporting their potential as markers reflective of patient immune status.

Considering the emerging role of tumour antigen-reactive T cells and the results of our prior study which demonstrated a survival advantage for patients possessing T cells reactive to HER2, we aimed to pinpoint the patient features associated with T cell responses to tumour antigens in breast cancer. We reasoned that multiple different factors could potentially influence whether a patient possesses T cells responsive to tumour antigens and thus clinical outcome. To increase the predictive utility of such biomarkers, here, we measured broad features including immunogenetic factors, soluble signalling molecules in serum as well as cellular components in the blood and also within the tumour, with the goal of defining a composite set of biomarkers predictive of TAA responses which may better correlate with clinical outcome than tumour antigen immune responses alone, and which may also allow more effective personalised application of cancer therapies.

Materials and methods

Patients

Blood samples from 50 patients with invasive carcinoma were collected at the St. Savas Cancer Hospital in Athens between February 2014 and May 2015, one day prior to surgery. The study includes women with an age range of 27–78 (median age 56 years). Peripheral blood mononuclear cells were isolated from blood using Ficoll–Hypaque gradient and stored in liquid nitrogen before being shipped to the Tübingen University Hospital for analysis. Written informed consent was approved by the Review Board at St. Savas Cancer Hospital. The patients all had non-metastatic invasive ductal carcinoma. Patients who had tumours of

any size but without extension to chest wall were included, i.e. patients were all of AJCC stages 1, 2A, 2B, 3A, 3C but not 3B and 4. None of these patients were treated with neoadjuvant chemotherapy or were enrolled to other research protocols, nor did they have any history of cancer or other serious health problems. Tumour expression of hormone receptors [progesterone (PR) and oestrogen receptors (ER)], the HER2 oncoprotein and the marker of cell proliferation Ki67 was available for all patients (Table 1).

Detection of antigen-reactive T cells

T cell responses to HER2, Mucin1 (MUC1) and Survivin (SUR) were measured after 12 days of in vitro culture. This procedure, including the detection of reactive T cells with flow cytometry, was performed as in our prior study [7] (see Online Resource 1 for full list of antibodies employed). Cytometer setup and tracking beads (BD Bioscience) were run before and after each sample measurement to ensure consistency in machine performance. The inclusion of a positive biological control (stimulation with influenza peptides) assured consistency in sample quality and the prevention of false-negative results.

Table 1 Characteristics of the breast cancer patients

Patient clinicopathological parameters ($n = 50$)	
Median age (range in years)	56 (27–78)
AJCC staging	
1	15
2A	10
2B	12
3A	12
3C	1
Receptor status	
Triple negative	5
ER+	42
PR+	33
HER2+	15
Ki67	
<10%	7
10–20%	21
>20%	22
T stage	
1	21
2	26
3	3
N stage	
0	21
1	17
2	11
3	1
Grade	
1	0
2	26
3	24

Phenotypic analysis of myeloid cells, T cells and Natural Killer cells

For characterisation of myeloid cells [including monocytes, MDSCs, plasmacytoid Dendritic Cells (pDCs) and myeloid DCs (mDCs)], T cells (including regulatory T cells) and Natural Killer cells (NK cells), PBMCs were thawed and stained as previously described [7], using the antibody panels in Online Resource 1. Antibody panels were established using fluorescence minus one controls. Cytometer setup and tracking beads were run before and after each sample measurement to ensure consistency in machine performance. The limited nature of patient material did not permit multiple testing of the same sample, but we performed multiple independent measurement of a healthy control donor ($n = 9$) in order to ensure consistency in measurement conditions.

Flow cytometry data analysis

Data were analysed with FlowJo software version 10.07 (immunophenotyping) or version 7.2.5 (antigen-reactive T cells). Flow cytometry data were analysed first by excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate (Online Resource 2). The assessment of T cell responses to tumour-associated antigens was performed using the same method as in our prior study [7]; we compared control (unstimulated) and peptide-stimulated cultures as described in the methods section "Detection of antigen-reactive T cells" and assigned a positive response when the frequency of T cells producing any cytokine in the stimulated sample was at least twice that of the control sample (Online Resource 4). Additionally, each response was visually assessed to ensure the presence of a clearly distinguishable population of positive events. T cell responses were considered categorically (present or absent) in addition to a quantitative assessment of the strength of response by calculating the ratio of the frequency of positive events in the stimulated sample by comparison with the unstimulated sample to give a stimulation index (SI). This method allows the detection of multiple cytokines from each patient, but does not directly assess whether production is from the same or different population of

cells. Antibody-stained leukocyte populations, including the assessment of T cell responses to TAAs, were gated according to the approaches shown in Online Resources 2–4.

Assessment of tumour-infiltrating leukocytes

Haematoxylin–Eosin-stained tumour slides were reviewed by two breast pathologists (M.S. and N.A.) to select the most representative slide of each tumour. Sections (4–5 μm) were stained individually with antibodies to CD4 (4B12, 1:40; Biogenex), CD8 (SP16, 1:80; Thermo Scientific), CD163 (10D6, 1:400; Biocare) and FoxP3 (236A/E7, 1:100; Abcam). Staining protocols for all antibodies were optimised using sections of tonsil tissues. Immunostaining was performed using the Leica Bond III automation (Leica Biosystems, Melbourne, Australia) and Leica detection kit (Leica Biosystems, Newcastle, UK). The staining protocol included a 30-min high pH epitope retrieval in the case of CD4, CD163 and FoxP3 antibodies, and a low pH retrieval for CD8, which was followed by a 30-min incubation with the primary antibody. Reactions were developed with diaminobenzidine, and sections were counterstained with haematoxylin.

Micrographs (3840 × 3072 pixels) of each slide stained with CD4, CD8, FoxP3 and CD163 were captured with a Nikon DXM-1200 Camera on a Nikon Eclipse E800 microscope with E Plan Achromat Objectives using Automatic Camera Tamer (ACT-1) Version 2 software. White balance was calibrated before image capture. The images were saved as JPG at 95% quality without image processing. Quantification of infiltrating cells was performed using Adobe Photoshop CS6 (used to select the exact colour of positive cells for each marker which was then converted to a grey scale image) and ImageJ (used to analyse the percentage of the surface covered by the stained cells). The surface coverage of stained cells was then converted to absolute number of infiltrating cells per mm^2. For each infiltrating subpopulation, we performed visual enumeration in ten representative regions by two independent researchers.

Patient HLA typing

DNA from peripheral blood cells was extracted using the automated Maxwell® 16 Blood DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. HLA genotyping was performed using rSSO-Luminex (LIFECODES® HLA SSO Typing—RAPID, Immucor Transplant Diagnostics, INC, Stamford, CT, USA), and the results were analysed with the MATCH IT! DNA software (Immucor Transplant Diagnostics, INC, Stamford CT).

Measurement of serum cytokines

Frozen sera from patients were thawed at 37 °C and subsequently mixed well. Measurement of IL-1Ra, IL-9 and IL-10 was simultaneously performed by Luminex using the human premixed multi-analyte kit (R&D systems) according to manufacturer's instructions. RANTES/CC-chemokine ligand 5 and TGF-β determinations were performed by separate Luminex-based kits (R&D systems). The full panel of cytokines investigated additionally included IL-1beta, IL-2, IL-4, IL-5, IL-8, IL-12 p70, IL-15, IL-17A, TNF, IFNγ and GM-CSF, but levels of these cytokines could not be detected.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Two independent groups were compared using the Mann–Whitney *U* test. Relationships across four grouping variables were assessed with Fisher's exact test. Correlations were assessed 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. For this reason, statistical analyses were not corrected using the Bonferroni method, and the results should be interpreted as such.

Results

Breast cancer patients frequently show in vitro responses to HER2, MUC1 and SUR

T cells reactive to the HER2, MUC1 and SUR tumour antigens were detected in the PBMC of 50 non-metastatic breast cancer patients using an established 12 day in vitro expansion protocol. Reactive T cells were found to be common in patients, with a high frequency of responses to HER2 (96%), MUC1 (80%) and SUR (72%) (Fig. 1a). Examining CD4+ and CD8+ T cell responses separately showed that CD4+ reactive T cells were more common (Fig. 1b) and produced a larger number of different cytokines than CD8+ cells (Fig. 1c).

T cell responses to tumour antigens are associated with clinical parameters in breast cancer

We investigated if T cell responses to HER2, MUC1 and SUR were associated with patient clinical features. This analysis showed that patients with a higher tumour grade had weaker CD4+ T cell responses to MUC1 ($p = 0.045$)

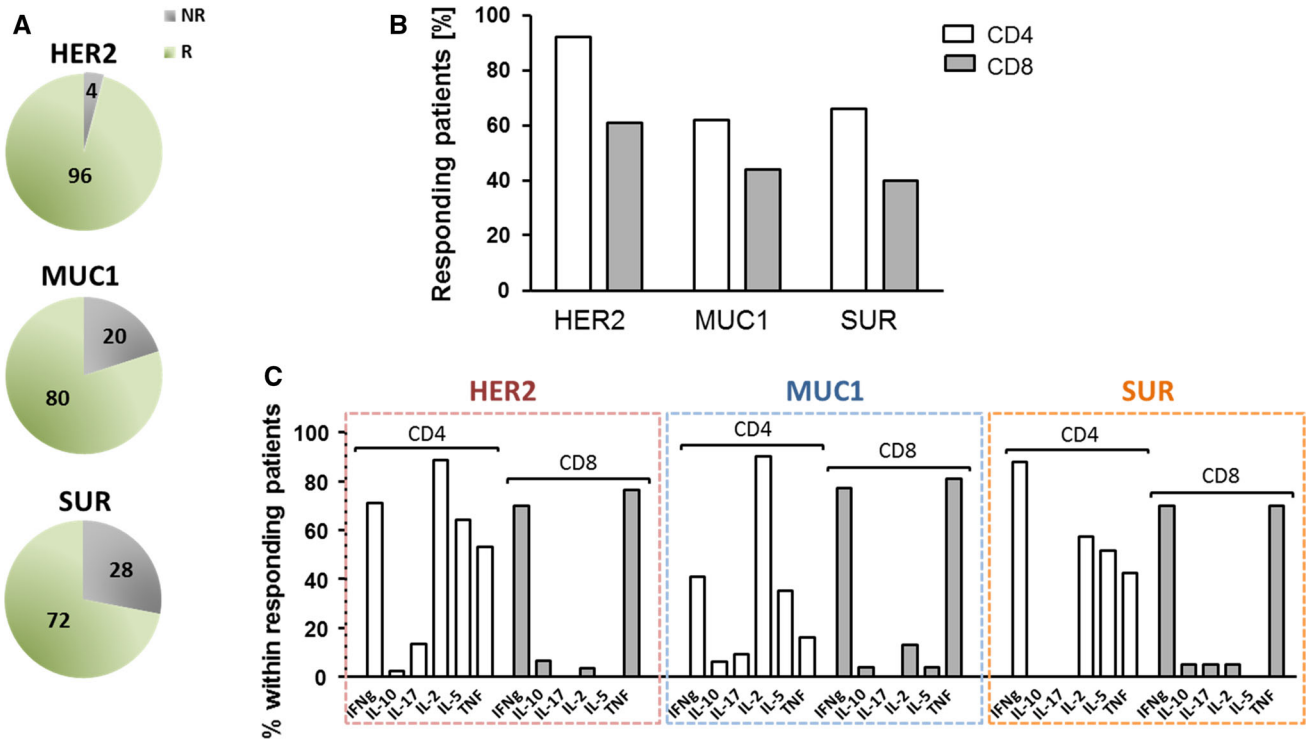

Fig. 1 T cell responses to tumour-associated antigens in breast cancer patients. CD4+ and CD8+ T cell responses measured by IL-2, IL-5, IL-10, IL-17, TNF and IFNγ production were detected in the PBMC of 50 breast cancer patients after stimulation with HER2, MUC1 or SUR TAAs. CD4+ and CD8+ T cells were considered responsive to an antigen if they showed a positive signal for any of the six measured cytokines. **a** Across these 50 breast cancer patients, HER2-reactive T cells were observed to be more common than MUC1- or SUR-reactive T cells. **b** Responses to all TAAs were more commonly seen for CD4+ than CD8+ T cells. **c** Differences in the cytokine production profile were observed for CD4+ but not for CD8+ T cells when comparing their responses to HER2, MUC1 and SUR. The percentage for each cytokine was calculated only considering patients who responded to HER2, MUC1 or SUR. *NR* non-responder; *R* Responder; *TAA* tumour-associated antigen; *HER2* human epidermal growth factor receptor 2; *MUC1* Mucin 1; *SUR* Survivin

(Fig. 2a), but those with oestrogen receptor (ER)-positive tumours had stronger CD8+ T cell responses to HER2 ($p = 0.031$) (Fig. 2b). No associations with SUR-responding T cells were found, nor were correlations with other clinical parameters including T, N or AJCC stage, total lymph node involvement as well as expression of Ki67, HER2 or progesterone receptor in the tumour.

Serum cytokines and patient HLA type are associated with T cell responses to TAAs

Immune features associated with responses to tumour antigens were sought in the form of serum cytokines and HLA type. We reasoned that a particular cytokine milieu or due to immunoediting, T cell responses to tumour antigens might be favoured or suppressed which might result in high frequencies of certain HLA types possessing TAA-reactive T cells. To this end, we measured serum levels of IL-1Ra, IL-9, IL-10, TGF-β1 and RANTES and performed HLA typing. We found that only in selected cases were these features related to responses to tumour antigens; a high proportion of HLA-A*01+ patients lacked T cells responsive to MUC1, whereas HLA-A*02+ patients were more likely to possess T cells reactive to this antigen than HLA-A*02-negative patients (Fig. 3a). Considering serum cytokines, we found higher serum IL-10 levels in patients who had CD8 HER2-(Fig. 3b) and SUR- ($p = 0.02$, data not shown) responsive T cells compared to those who did not have these cells. Aside from these associations no other relationships between T cell responses and serum cytokines or HLA type were found.

Relationships between tumour leukocyte infiltration and in vitro T cell responses to tumour antigens

Based on the notion that TAA-reactive T cells are active against tumour cells in situ, we investigated if they were associated with tumour leukocyte infiltration, namely by CD4+, CD8+, FoxP3+ and CD163+ cells, which may act as local regulators of tumour immunity. These analyses revealed that there was no association between tumour leukocyte infiltration and in vitro T cell responses to HER2

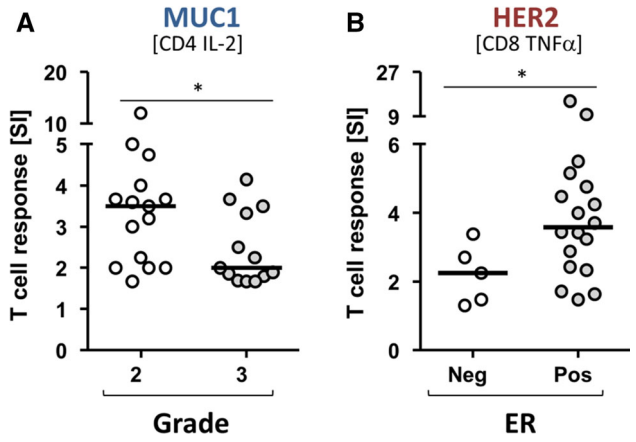

Fig. 2 T cell responses to MUC1 and HER2 correlate with tumour grade and ER receptor expression. T cell responses to the HER2, MUC1 or SUR TAAs were measured in 50 breast cancer patients following 12 days of in vitro expansion. The SI (ratio of cytokine positive cells between peptide-stimulated and unstimulated T cells) of responding CD4+ or CD8+ T cells were correlated with the patient clinical parameters grade, T, N, AJCC stage, total lymph node involvement as well as tumour expression of Ki67, HER2, ER and PR. **a** Patients with more advanced tumour grade showed weaker responses to MUC1. **b** ER-positive patients responded more strongly to HER2 than ER-negative patients. *SI* stimulation index; *ER* oestrogen receptor; *HER2* human epidermal growth factor receptor 2; *MUC1* mucin 1; $*p < 0.05$ (Mann–Whitney *U* test)

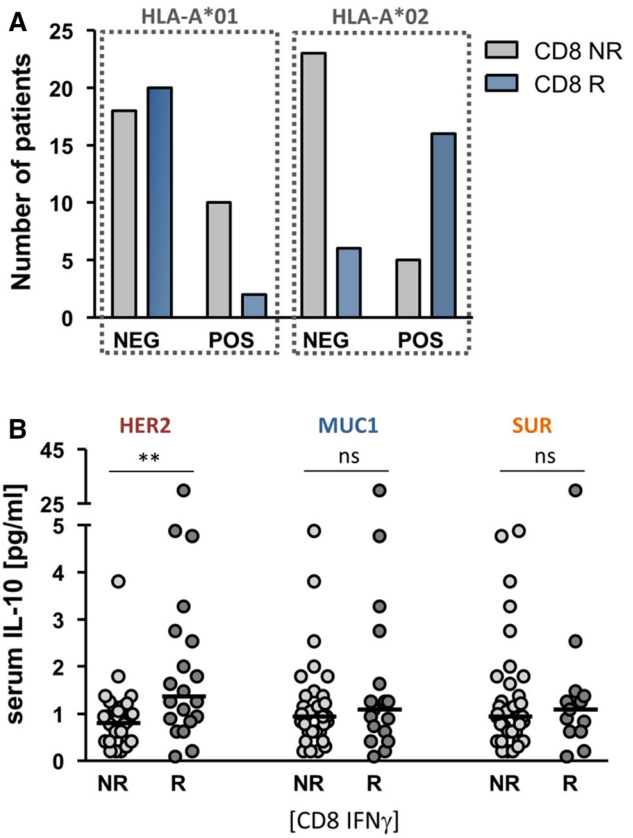

Fig. 3 Patient HLA type and serum cytokine levels correlate with T cell responses to tumour antigens. Patients with or without HER2-, MUC1- or SUR-reactive T cells were compared in terms of HLA type and serum cytokines ($n = 50$). **a** HLA-A*01 ($p = 0.045$) and HLA-A*02 ($p = 0.0001$) status was associated with MUC1-reactive T cells (Fisher's exact test). CD8+ T cells were considered responsive to an antigen if they showed a positive signal for any of the six measured cytokines. **b** Serum IL-10 levels were higher in patients who had IFNγ producing CD8 HER2-reactive T cells (Mann–Whitney *U* test) compared to patients without these cells. *R* responder; *NR* non-responder; $**p < 0.01$; *NS* not significant; *HER2* human epidermal growth factor receptor 2; *MUC1* mucin 1; *SUR* survivin

(Fig. 4a), but patients whose tumours were more densely infiltrated by CD163+ , CD4+ T cells (Fig. 4b) as well as combined cell counts for both CD4+ and CD8+ T cells (data not shown) more commonly possessed CD4+ T cells responding to the MUC1 antigen. Conversely, patients who showed less tumour infiltration by CD8+ T cells more commonly had CD8+ T cells responding to SUR (Fig. 4c). No other associations between TIL and antigen-reactive T cells were found.

Blood leukocyte populations as surrogate markers for anti-tumour T cell responses

The immunogenetic, molecular and cellular features investigated so far were only occasionally associated with in vitro T cell responses to tumour antigens, which led us to pursue more robust markers of tumour antigen responses in the form of blood leukocytes. To this end, 30 different myeloid and 23 lymphoid populations comprising T cells, monocytes, dendritic cells, natural killer cells, regulatory T cells and myeloid-derived suppressor cell phenotypes were tested for association with T cell responses to the HER2, MUC1 or SUR antigens (see Online Resource 5 for a complete list of tested phenotypes). We were particularly interested in exploring these relationships for leukocytes with suppressor phenotypes, namely Tregs and MDSCs. Furthermore, to account for potential location-dependent differences, we also re-tested a number of cell types that were investigated in tissue. This analysis revealed that many blood leukocyte populations were associated with T cell responses to HER2 and MUC1 and SUR. It was surprising that compared with phenotypically non-suppressive leukocyte populations, we found relatively few correlations for Tregs (two correlations) and MDSC phenotypes (nine correlations) (Table 2). Noteworthy was that patients with higher levels of blood monocytes presented with absent or weaker CD4+ and CD8+ T cells responsive to HER2 and MUC1 than patients with lower levels of monocytes. Similar inverse relationships were also observed for NK cells, mDCs and CD4+ and CD8+ T cells expressing markers of proliferation (Fig. 5), whereas other populations were positively associated with antigen-reactive T cells (Table 2). Interestingly, we observed that within monocytes, the HLA-DR+ fraction positively correlated with

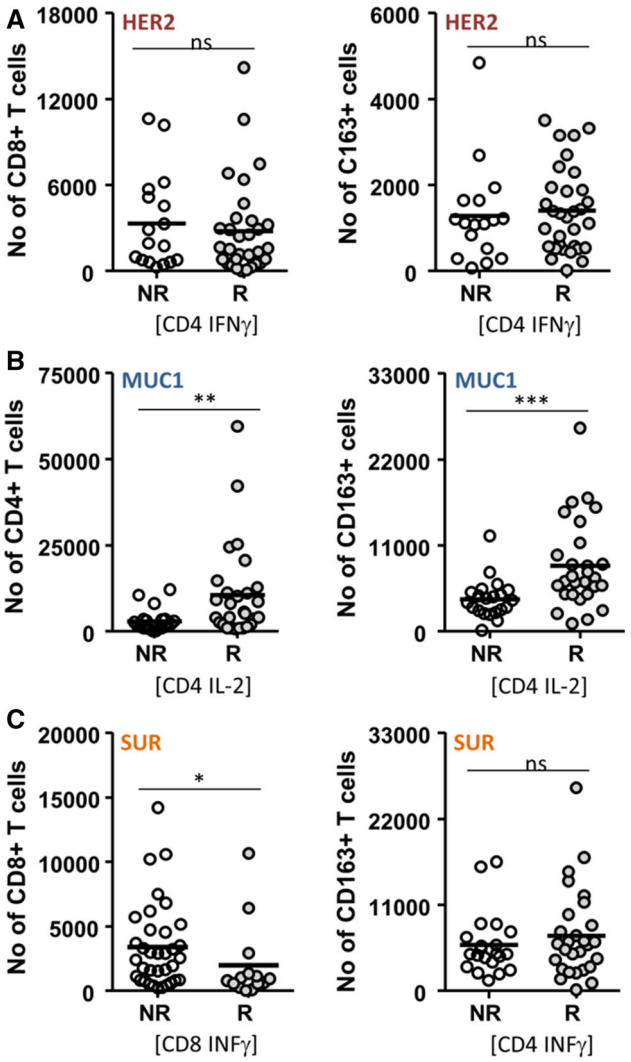

Fig. 4 Tumour-infiltrating leukocytes are associated with peripheral T cell responses to TAAs. The level of tumour leukocyte infiltration was compared for patients possessing T cells reactive to the HER2, MUC1 or SUR TAAs with those not possessing antigen-reactive T cells ($n = 50$). **a** Patients with HER2-responsive T cells showed no difference in their level of TILs. **b** Patients with MUC1-reactive T cells showed greater tumour infiltration by CD4+ T cells and CD163+ cells. **c** Patients with T cells reactive to the SUR antigen had less tumour infiltration by CD8+ T cells. Groups were divided according to patients who possessed the indicated T cell response and those who lacked these cells. *NR* non-responder; *R* responder; *TIL*, tumour-infiltrating leukocytes; $***p < 0.001$; $**p < 0.004$; $*p < 0.04$; *NS* not significant (Mann–Whitney *U* test); *TAA* tumour-associated antigen; *HER2* human epidermal growth factor receptor 2; *MUC1* mucin 1; *SUR* survivin

tumour antigen-reactive T cells, while the HLA-DR− fraction was negatively associated with TAA-reactive T cells, indicating that the balance between monocyte maturation states may be relevant for the presence of anti-tumour T cells. The location of these leukocytes, whether in tissue or in blood, was also relevant to their relationship with tumour antigen-reactive T cells; correlations for a particular cell type in blood were not found when the same cell type was present in tissue and vice versa (Fig. 4; Table 2), thereby demonstrating location-dependent differences. Figure 5 shows representative relationships between blood leukocyte frequencies and TAA T cell responses, and Table 2 provides an overview of all correlations identified. Similar relationships were obtained whether considering the strength of T cell response (stimulation index) or categorically comparing patients who had reactive T cells for each cytokine with those who did not.

Combining immune parameters improves the accuracy of predicting patients with TAA-reactive T cells

We sought to improve upon the accuracy of the parameters found to be associated with TAA-reactive T cells by analysing multiple immunological features. By combining different immune parameters, we were able to achieve superior prediction in the specificity and sensitivity in identifying patients possessing T cells reactive to HER2, MUC1 or SUR (Fig. 6a, selected results shown). Furthermore, these combinations of immune features allowed us to better stratify patients by the strength and frequency of T cell responses, i.e. into subgroups with stronger and more frequent T cell responses or with weaker and less frequent responses (Fig. 6b, selected results shown). For example, the group of patients with below median levels of monocytes and NK cells only contained HER2 responders, with an average SI of 14.5. In comparison, the group with above median levels of these cells was made up of roughly equal numbers of responding and non-responding patients and had an average SI of 2.3 (Fig. 6b, left panel). We observed that combining different types of immunological parameters, for example, tumour-infiltrating cells and blood leukocyte populations, also resulted in better prediction of TAA responses. Patients with high levels of tumour infiltration by T cells and low levels of CD16+ mDCs in peripheral blood were more likely to have T cells reactive to MUC1 compared with patients without this profile (Fig. 6b, middle panel).

Discussion

This study was conducted to identify composite immunological features including tumour-infiltrating leukocytes, HLA type as well as peripheral immune cell types and cytokines, with the goal of identifying biomarkers which can accurately select breast cancer patients with responses to tumour antigens, predict clinical outcome or facilitate improved therapeutic customisation to individual patients.

Table 2 Correlations between blood leukocytes and T cell responses to HER2, MUC1 and SUR

T cell response	Leukocyte population	*p* value	*r* value
CD4 IL-2	Monocytes	0.0001	−0.54
	NK cells	0.004	−0.42
	CD4+ Ki67+, CD8+ Ki67+	0.04, 0.02	−0.29, −0.33
CD4 IFN	Monocytes	0.0005	−0.49
	CD14+ CD124+	0.024	0.33
CD4 IL-5	Monocytes	0.0002	−0.53
	CD14+ CD124+	0.007	0.39
	mDCs	0.010	−0.37
	NK cells	0.026	−0.33
CD4 TNF	Monocytes	<0.0001	−0.60
	CD14+ CD124+	0.0009	0.47
	mDCs	0.045	−0.30
	NK cells	0.024	−0.33
CD8 TNF	Monocytes	0.038	−0.30
	pDCs	0.016	−0.35
CD4 IFN	CD14+ HLA-DR−	0.013	−0.36
	CD14+ CD124+	0.049	0.29
	CD4+ CD25+ FoxP3+ Ki67+ CD45RA+	0.033	0.33
CD4 IL-2	CD16+ mDCs	0.036	−0.31
CD4 IL-5	Monocytes	0.0005	−0.49
	CD14+ CD124+	0.006	0.40
	mDCs	0.027	−0.32
	CD8+ Ki67+, CD4+ Ki67+	0.002, 0.04	−0.42, −0.29
CD8 IFN	CD4+ CD25+ FoxP3+ Ki67+ CD45RA+	0.049	−0.31
	mDCs	0.028	−0.32
	CD4+, CD8+	0.015, 0.017	−0.34, 0.34
	ratio CD4/CD8	0.0093	−0.36
CD8 IL-2			
CD8 IL-2	Lin-CD14+ HLA-DR∓	<0.05, <0.05	−0.29, 0.29
CD8 TNF	Monocytes	0.02	−0.34
	mDCs, pDCs	0.024, 0.038	−0.33, −0.30
CD4 IL-2	CD8+ Ki67+	0.012	−0.35
CD8 IFN	Lin-CD14+ HLA-DR∓	0.022, 0.015	−0.34, 0.35
CD8 TNF	Lin-CD14+ HLA-DR∓	0.021, 0.017	−0.34, 0.35

Lin, lineage markers CD3 CD19 and CD56. Correlations were assessed using the SI

We assessed T cells responding to HER2, MUC1 or SUR in 50 breast cancer patients and found certain associations between T cell responses with HLA type, serum cytokines and tumour-infiltrating leukocytes. In contrast to these limited associations, a number of different leukocytes in blood, including monocytes, natural killer cells, dendritic cells and T cells broadly correlated with tumour antigen-reactive T cells. Moreover, accuracy in selecting patients with tumour antigen-reactive T cells was improved when considering multiple patient parameters. The cluster of biomarkers identified here reveal immune profiles correlating with the presence of TAA-reactive T cells that may allow for the selection of patients with immune systems most capable of mounting an anti-tumour response, thus potentially identifying patients who possess a wider repertoire of TAA-reactive T cells not limited to those which can be feasibly measured. Ongoing clinical follow-up of these prospectively recruited patients will reveal whether the composite set of biomarkers identified here, including TAA-reactive T cells, will allow superior survival prediction. Selecting patients using these biomarker combinations may allow personalised tailoring of patient treatments, for example, sparing patients with a favourable prognosis from otherwise unnecessary treatment, or the

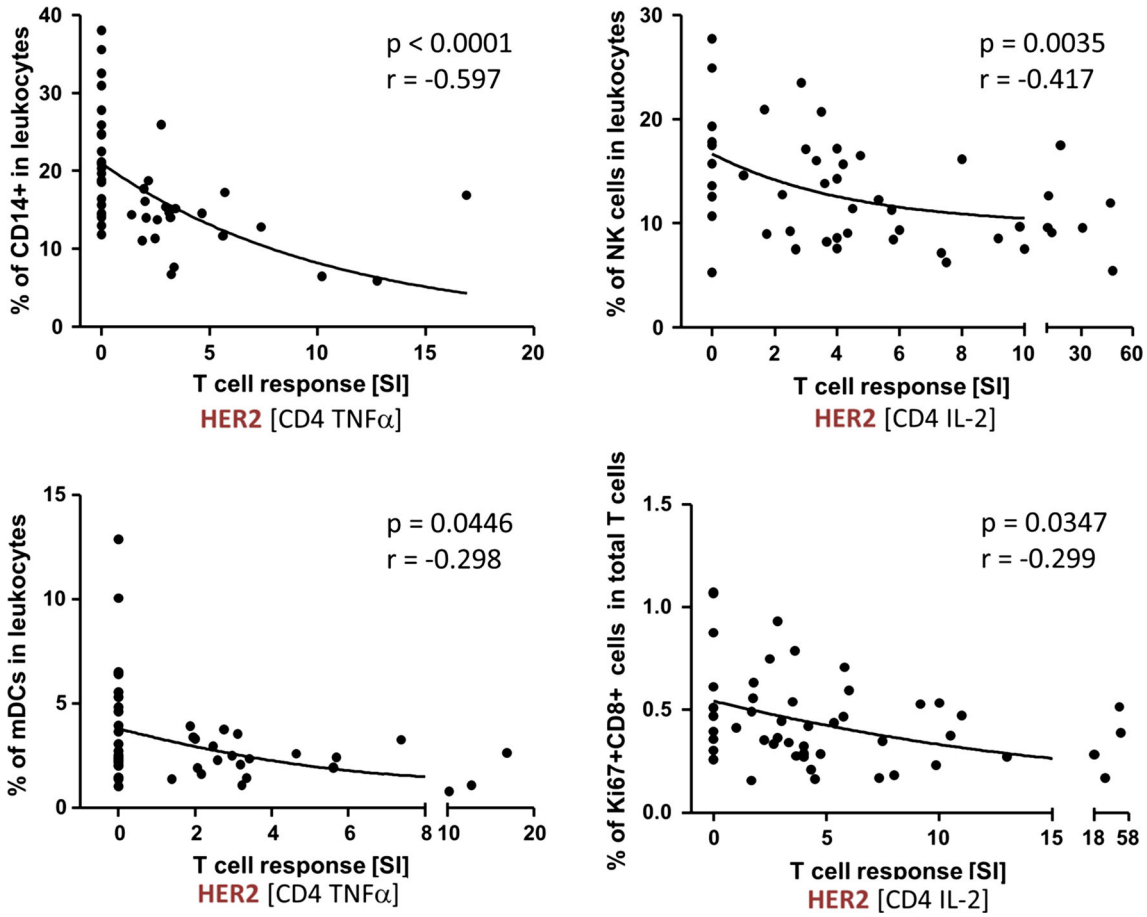

Fig. 5 Correlations between blood leukocytes and TAA-reactive T cells. Levels of human blood leukocytes correlate with T cells responding to the HER2, MUC1 or SUR TAAs ($n = 50$). Figure shows representative examples for monocytes, natural killer cells, myeloid dendritic cells and CD8+ T cells, which were inversely associated with TAA-reactive T cells (Spearman correlation). The SI (ratio of cytokine positive cells between peptide-stimulated and unstimulated T cells) of responding CD4+ or CD8+ T cells were correlated with the frequency of blood leukocytes. *TAA* tumour-associated antigen; *HER2* human epidermal growth factor receptor 2; *SI* stimulation index

administration of more aggressive therapies for patients with a poorer prognosis.

The results from this study may also have implications for the treatment of breast cancer patients with immunotherapies. The clinical benefit of immunomodulatory antibodies (so-called check point inhibitors) relies on the presence or emergence of anti-tumour T cells, suggesting that patients with pre-existing tumour immunity in the form of TAA-reactive T cells may be more likely to respond to these drugs [25, 26]. As such, the results presented here may provide a basis for the selection of breast cancer patients more likely to benefit from treatment with immunomodulatory antibodies. Furthermore, patients without TAA-reactive T cells but who show similar immune profiles to patients with them may also benefit from treatment with these drugs due to the existence of conditions more permissible to the presence TAA-reactive T cells. These results may also extend to the use of cancer vaccines, whereby patients who have immune profiles favourably associated with the presence of anti-tumour T cells may also be more likely to develop an immune response to the vaccine and to benefit clinically [5, 6, 27–29]. Furthermore, our results allow the selection of patients with antigen-reactive T cells producing specific cytokines. Because our prior study found that not only the general presence of TAA-reactive T cells but also the production of certain cytokines by these cells to be relevant for patient survival [7], the immune profiles identified here therefore allow a high degree of specificity in identifying those most likely to respond to immunotherapy or to have a favourable prognosis.

Despite numerous associations between blood leukocytes and TAA-reactive T cells, it was surprising that few correlations were found for populations of cells with suppressor phenotypes, namely Tregs and MDSCs. This suggests that cells corresponding to these putatively

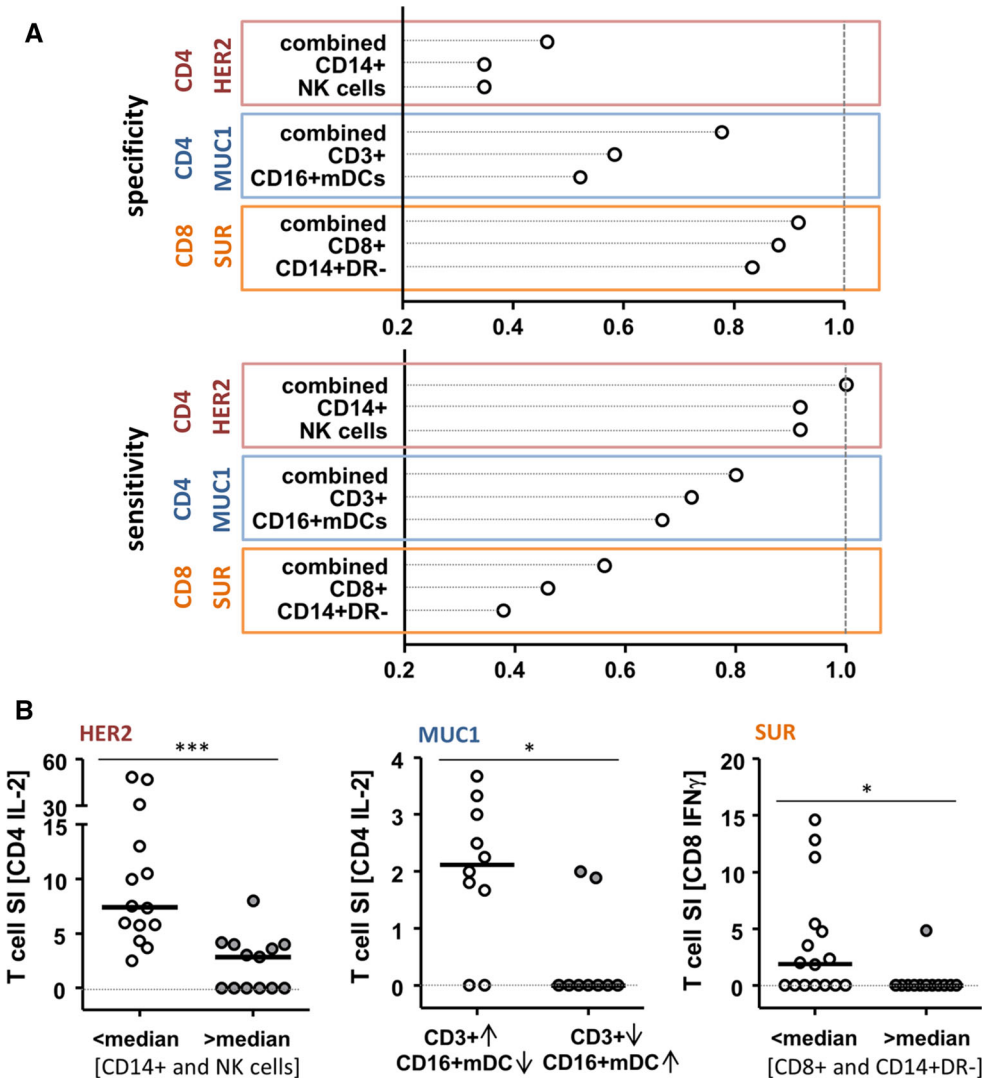

Fig. 6 Combining multiple immunological parameters improves the accuracy of predicting patients with anti-tumour reactive T cells. **a** Greater specificity and sensitivity in identifying patients with tumour antigen-reactive T cells was achieved by combining immune parameters. The sensitivity and specificity was calculated based on the presence or absence of the following T cells: CD8 IFNγ ("CD8 SUR"), CD4 IL-2 ("CD4 MUC1") and CD4 IL-2 ("CD4 HER2"). **b** Combining multiple immune parameters also allowed the sorting of patients into groups who have stronger or weaker T cell responses to HER2, MUC1 or SUR. Representative examples shown (Mann–Whitney *U* test). The SI was used to represent the strength of T cell responses to HER2, MUC2 or SUR. *SI* stimulation index; *HER2* human epidermal growth factor receptor 2; *MUC1* mucin 1; *SUR* survivin; $n = 50$; ***$p < 0.0005$; *$p < 0.02$

suppressive phenotypes are not suppressive in breast cancer patients, or if they are suppressive, that they do not exert suppression against antigen-reactive T cells in blood. However, the frequencies of a number of cell populations related to MDSCs and Tregs, namely monocytes and CD4+ T cells, were found to be inversely related to the presence of TAA-reactive T cells. These observations suggest that there are leukocyte populations in blood which suppress T cell responses to tumour antigens, but that they remain imperfectly defined on a phenotypic level. A precise set of phenotypic markers capable of accurately identifying suppressive cells may in turn allow more accurate biomarkers for the identification of patients with tumour antigen-responsive T cells, but such markers remain elusive [21]. One obstacle to the identification of such specific markers is highlighted by the observation in this study that there are differences in the association with TAA-reactive T cells and leukocyte populations that are dependent on the location of the cell population in question (whether in tissue or blood). This suggests that cellular location and phenotype are both relevant to the function of any given leukocyte population, and unless location-specific markers are found, may further limit the identification of marker proteins which accurately reflect cellular function.

Conclusion

This study identified composite sets of immune features which predict patients with TAA-reactive T cells. Considering that T cell responses to tumour antigens correlate with breast cancer patient survival, the biomarkers identified here which include HLA type, serum cytokines, tumour-infiltrating and blood leukocytes in addition to in vitro TAA-reactive T cells may allow superior prediction of patient survival or more personalised therapeutic management of breast cancer patients.

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Compliance with ethical standards

Conflict of interest Nicole Janssen, Sotirios P. Fortis, Lisa Speigl, Christoforos Haritos, Nectaria N. Sotiriadou, Michael Sofopoulos, Niki Arnogiannaki, Catherine Stavropoulos-Giokas, Amalia Dinou, Sonia Perez, Graham Pawelec, Constantin N. Baxevanis and Christopher Shipp 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.

Informed consent Informed consent was obtained from all individual participants included in the study.

Research involving animal and human rights This article does not contain any studies with animals performed by any of the authors

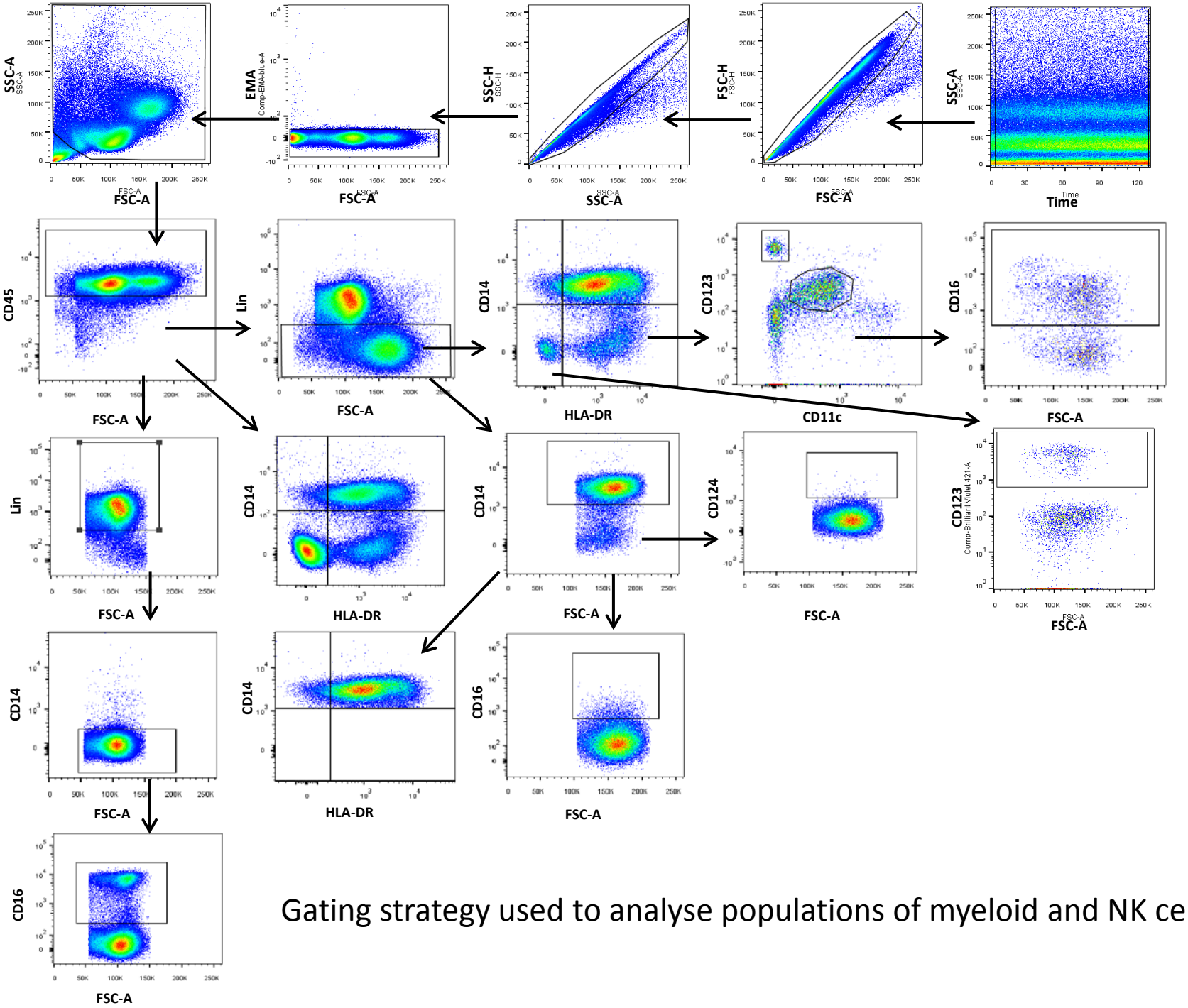
References

1. Ugurel S, Rohmel J, Ascierto PA, Flaherty KT, Grob JJ, Hauschild A, Larkin J, Long GV, Lorigan P, McArthur GA, Ribas A, Robert C, Schadendorf D, Garbe C (2016) Survival of patients with advanced metastatic melanoma: the impact of novel therapies. Eur J Cancer 53:125–134. doi:10.1016/j.ejca.2015.09.013
2. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, Barlesi F, Kohlhaufl M, Arrieta O, Burgio MA, Fayette J, Lena H, Poddubskaya E, Gerber DE, Gettinger SN, Rudin CM, Rizvi N, Crino L, Blumenschein GR Jr, Antonia SJ, Dorange C, Harbison CT, Graf Finckenstein F, Brahmer JR (2015) Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med 373:1627–1639. doi:10.1056/NEJMoa1507643
3. Weber JS, D'Angelo SP, Minor D, Hodi FS, Gutzmer R, Neyns B, Hoeller C, Khushalani NI, Miller WH Jr, Lao CD, Linette GP, Thomas L, Lorigan P, Grossmann KF, Hassel JC, Maio M, Sznol M, Ascierto PA, Mohr P, Chmielowski B, Bryce A, Svane IM, Grob JJ, Krackhardt AM, Horak C, Lambert A, Yang AS, Larkin J (2015) Nivolumab versus chemotherapy in patients with advanced melanoma who progressed after anti-CTLA-4 treatment (CheckMate 037): a randomised, controlled, open-label, phase 3 trial. Lancet Oncol 16:375–384. doi:10.1016/S1470-2045(15)70076-8
4. Postow MA, Chesney J, Pavlick AC, Robert C, Grossmann K, McDermott D, Linette GP, Meyer N, Giguere JK, Agarwala SS, Shaheen M, Ernstoff MS, Minor D, Salama AK, Taylor M, Ott PA, Rollin LM, Horak C, Gagnier P, Wolchok JD, Hodi FS (2015) Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. N Engl J Med 372:2006–2017. doi:10.1056/NEJMoa1414428
5. Belli F, Testori A, Rivoltini L, Maio M, Andreola G, Sertoli MR, Gallino G, Piris A, Cattelan A, Lazzari I, Carrabba M, Scita G, Santantonio C, Pilla L, Tragni G, Lombardo C, Arienti F, Marchiano A, Queirolo P, Bertolini F, Cova A, Lamaj E, Ascani L, Camerini R, Corsi M, Cascinelli N, Lewis JJ, Srivastava P, Parmiani G (2002) Vaccination of metastatic melanoma patients with autologous tumor-derived heat shock protein gp96-peptide complexes: clinical and immunologic findings. J Clin Oncol 20:4169–4180
6. Coulie PG, Karanikas V, Colau D, Lurquin C, Landry C, Marchand M, Dorval T, Brichard V, Boon T (2001) A monoclonal cytolytic T-lymphocyte response observed in a melanoma patient vaccinated with a tumor-specific antigenic peptide encoded by gene MAGE-3. Proc Natl Acad Sci USA 98:10290–10295. doi:10.1073/pnas.161260098
7. Bailur JK, Gueckel B, Derhovanessian E, Pawelec G (2015) Presence of circulating Her2-reactive CD8+ T-cells is associated with lower frequencies of myeloid-derived suppressor cells and regulatory T cells, and better survival in older breast cancer patients. Breast Cancer Res 17:34. doi:10.1186/s13058-015-0541-z
8. Peoples GE, Gurney JM, Hueman MT, Woll MM, Ryan GB, Storrer CE, Fisher C, Shriver CD, Ioannides CG, Ponniah S (2005) Clinical trial results of a HER2/neu (E75) vaccine to prevent recurrence in high-risk breast cancer patients. J Clin Oncol 23:7536–7545. doi:10.1200/JCO.2005.03.047
9. Disis ML, Dang Y, Coveler AL, Marzbani E, Kou ZC, Childs JS, Fintak P, Higgins DM, Reichow J, Waisman J, Salazar LG (2014) HER-2/neu vaccine-primed autologous T-cell infusions for the treatment of advanced stage HER-2/neu expressing cancers. Cancer Immunol Immunother 63:101–109. doi:10.1007/s00262-013-1489-4
10. Mittendorf EA, Clifton GT, Holmes JP, Schneble E, van Echo D, Ponniah S, Peoples GE (2014) Final report of the phase I/II clinical trial of the E75 (nelipepimut-S) vaccine with booster inoculations to prevent disease recurrence in high-risk breast cancer patients. Ann Oncol 25:1735–1742. doi:10.1093/annonc/mdu211
11. Bonertz A, Weitz J, Pietsch DH, Rahbari NN, Schlude C, Ge Y, Juenger S, Vlodavsky I, Khazaie K, Jaeger D, Reissfelder C, Antolovic D, Aigner M, Koch M, Beckhove P (2009) Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. J Clin Invest 119:3311–3321. doi:10.1172/JCI39608
12. Horn T, Grab J, Schusdziarra J, Schmid S, Maurer T, Nawroth R, Wolf P, Pritsch M, Gschwend JE, Kubler HR, Beckhove P (2013) Antitumor T cell responses in bladder cancer are directed against a limited set of antigens and are modulated by regulatory T cells and routine treatment approaches. Int J Cancer 133:2145–2156. doi:10.1002/ijc.28233
13. Hadaschik B, Su Y, Huter E, Ge Y, Hohenfellner M, Beckhove P (2012) Antigen specific T-cell responses against tumor antigens are controlled by regulatory T cells in patients with prostate cancer. J Urol 187:1458–1465. doi:10.1016/j.juro.2011.11.083
14. Schmidt HH, Ge Y, Hartmann FJ, Conrad H, Klug F, Nittel S, Bernhard H, Domschke C, Schuetz F, Sohn C, Beckhove P (2013) HLA Class II tetramers reveal tissue-specific regulatory T cells that suppress T-cell responses in breast carcinoma patients. Oncoimmunology 2:e24962. doi:10.4161/onci.24962
15. Brudvik KW, Henjum K, Aandahl EM, Bjornbeth BA, Tasken K (2012) Regulatory T-cell-mediated inhibition of antitumor immune responses is associated with clinical outcome in patients with liver metastasis from colorectal cancer. Cancer Immunol Immunother 61:1045–1053. doi:10.1007/s00262-011-1174-4
16. Weed DT, Vella JL, Reis IM, De la Fuente AC, Gomez C, Sargi Z, Nazarian R, Califano J, Borrello I, Serafini P (2015) Tadalafil reduces myeloid-derived suppressor cells and regulatory T cells and promotes tumor immunity in patients with head and neck squamous cell carcinoma. Clin Cancer Res 21:39–48. doi:10.1158/1078-0432.CCR-14-1711
17. Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC, Carbone DP, Gabrilovich DI (2001) Increased

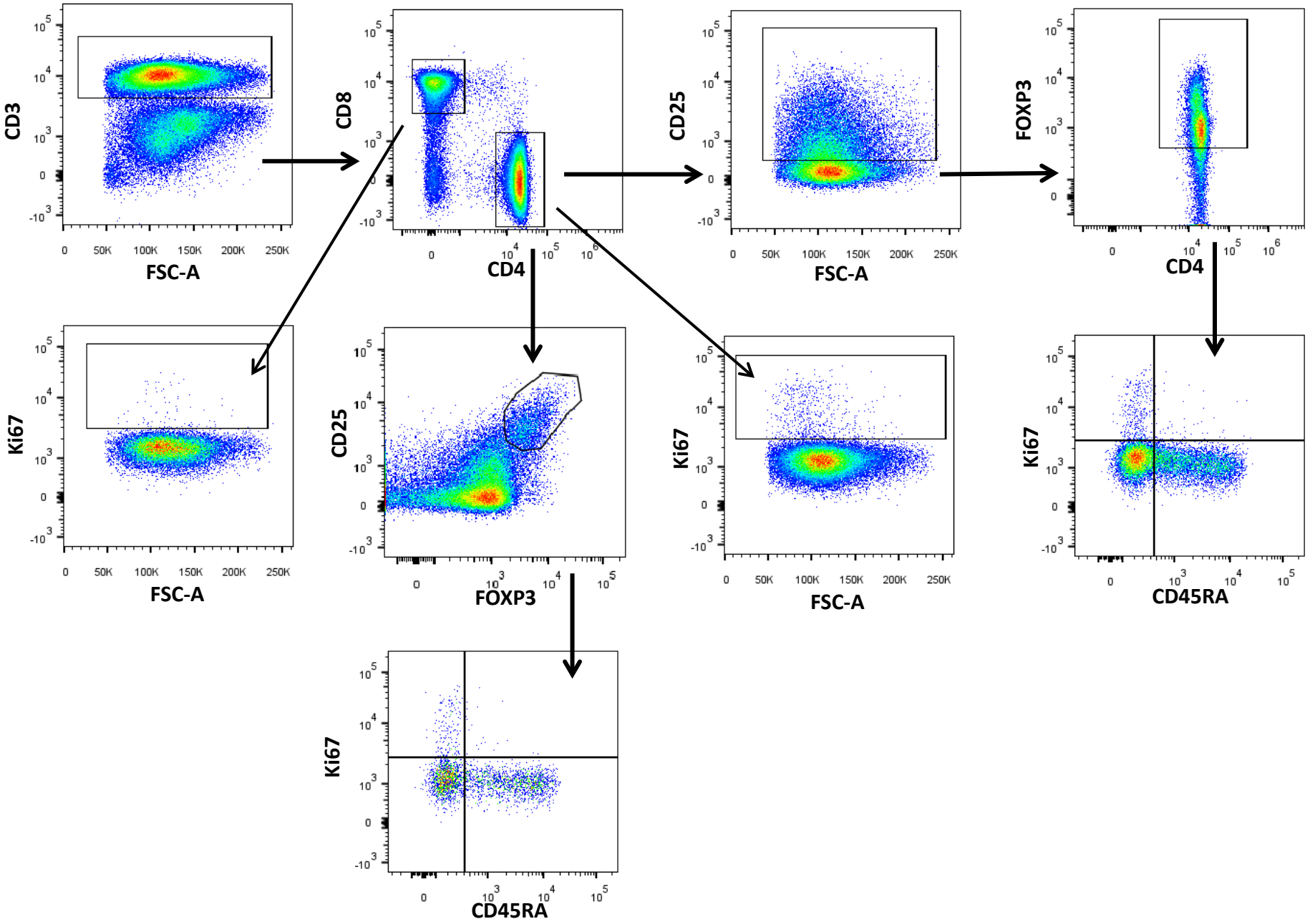
production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol 166:678–689
18. Solito S, Bronte V, Mandruzzato S (2011) Antigen specificity of immune suppression by myeloid-derived suppressor cells. J Leukoc Biol 90:31–36. doi:10.1189/jlb.0111021
19. Messmer MN, Netherby CS, Banik D, Abrams SI (2015) Tumor-induced myeloid dysfunction and its implications for cancer immunotherapy. Cancer Immunol Immunother 64:1–13. doi:10.1007/s00262-014-1639-3
20. Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. Nat Rev Immunol 8:523–532. doi:10.1038/nri2343
21. Shipp C, Speigl L, Janssen N, Martens A, Pawelec G (2016) A clinical and biological perspective of human myeloid-derived suppressor cells in cancer. Cell Mol Life Sci. doi:10.1007/s00018-016-2278-y
22. Castelli C, Rivoltini L, Rodolfo M, Tazzari M, Belgiovine C, Allavena P (2015) Modulation of the myeloid compartment of the immune system by angiogenic- and kinase inhibitor-targeted anticancer therapies. Cancer Immunol Immunother 64:83–89. doi:10.1007/s00262-014-1576-1
23. Vayrynen JP, Kantola T, Vayrynen SA, Klintrup K, Bloigu R, Karhu T, Makela J, Herzig KH, Karttunen TJ, Tuomisto A, Makinen MJ (2016) The relationships between serum cytokine levels and tumor infiltrating immune cells and their clinical significance in colorectal cancer. Int J Cancer 139:112–121. doi:10.1002/ijc.30040
24. Torres C, Linares A, Alejandre MJ, Palomino-Morales RJ, Caba O, Prados J, Aranega A, Delgado JR, Irigoyen A, Martinez-Galan J, Ortuno FM, Rojas I, Perales S (2015) Prognosis relevance of serum cytokines in pancreatic cancer. Biomed Res Int 2015:518284. doi:10.1155/2015/518284
25. McGranahan N, Furness AJ, Rosenthal R, Ramskov S, Lyngaa R, Saini SK, Jamal-Hanjani M, Wilson GA, Birkbak NJ, Hiley CT, Watkins TB, Shafi S, Murugaesu N, Mitter R, Akarca AU, Linares J, Marafioti T, Henry JY, Van Allen EM, Miao D, Schilling B, Schadendorf D, Garraway LA, Makarov V, Rizvi NA, Snyder A, Hellmann MD, Merghoub T, Wolchok JD, Shukla SA, Wu CJ, Peggs KS, Chan TA, Hadrup SR, Quezada SA, Swanton C (2016) Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. Science 351:1463–1469. doi:10.1126/science.aaf1490
26. Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, Xu Y, Pogoriler E, Terzulli SL, Kuk D, Panageas KS, Ritter G, Sznol M, Halaban R, Jungbluth AA, Allison JP, Old LJ, Wolchok JD, Gnjatic S (2011) Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. Proc Natl Acad Sci USA 108:16723–16728. doi:10.1073/pnas.1110814108
27. Lonchay C, van der Bruggen P, Connerotte T, Hanagiri T, Coulie P, Colau D, Lucas S, Van Pel A, Thielemans K, van Baren N, Boon T (2004) Correlation between tumor regression and T cell responses in melanoma patients vaccinated with a MAGE antigen. Proc Natl Acad Sci USA 101(Suppl 2):14631–14638. doi:10.1073/pnas.0405743101
28. Karanikas V, Lurquin C, Colau D, van Baren N, De Smet C, Lethe B, Connerotte T, Corbiere V, Demoitie MA, Lienard D, Dreno B, Velu T, Boon T, Coulie PG (2003) Monoclonal anti-MAGE-3 CTL responses in melanoma patients displaying tumor regression after vaccination with a recombinant canarypox virus. J Immunol 171:4898–4904
29. Peper JK, Stevanovic S (2015) A combined approach of human leukocyte antigen ligandomics and immunogenicity analysis to improve peptide-based cancer immunotherapy. Cancer Immunol Immunother 64:1295–1303. doi:10.1007/s00262-015-1682-8

TAA reactive T cells	
Cell surface	
CD3-BV605	BioLegend (San Diego, CA, USA)
CD4-Pacific Blue	BioLegend
CD8-APC-H7	BD Biosciences (Frahnklin Lakes, NJ, USA)
Intracellullar	
IL-2- Alexa Flour 700	BioLegend
IL-5-PE	BioLegend
IL-10-APC	Miltenyi Biotec (Bergisch Gladbach, Germany)
IL-17-PerCP-Cy5.5	eBioscience (SanDiego, CA, USA)
IFNγ-PECy7	BD Biosciences
TNF-FITC	BioLegend
Myeloid cells and NK cells	
Cell surface	
CD14-BV711	BioLegend
CD123-BV421	BD Horizon
CD45-V500	BD Horizon
HLA-DR-PerCP-Cy5.5	BD Pharmingen
CD11c-PECy7	BioLegend
CD124-APC	R&D Systems (Minneapolis, MN, USA)
CD86-PE	BioLegend
CD16-FITC	BioLegend
Lineage cocktail containing: CD3-,CD19- and CD56-BV605	BioLegend, BD Horizon
T cells	
Cell surface	
CD45RA-BV421	BioLegend
CD103-BV711	BD Horizon
CD25-PE	BD Pharmingen
CD4-PECy7	BioLegend
CD8-APC-H7	BD Pharmingen
Intracellullar	
CD3- Alexa Flour 700	BD Pharmingen
Ki67-FITC	eBioscience
FoxP3- Alexa Flour 647	BD Pharmingen

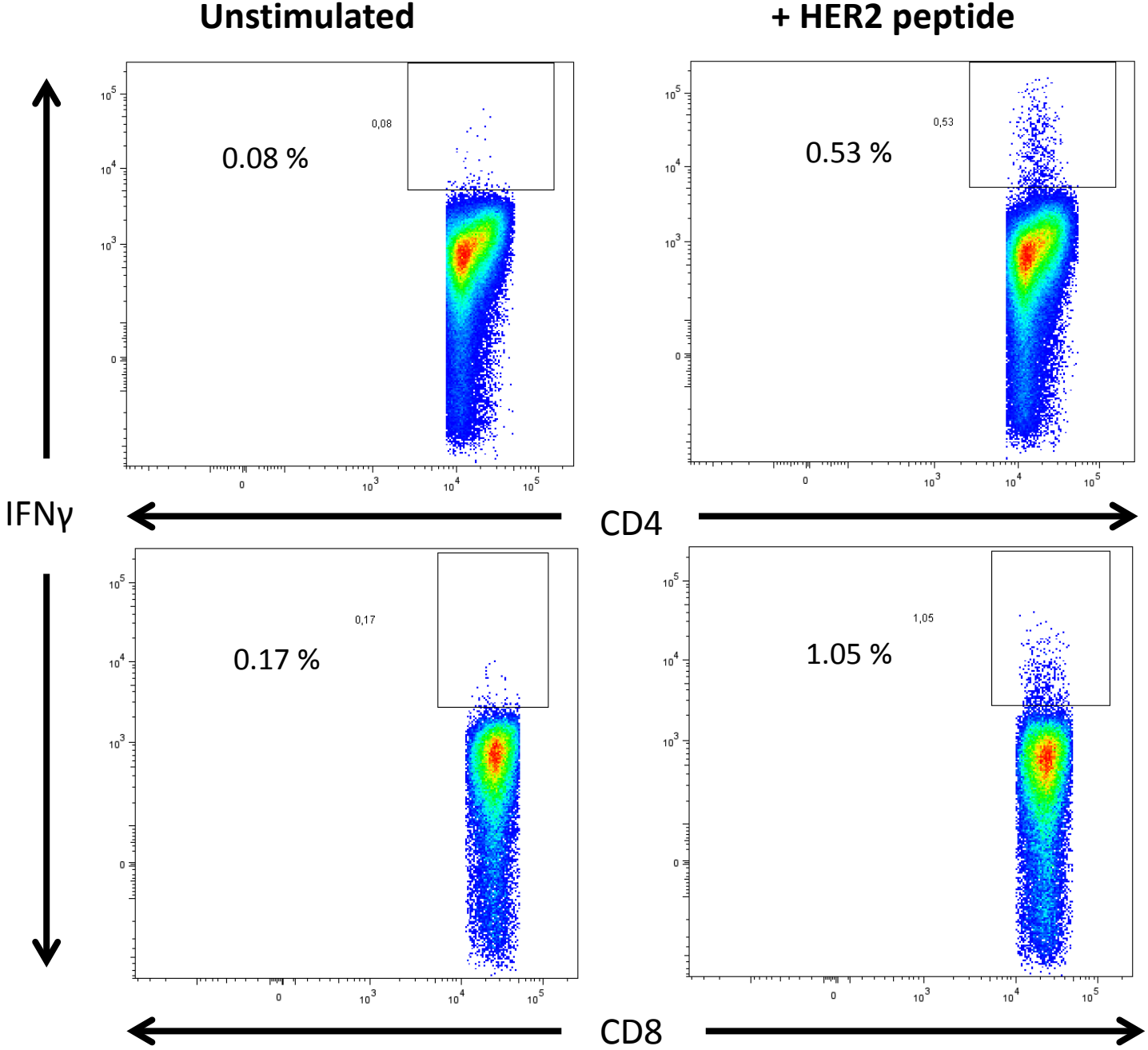
"Cell surface" refers to antibodies applied prior to cell permeabilisation and "intracellular" indicates those added following permeabilisation.



Gating strategy used to analyse populations of myeloid and NK cells



Gating strategy used to assess populations of lymphocytes including regulatory T cells



Control (left panels) and stimulated (right panels) CD4 (upper panels) and CD8 (lower panels) example showing a positive response for IFNγ to HER2

Myeloid phenotypes	Lymphocyte phenotypes
CD14+	CD3+CD4+
CD14+HLA-DR-	CD4+CD25+
Lin-CD14+	CD4+CD25+FoxP3+
Lin-CD14+HLA-DR-	CD4+CD25+FoxP3+Ki67+/-CD45RA+/-
CD14+CD16+	CD4+Ki67+
CD14+CD124+	CD3+CD8+
Lin-CD14-HLA-DR+CD123+	CD8+Ki67
Lin-CD14-HLA-DR-CD123+	Lin+CD16+
Lin-CD14-HLA-DR+CD11c+	
Lin-CD14-HLA-DR+CD11c+CD16+	

Leukocyte populations measured in peripheral blood of breast cancer patients

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Research paper

Inhibiting HSP90 prevents the induction of myeloid-derived suppressor cells by melanoma cells

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ABSTRACT

Metastatic melanoma is the most dangerous form of skin cancer, with an ever-increasing incidence worldwide. Despite encouraging results with immunotherapeutic approaches, long-term survival is still poor. This is likely partly due to tumour-induced immune suppression mediated by myeloid-derived suppressor cells (MDSCs), which were shown to be associated with response to therapy and survival. Thus, identifying pathways responsible for MDSC differentiation may provide new therapeutic targets and improve efficacy of existing immunotherapies. Therefore, we've analysed mechanisms by which tumour cells contribute to the induction of MDSCs. Established melanoma cell lines were pre-treated with inhibitors of different pathways and tested for their capacity to alleviate T cell suppression via MDSC differentiation *in vitro*. Targeting HSP70/90 in melanoma cells resulted in reduced induction of immune suppressive cells on a phenotypic and functional basis, for which a more potent effect was observed when HSP90 was inhibited under hypoxic conditions. This initial study suggests a novel mechanism in tumour cells responsible for the induction of MDSC in melanoma.

1. Introduction

Melanoma is the most dangerous form of skin cancer with an ever increasing incidence worldwide. Despite the recent introduction of promising immunotherapies, the majority of patients with metastatic disease still face a poor prognosis. Relapse or non-response to these drugs is likely or at least partly due to tumour-induced immune suppression, mediated by myeloid derived suppressor cells (MDSCs) and regulatory T cells. MDSCs in particular represent one of the major barriers preventing effective cancer treatment. These cells dampen beneficial anti-tumour immune responses by both innate and adaptive immune cells [1,2]. Supporting their relevance for cancer immunotherapy, MDSCs are elevated in the blood of melanoma patients compared with healthy individuals [3,4], and patients with high relative levels experience shortened survival [5,6] and respond more poorly to immunotherapy with the anti-CTLA-4 antibody ipilimumab [7]. In addition to *in vitro* studies which show that melanoma-induced MDSCs suppress activated T and NK cells [1,2], a lower proportion of melanoma patients with high MDSC levels exhibits anti-tumour antigen T cell reactivity [8]. That *in vitro* model suggested that the negative association between high MDSC levels and patient prognosis may be through suppression of tumour immunity. Several mechanism of MDSC-mediated immnunosuppresion have already been identified including the induction of regulatory T cells, impairing NK cell function [9] and inhibition of T cell activation [10–12]. On a molecular level, T cell suppression by MDSCs can occur through a variety of secreted factors such as reactive oxygen species (ROS), arginase and iNOS, TGFb and indoleamine 2,3.dioxygenase (IDO) [10–12].

Factors that are involved in the expansion and activation of MDSCs, in addition to cell-cell contact, could either be secreted by tumour cells themselves, or by stromal cells present in the tumour microenvironment, such as activated T cells. These interactions have been shown to result in an elevation of MDSCs in melanoma patients compared with healthy individuals [3,4]. These observations are complemented by *in vitro* co-culture experiments with tumour cell lines which show the induction of MDSC-like cells from healthy human monocytes mediated by tumour cells [13]. Although several soluble factors including GM-CSF, IL-6 and PGE2 have been shown to be involved in the differentiation of monocytes into suppressive MDSCs [2,3,14–18], the precise molecular pathways responsible for their differentiation as well as

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pathways in tumour cells responsible for this remain to be elucidated. The identification of these mechanisms could reveal novel therapeutic targets because they contribute to the maintenance of immunosuppressive pathways. As such, MDSCs have been proposed as a major barrier to effective immunotherapy and thus drugs which aim to affect tumour cells and block their ability to cause differentiation of myeloid cells into MDSCs may improve the efficacy of other forms of immunotherapy. Notably, both Obermayer et al. and Mao et al. demonstrated the importance of COX2/PGE2 and STAT3 in the differentiation and suppressive function of MDSCs [1,2,14]. Treating T cell/monocyte cultures with inhibitors targeting the COX2/PGE2 and STAT3 pathway resulted in alleviation of T cell suppression by such melanoma-induced MDSC-like cells differentiated from normal monocytes. Those studies focused on the immune-suppressive pathways in monocytes, whereas the present study was designed to better understand the molecular pathways in tumour cells responsible for re-programming normal monocytes to become suppressive MDSCs. Furthermore, few prior studies have addressed physical characteristics of the tumour microenvironment that might influence these processes. The tumour mass has distinctive features when compared with surrounding healthy tissue. These typically include reduced oxygen tension, acidic pH and aberrant angiogenesis which contribute to limited oxygen and nutrient delivery [19–22]. One of the major responses to cellular stress such as hypoxia is the up-regulation of heat shock proteins (hsps) and induction of the hypoxia inducible factor 1 α (HIF-1α) transcription factor. HIF-1α has been shown to regulate the expression of hsps and to be involved in the differentiation of MDSCs [23], while hypoxia itself has been linked to aberrant cytokine expression that may support MDSC differentiation. Considering this, the present study was designed to investigate the role that hsps, pH regulatory proteins as well as other molecular pathways may play in the differentiation of MDSCs in melanoma, with the aim of identifying pathways in tumour cells which are responsible for inducing the differentiation of MDSCs.

To investigate MDSC induction by tumour cells, we developed an *in vitro* co-culture model which allowed us to reconstruct melanoma-monocyte interactions (Fig. 1). To consider the distinctive features found in the tumour mass, we also considered the influence of low oxygen tension in our *in vitro* model. To pin-point the molecular pathways in tumour cells responsible for inducing immune suppression, we pre-treated melanoma cells with a panel of inhibitors including those against molecular hsps, pH regulatory proteins and inhibitor of COX and JAK/STAT signaling. The primary goal of this study was to uncover mechanisms in tumour cells responsible for causing the differentiation of monocytes into MDSCs. We identified inhibitors preventing the induction of MDSCs by targeting certain molecular pathways in melanoma cells, and tested whether this inhibition would be effective under hypoxic conditions, in order to give a better indication of the efficacy of such approaches *in vivo*. The identification of suppressive pathways in melanoma cells may point to new therapeutic targets and improve the efficacy of existing immunotherapies by alleviating tumour-induced immune suppression.

2. Materials and methods

2.1. Cell isolation and patient materials

Peripheral blood mononuclear cells were isolated from consenting healthy donors of whole-blood leukoreduction filters (“Kegel”, Tübingen University Hospital) using Ficoll-Hypaque gradient centrifugation and stored in freezing medium containing 10% DMSO, 20% FCS and 70% RPMI in liquid nitrogen until use. Monocytes were isolated using magnetic bead separation with a CD14+ monocyte isolation kit using LS separation columns (Miltenyi Biotech, Teterow, Germany). Autologous T cells were isolated from the negative fraction of the monocyte isolation by CD3+ T cell isolation kit according to the manufacturer’s protocol (Miltenyi Biotech).

2.2. Tumour cell line culture

The EST human melanoma cell lines were sourced from the European Searchable Tumor Line Database (ESTDAB; http://www.ebi.ac.uk/ipd/estdab). 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). These cell lines have been tested for mycoplasma and verified with DNA finger printing. For melanoma-monocyte co-culture experiments 2.0×10^5 melanoma cells were cultured in 2 ml IMDM medium supplemented with 10% FCS per well in a 6-well plate and allowed to rest overnight before being treated with either 5 μM geldanamycin (an HSP90 inhibitor, Invitrogen, San Diego, USA), 4 μM methylene blue (inhibition of HSP70/NO synthase, which affects protein function and ubiquitination as well as modulation of polyglutamine protein degradation, Sigma-Aldrich), 750 μMa acetylsalicylic acid (COX inhibitor, Sigma-Aldrich, Steinheim, Germany), 35 μM AG490 (JAK/STAT inhibitor), 300 μM U-104 (CA inhibitor, Sigma-Aldrich) for 18 h. The concentration of each drug was titrated so that melanoma cell line viability and growth was not impaired (data not shown). Melanoma cells were additionally left untreated as a control. Following the 18 h treatment, inhibitors (or medium in the case of control wells) were removed by three gentle washes with Hank’s Balanced Salt Solution (HBSS) (Sigma-Aldrich). Treated and control melanoma cells were subsequently cultured in IMDM with 10% FCS until the addition of isolated CD3+ and CD14+ cells (typically 3 h). To further reduce the chance that the inhibitors could directly interact with the isolated

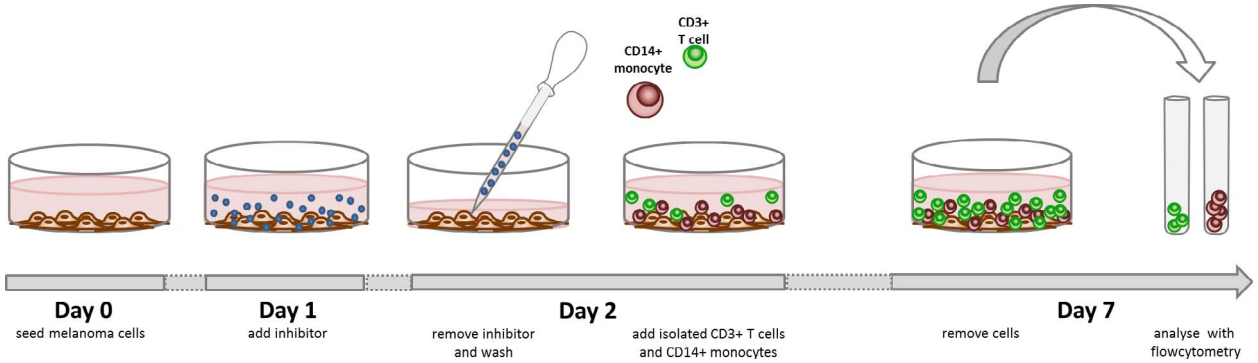

Fig. 1. *In vitro* model of immune suppression by melanoma cells. Established melanoma cell lines were cultured overnight in IMDM with 10% FCS before being treated for 18 h with either GA, MB, U-104, acetylsalicylic acid, AG490 or left untreated as a control. Inhibitors were removed after 18 h and melanoma cells were subsequently co-cultured with isolated CD14+ and activated CFSE-labelled CD3+ cells at a ratio of 5:1 for 5 days. Cells were then harvested and stained for CD3, CD4, CD8 (T cells) or CD14 and HLA-DR (monocytes) and analysed using flow cytometry. GA: Geldanamycin (HSP90 inhibitor); MB: methylene blue (HSP70/NO synthase inhibitor), U-104 (CA inhibitor); acetylsalicylic acid (COX inhibitor); AG490 (JAK/STAT inhibitor).

CD3+ and CD14+ cells, melanoma cultures were washed once more with HBSS before the addition of CD3+ and CD14+ cells. Isolated CD3+ and CD14+ cells were added to melanoma cells at a ratio of 1:5 T cells : monocytes (5.0×10^5 T cells to 2.5×10^6 monocytes). Isolated T cells and monocytes were added to 6 well plates and co-cultured for 5 days under the following conditions: 1) pre-treated melanoma cells (Fig. 1), 2) non-treated melanoma cells (Figs. 1 and 3) without melanoma cells (positive control).

2.3. T cell proliferation

T cell proliferation was used to estimate the degree of immune suppression in melanoma-monocyte co-culture experiments. Isolated (CD3+) T cells were labelled with carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) at a concentration of 1.4 μmol/L in 1 ml PB buffer for 5 min at room temperature. Non-bound CFSE was removed by three rounds of washing (X-Vivo 15, Lonza, Verviers, Belgium) followed by centrifugation at 300*g* for 5 min. 5.0×10^5 CFSE-labelled T cells were added to 6 well plates either with monocytes as a positive control (2.5×10^6 monocytes giving a ratio of 1:5) or with monocytes and melanoma cells (2.0×10^5) to investigate suppressive effects of melanoma-educated monocytes. Additional controls consisting of melanoma cells only and T cells only were included in selected cases. All co-culture experiments were performed for 5 days in 6 well plates with 7 ml IMDM containing 10% FCS unless otherwise stated. T cells were activated with anti-CD3/CD28 mAb coated beads (2 μL per well). After 6 days of culture, the degree of T cell proliferation by CD4+ and CD8+ T cells was measured by flow cytometry.

2.4. Phenotypic analysis of T cells and monocytes

Co-cultured T cells and monocytes were removed from 6 well plates by gentle resuspension and aspiration of the culture medium followed by washing with HBSS. Cells were then stained for cell surface markers as previously described [24], but without exposure to light. Details relating to the panel of antibodies appear in Supplementary data S1. Antibody panels were established using fluorescence minus one controls. Cytometer setup and tracking beads were run before and after each sample measurement to ensure consistency in machine performance.

2.5. Flow cytometry data analysis

Flow cytometry data were analysed by first excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of cell debris with the use of a morphological gate. The full gating strategy employed to assess T cell proliferation and to identify T cells and monocytes is shown in Supplementary data S2.

3. Results

Mao et al. has previously shown that MDSC-like cells can be induced by culturing monocytes from healthy individuals with melanoma cells [1]. Based on this observation, here we established a model of tumour-induced immune suppression that allows interactions between three cell types to be studied: tumour cells, monocytes and T cells. Preliminary experiments determined the optimum ratio of these different cell types (data now shown), and thereafter we sought to characterise how these cells interact across time. For these experiments, we cultured peripheral monocytes from a healthy donor with autologous CFSE-labeled T cells and the EST-200 melanoma cell line for 24, 48, 72, 96 or 120 h *in vitro*. We observed that CD4+ T cell suppression plateaued after 96 h, whereas CD8+ T cell suppression increased with time (Fig. 2A, left panel). An example of inhibited proliferation of CD8+ T cells according to CFSE dilution assay is shown in Fig. 2A (right panel). These results suggest that interactions between melanoma cells and monocytes result in T cell suppression, because T cell proliferation was not inhibited when T cells were cultured directly with melanoma cells (Supplementary data S3 and S4). To investigate these observations in more detail we tested if T cell suppression was associated with changes in monocyte phenotype over time. Low levels of HLA-DR have been proposed as a marker for suppressive myeloid cells, thus we tested the expression of this molecule in our *in vitro* culture model (Fig. 2B). In accordance with the observation of greater T cell suppression over time, we found that HLA-DR expression on monocytes cultured with melanoma cells was also reduced with time when compared to HLA-DR expression on monocytes cultured only with T cells. Collectively, these results suggest that melanoma cells cause monocytes to differentiate into cells that phenotypically and functionally resemble myeloid-derived suppressor cells.

To validate these initial results obtained in our *in vitro* model of MDSC differentiation using the EST-200 melanoma cell line, we tested 6 healthy donors with a greater number of established melanoma cell lines (EST-41, EST-83, EST-145, EST-152 and EST-200). We observed similar results with these cell lines as with EST-200 – T cell proliferation was suppressed when monocytes from healthy donors were co-cultured with melanoma cells compared to T cells and monocytes without melanoma cells. Of the cell lines tested, EST-145, EST-152 and EST-200 were found to induce the greatest degree of suppression with between 90 and 70% T cell suppression compared to T cells and monocytes cultured without melanoma cells (Fig. 3, top panel). Generally speaking, these results are in accordance with the changes previously reported to occur when monocytes undergo differentiation into immune suppressive cells (Fig. 3, bottom panel) [2], namely HLA-DR down-regulation and CD14 up-regulation. To exclude direct effects of melanoma cells on T cells, we co-cultured T cells with melanoma cells without monocytes; T cell proliferation was not inhibited under these conditions ($n = 2$, data not shown). It should be noted that some melanoma cell lines ($n = 3$, data not shown) failed to induce monocytes to become suppressive for T cell proliferation, suggesting that there is heterogeneity in the induction of immunosuppressive capacity or in the immune suppressive mechanisms employed by melanoma cells. Furthermore, this observation demonstrates that the observed immune suppressive effects in our co-culture model are not caused by non-specific interactions between monocytes and the presence of any type of cell.

Having developed a model that allowed us to generate MDSC-like cells *in vitro*, we then attempted to identify molecular pathways in melanoma cells responsible for converting normal monocytes into immune suppressive cells. To achieve this, we pre-treated melanoma cells for 18 h with a panel of inhibitors targeting a range of different molecular pathways including geldanamycin (HSP90 inhibitor), methylene blue (inhibitor of HSP70/NO synthase), acetylsalicylic acid (COX inhibitor), AG490 (JAK/STAT inhibitor) and U-104 (CA inhibitor). Prior to the addition of isolated monocytes and T cells, pre-treated melanoma cultures were thoroughly washed several times, allowed to rest and washed once more. This was performed to minimise the potential for direct contact between soluble drugs and the isolated immune cells. In order to test the effect of the remaining drug on T cell proliferation, T cells were directly cultured with melanoma cells (i.e. without monocytes). Here, proliferation was found to be comparable whether the melanoma cells had been treated with inhibitors or not, suggesting that either no traces of the drugs remained, or the levels were so low that they did not affect the functioning of the immune cells (data not shown). Of the panel of inhibitors tested, we observed that selected drugs alleviated the induction of immune suppression, with geldanamycin being most effective in this respect. This was followed by the other hsp inhibitor, methylene blue, while occasional but less consistent effects were seen for U-104. No significant effect was found for acetylsalicylic acid or AG490. The effect of inhibitors was not the same for all melanoma cell lines, but for geldanamycin an effect was observed in

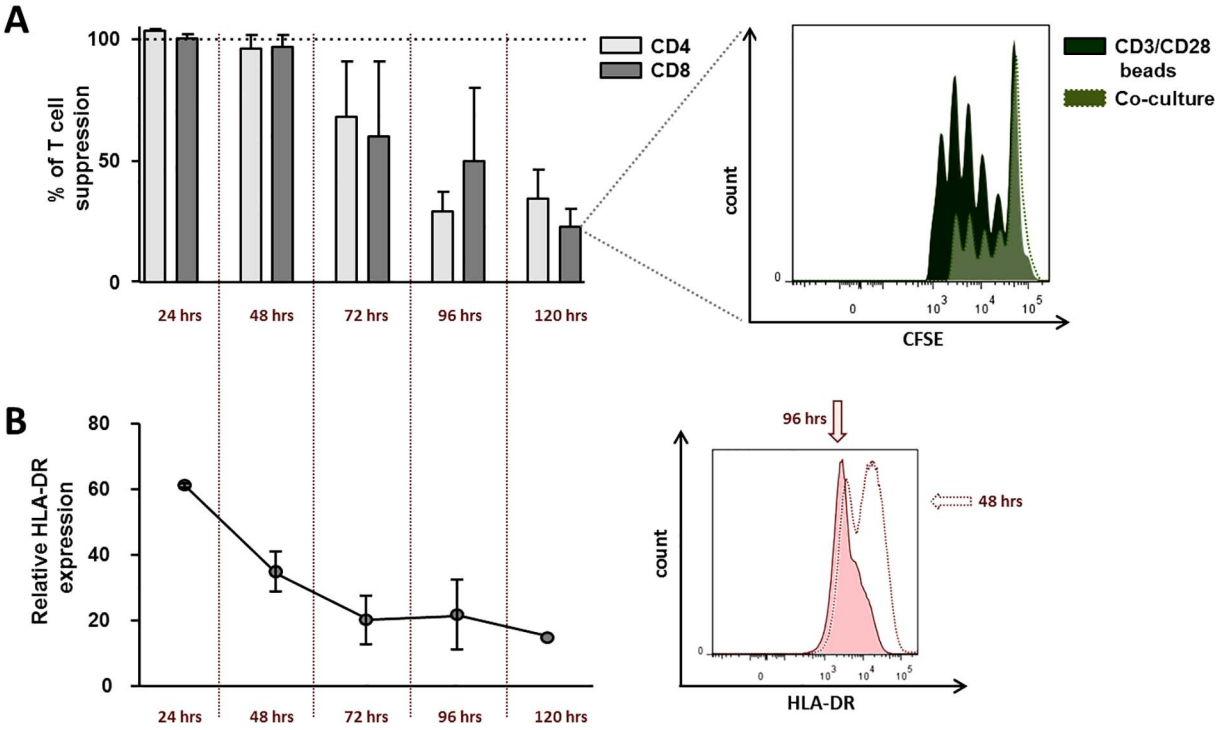

Fig. 2. Monocytes co-cultured with melanoma cells resemble MDSC cells in phenotype and function. Isolated monocytes and T cells were co-cultured with the melanoma cell line EST-200 for the time shown (A). The level of T cell suppression was determined using CFSE-stained autologous T cells. T cell suppression by melanoma-educated monocytes increased over time with a maximum of suppression observed on or after 96 h. (B) Phenotypic changes (HLA-DR) of melanoma-educated monocytes (identified as CD14+) were analysed using flow cytometry.

3 of 4 tested melanoma cell lines (EST-145, EST-152, EST-200, Fig. 4B–D). This was followed by the less effective inhibitors methylene blue and U-104 (Fig. 4B–D). The one exception to this trend was for the cell line EST-41 (Fig. 4A). Here, the most effective inhibitor was methylene blue, followed by geldanamycin and U-104 with similar effects. Inhibitor-induced alleviation of T cell suppression induction was again reflected by changes in monocyte phenotype; we observed increased expression of HLA-DR when T cell suppression was reduced (Fig. 4D, bottom panel). Again, this association was not observed with cell line EST-41: there was no association between HLA-DR expression on melanoma-educated monocytes and the degree of T cell proliferation when monocytes and T cells were co-cultured with EST-41.

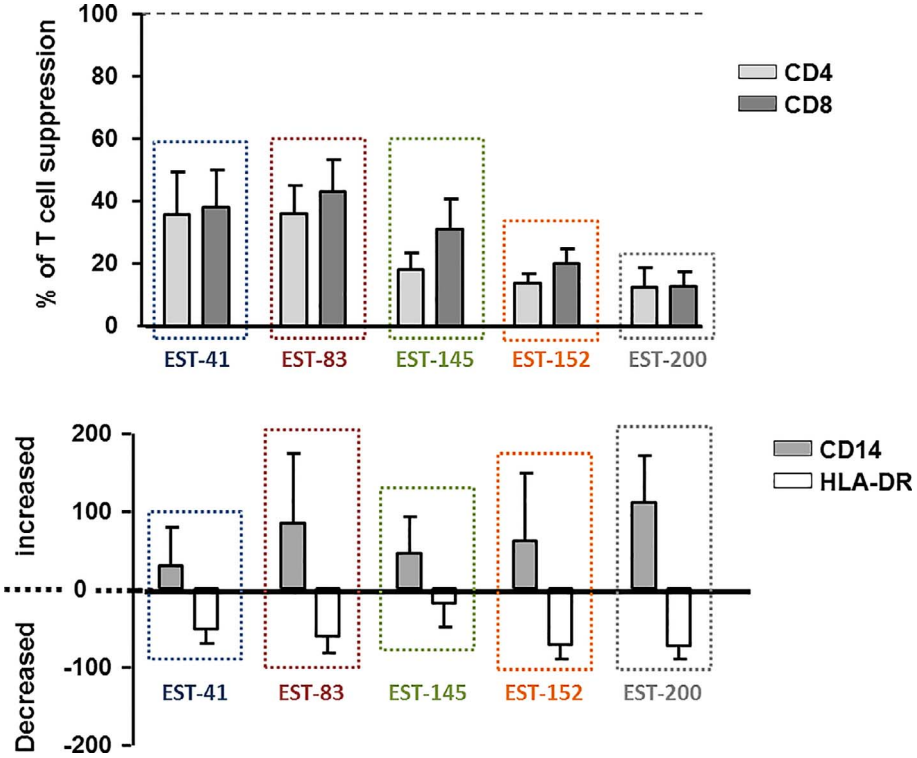

Fig. 3. Monocytes co-cultured with melanoma cells suppress T cell proliferation and show a phenotype resembling MDSCs. Monocytes and T cells were co-cultured with the established melanoma cell lines EST-41, EST-83, EST-145, EST-152 or EST-200. T cell proliferation as well as phenotypic analysis of monocytes (CD14 and HLA-DR expression) was analysed using flow cytometry (n = 6). T cell proliferation was suppressed when monocytes were co-cultured with melanoma cell lines EST-41, EST-83, EST-145 and EST-200 (top panel) (relative degree of suppression obtained by comparing proliferation of T cells and monocytes cultured without melanoma cells). In line with the observed suppressive capacity of monocytes co-cultured with melanoma cells, phenotypic analysis revealed an up-regulation of CD14 and down-regulation of HLA-DR expression on melanoma-educated monocytes (bottom panel).

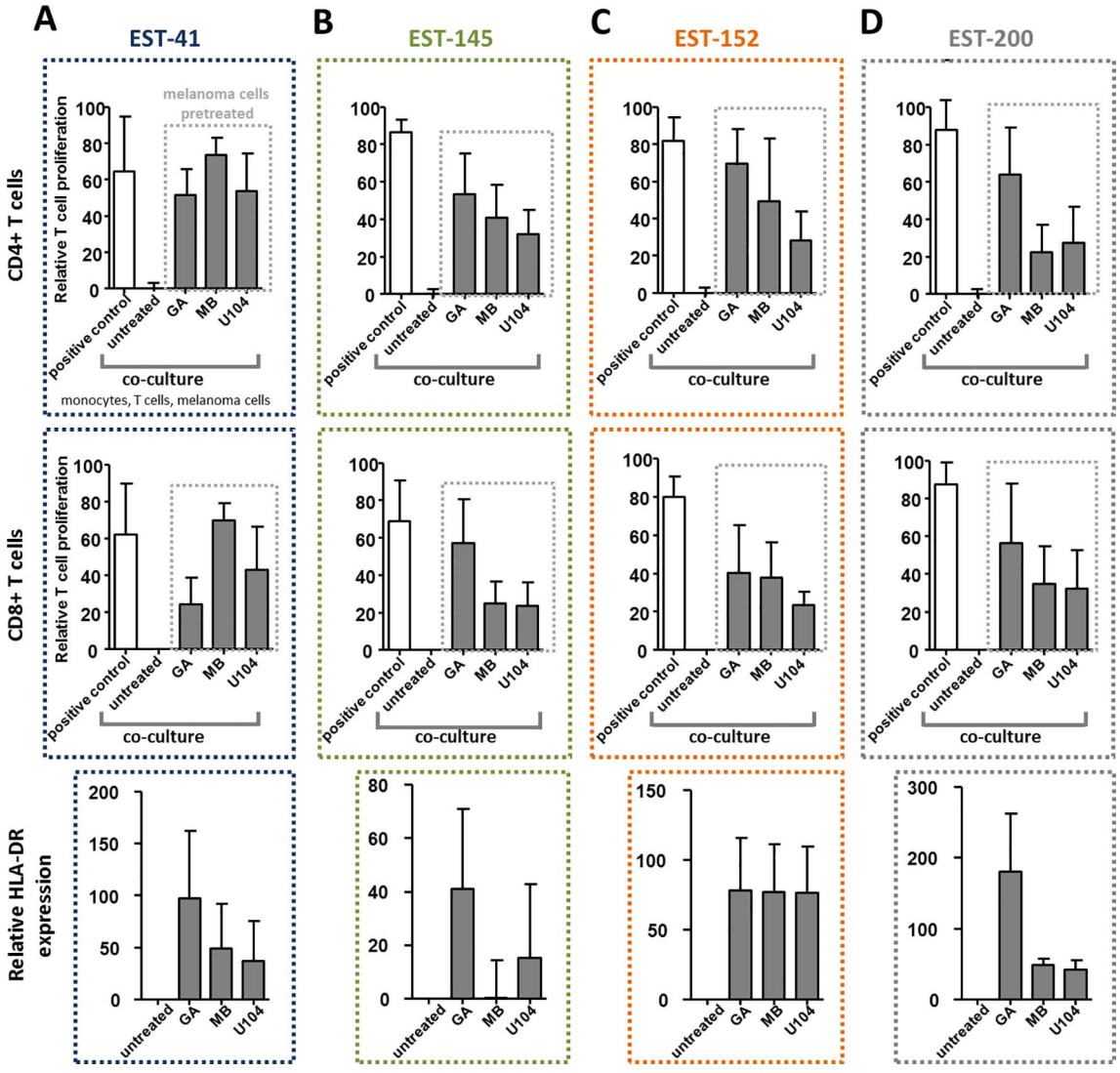

Fig. 4. Pre-treating melanoma cell lines with GA or MB alleviates T-cell suppression in melanoma-monocyte co-cultures. Melanoma cell lines EST-41, EST-145, EST-152 and EST-200 were pre-treated for 18 h with a panel of inhibitors targeting different molecular pathways (GA – HSP90, MB – HSP70 and U-104 – CA) (n = 6). Suppression of CD4+ and CD8+ T cells was alleviated when melanoma cells were pre-treated with these inhibitors (upper two panels). GA and MB were most effective in reducing T cell suppression. Phenotypic analysis of melanoma-educated monocytes agreed with functional analysis observed here; HLA-DR expression was up-regulated on monocytes co-cultured with pre-treated melanoma cells relative to control monocytes co-cultured with untreated melanoma cells (bottom panel).

To exclude the possibility that alleviation of T cell suppression in inhibitor pre-treated co-cultures was related to the inhibitor affecting the growth of melanoma cells, we performed experiments with double the number of melanoma cells (4.0×10^5). Here, we did not observe differences related to the number of melanoma cells (data not shown). Additionally, we also titrated drug concentrations so growth was not adversely affected by the concentrations of these drugs used. Together these results suggest that the effect of these drugs at alleviating T cell suppression occurs through molecular pathways in melanoma cells, not through indirect effects on the growth of melanoma cells.

Having shown that geldanamycin was most effective in reducing T cell suppression induction, we then attempted to determine the time-dependent effect of this drug on preventing the conversion of monocytes into immune suppressive cells. To achieve this, the melanoma cell line EST-200 was pre-treated for 18 h with geldanamycin as before, and then co-cultured with monocytes and autologous CFSE-labeled T cells from two healthy donors for 24–120 h. We observed a decrease in T cell suppression over time when monocytes were co-cultured with geldanamycin pre-treated melanoma cells compared to co-cultures with untreated melanoma cells (Fig. 5, left panel). Changes in CD4+ and CD8+ T cell proliferation across time are shown in Fig. 5 (right panel). To investigate whether these observations are associated with changes in monocyte phenotype over time, we also analysed the expression of the myeloid maturation marker HLA-DR. In accordance with the observation of decreased T cell suppression over time, we found that HLA-DR expression on monocytes cultured with geldanamycin pre-treated melanoma cells was increased as well. Additionally, because several soluble factors including GM-CSF have been shown to be involved in the differentiation of monocytes into suppressive MDSCs, we analysed the expression of GM-CSF in monocyte-melanoma co-cultures in preliminary experiments. Here we observed decreased expression of GM-CSF when melanoma cells were pre-treated with geldanamycin (data not shown). Collectively, these results suggest that pre-treating melanoma cells with geldanamycin (blockade of HSP 90) results in a long lasting effect at preventing the conversion of normal monocytes into immune suppressive cells.

Having shown that geldanamycin and methylene blue were the most effective drugs at preventing the induction of immune suppression, we investigated whether they are effective under conditions which more closely mimic the tumour microenvironment. Many drugs are less

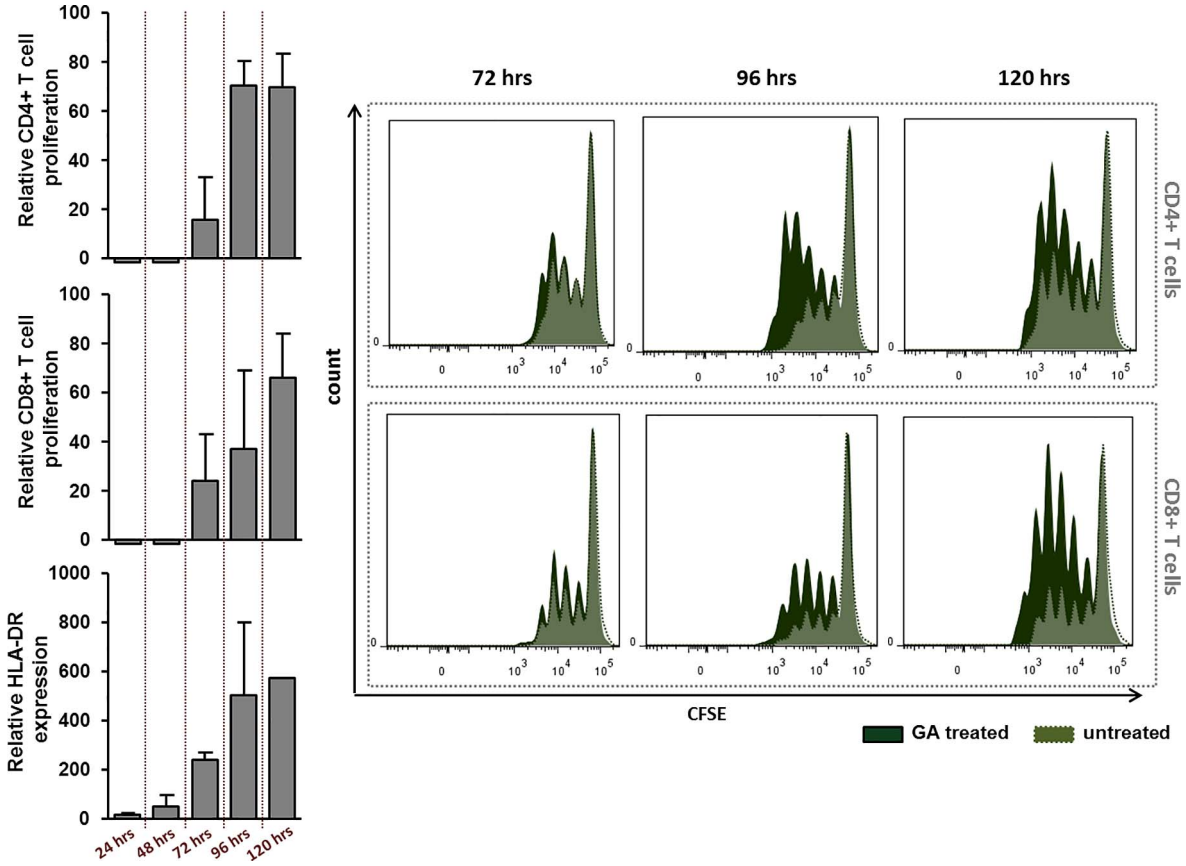

Fig. 5. Time-dependent effects of GA in co-cultures of monocytes, T cells and melanoma cells. The melanoma cell line EST-200 was pretreated with GA for 18 h before being removed and co-cultured with isolated CD14+ cells plus activated CFSE-stained CD3+ T cells from 2 healthy individuals for either 24–120 h. We observed an increase in T cell proliferation of CD4+ and CD8+ cells over time when monocytes were co-cultured with GA pre-treated melanoma cells. In accordance with changes in T cell proliferation, we also found an increase in HLA-DR expression on monocytes in GA-pretreated co-cultures (left panel). An example of CD4+ and CD8+ T cell proliferation according to CFSE is shown in the right-hand panel.

effective due to the conditions found in the tumour microenvironment [25,26]; however, because these two inhibitors target proteins which are up-regulated under the stressful conditions of the tumour microenvironment we hypothesised that they may be more effective under these conditions. To achieve this, we utilised our established *in vitro* model to induce MDSC-like cells under conventional culture conditions and compared the results obtained under hypoxia (2% O_2). As shown above, we observed that geldanamycin and methylene blue reduced T cell suppression in co-cultures performed under conventional culture conditions. Comparing the effect of these drugs between the conventional and 2% O_2 conditions showed that the effect of alleviating immune suppression was not lost under hypoxic conditions. On the contrary, a trend of greater activity under hypoxia was observed (Fig. 6). Similar to GA, MB was found to be equally effective under hypoxic and hyperoxic conditions (Fig. 6). This suggests that hypoxia in the tumour microenvironment *in vivo* would not diminish the efficacy of drugs against HSP90.

4. Discussion

Through their immune suppressive activity, MDSCs are thought to represent one of the major barriers preventing effective cancer treatment [5]. A number of mechanisms by which MDSCs suppress the immune system have been elucidated, and several extracellular mechanisms of MDSC induction have been reported [16,18]. In contrast, little is known about whether intra-cellular pathways in tumour cells are responsible for converting monocytes into immune suppressive cells. In the present study, using a new approach for *in vitro* generation of tumour-associated human MDSCs, we describe the blocking of MDSC induction by pretreating melanoma cells with hsp inhibitors. We report that MDSC-like cells could be induced by co-culturing monocytes with several melanoma cell lines, suggesting that melanoma cells are able to direct monocytes from healthy individuals to differentiate into cells capable of suppressing T cell proliferation. Furthermore, this observation suggests that tumour-induced MDSC differentiation is a mechanism of immune suppression that could also occur in melanoma patients [1]. Although several soluble factors including GM-CSF, IL-6 and COX-2/PGE2 have been shown to be involved in the differentiation of monocytes into suppressive MDSCs [16], the molecular pathways in tumour cells responsible for the induction of MDSC-like cells remain to be elucidated. In preliminary experiments on isolated monocyte-melanoma cell co-cultures we observed reduced GM-CSF expression in co-cultures pre-treated with geldanamycin, suggesting that Hsp90 is involved in the production of GM-CSF. Because GM-CSF has been shown to be involved in the differentiation of MDSCs, we pretreated melanoma cells with a panel of inhibitors, in contrast to prior studies which directly treated melanoma-educated monocytes with inhibitors, in order to identify pathways in the tumour cells themselves that were responsible for maintaining immunosuppressive networks. Lechner et al. has shown that neutralisation of GM-CSF, IL-6 and IL-1beta in tumour cell line-PBMC co-cultures abrogated induction of CD33+ suppressor cell function and restored T cell proliferation to a level comparable to controls [18]. Here, we have tested the potential of a panel of inhibitors including those against hsps, pH regulatory proteins and inhibitors of COX and JAK/STAT signaling. Co-culturing monocytes from healthy individuals with melanoma cells pre-treated with geldanamycin (targeting HSP90) or methylene blue (targeting HSP70/NO synthase) resulted in increased HLA-DR expression on CD14+ monocytes and decreased suppression of T cell proliferation. Interestingly, comparing the effect of the different drugs between melanoma cell lines, we observed

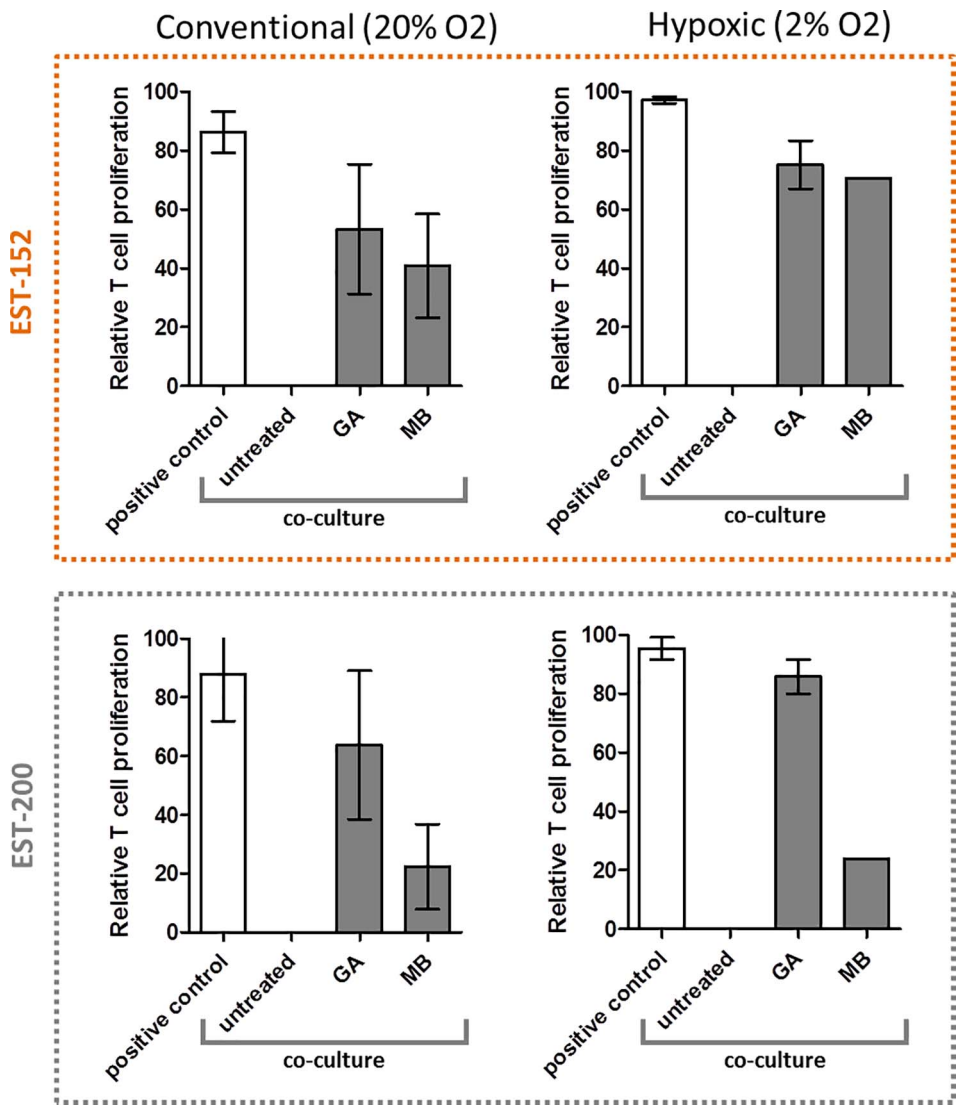

Fig. 6. GA is more effective in alleviating T cell suppression under hypoxic than under conventional culture conditions. The melanoma cell lines EST-152 and EST-200 were pretreated with GA or MB and co-cultured with isolated monocytes and activated CFSE-stained CD3+ T cells for 120 h under either conventional (20% O_2) or hypoxic (2% O_2) conditions. GA pretreated melanoma cells co-cultured with monocytes and T cells under hypoxic conditions were found to be more sensitive to GA treatment, resulting in less T cell suppression compared to the conventional setting. The effect of MB was not inhibited by hypoxic either, and was found to be at least as effective under these conditions.

differences in the capacity to alleviate T cell proliferation and this differences in preventing melanoma cells from inducing MDSCs. This suggests that different melanoma cell lines may use different mechanism to convert monocytes into suppressive MDSCs and thus resulting in different effects of the drugs on alleviating T cell proliferation. However, the majority of cell lines were susceptible to treatment with GA followed by less effective inhibitor methylene blue. Therefore, these results show that targeting HSP70 and HSP90 within tumour cells prevents the induction of monocyte differentiation into suppressive immune cells on both at a phenotypic and functional level [27–30]. The fraction of cell lines which did not respond to geldanamycin treatment may use HSP90-independent mechanisms of MDSC differentiation, because every melanoma cell line examined to date has been shown to express HSP90 [31]. HSP70 and HSP90 are two major types of molecular chaperones that have central roles in cellular functioning by maintaining the integrity of intracellular proteins [32]. These proteins are highly conserved and present in all cells of every organism [33], thus our results obtained *in vitro* using established cell lines are likely to represent the roles that these proteins play *in vivo*. These molecules act in a concerted fashion together with co-chaperones to assist in the folding, function and degradation of a wide range of client proteins. Furthermore, they play key roles in cancer; their up-regulation in response to stress assists the functioning of cancer cells under the harsh conditions of the tumour microenvironment, while their chaperoning action maintains key signalling networks responsible for aberrant cell division [34]. Because inhibiting the function of hsps alleviated tumour-induced immune suppression, this suggests that there are overlapping networks between those traditionally thought to be responsible for cancer maintenance (i.e. anti-apoptotic and signalling pathways) and those more recently appreciated to influence cancer progression such as immune suppression. Indeed this is reflected in the realisation that traditional chemotherapeutic drugs exert much of their beneficial effect through immune modulatory mechanisms [35]. Given that MDSCs have been shown to be induced through pro-inflammatory pathways, it seems plausible that inhibiting HSP70 and HSP90 in tumour cells affects the production of pro-inflammatory cytokines. Supporting this notion, preliminary results showed reduced GM-CSF production in monocyte-melanoma cell co-cultures when melanoma cells were pre-treated with the HSP90 inhibitor geldanamycin.

Because hypoxia is a widespread feature of the tumour microenvironment that is also clinically important [25,26], culturing tumour cells under high oxygen conditions (as is the case with cultures in air)

may result in the generation of misleading results. The tumour mass consists of areas with distinctive conditions when compared with surrounding healthy tissue, including reduced oxygen tension, acidic pH and aberrant angiogenesis which contributes to limited oxygen and nutrient delivery. The major mechanism by which cells respond to hypoxia is through modulation of the HIF transcription factor [36]. HIF has been shown to regulate the expression of hsps, while HSP90 itself stabilises HIF in melanoma cells under hypoxic conditions [37,38]. It has been shown that hypoxia is capable of up-regulating the expression of HSP70 and HSP90 [31,39]. Furthermore, Hsp90 is important for hypoxic tolerance as measured by the growth rate and viability of melanoma cells [31]. As such, given the importance of hsps under hypoxic conditions, hsp-inhibiting drugs may be relatively more effective in hypoxic tumours, for which many standard therapies are known to be less effective [25]. Considering this, we developed the hypothesis that targeting these molecules in tumour cells under low oxygen tension will not result in diminished efficacy that is associated with many drugs. On the contrary we predicted that drugs against these molecules may be more effective under low oxygen tension. Indeed, our results demonstrate that drugs targeting HSP70 and HSP90 do not suffer from reduced efficacy under hypoxic conditions, while we observed a trend of a more potent effect when inhibiting HSP90 under these conditions. These results suggest that melanoma cells rely more heavily on these molecules under hypoxic conditions, in turn resulting in a similar or greater effect when measuring consequences of inhibiting their action under these conditions. These observations suggest that targeting molecules such as hsps that tumour cells more heavily rely on under hypoxic conditions may avoid the issue of weakened activity under these conditions.

Aside from the observation that the beneficial effects on preventing immune suppression when targeting HSP70 and HSP90 in tumour cells is not diminished by hypoxic conditions, further possible implications of our results relate to the long duration of effect that we observed. Tumour cells were exposed transiently to these drugs (18 h), before being co-cultured for another 5 days in our co-culture model with immune cells. We observed a maintenance of the effect for CD4+ cells after 5 days post-exposure, while an increasing strength of alleviating suppression of proliferation of CD8+ T cells was seen up until 5 days. These results suggest that a single exposure to these drugs is sufficient to result in a long-lasting therapeutic effect, potentially saving the patient from unnecessary drug side effects. Hsp inhibitors have been trialed clinically, but not in dosing schedules similar to the conditions used here in which sub-lethal doses that do not directly kill tumour cells were employed followed by a long duration without exposure to the drug.

5. Conclusion

Collectively, this study demonstrates a novel mechanism in tumour cells responsible for the induction of MDSC-like cells in melanoma. A single, sub-lethal dose was sufficient to provide protection against the induction of immune suppression. Furthermore, unlike many standard therapies the activity of these drugs was not diminished by hypoxic conditions. This initial study into hsp-mediated immune suppression warrants further investigation to validate the proposition of hsps as immunomodulatory agents.

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Declaration of interest

None.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.cellimm.2018.02.012.

References

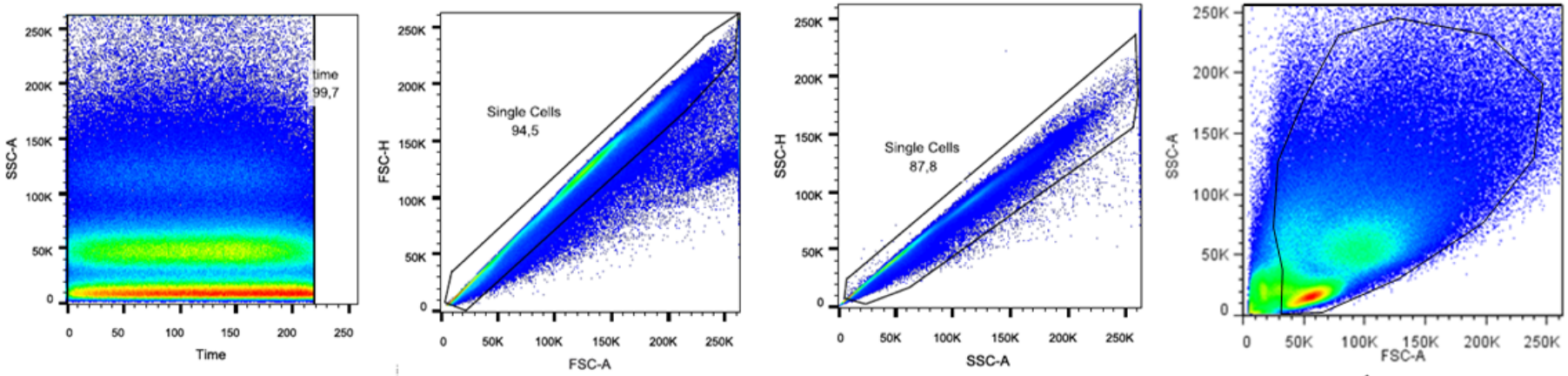
[1] Y. Mao, I. Poschke, E. Wennerberg, Y. Pico de Coana, S. Egyhazi Brage, I. Schultz, J. Hansson, G. Masucci, A. Lundqvist, R. Kiessling, Melanoma-educated CD14+ cells acquire a myeloid-derived suppressor cell phenotype through COX-2-dependent mechanisms, Cancer Res. 73 (2013) 3877–3887.
[2] Y. Mao, D. Sarhan, A. Steven, B. Seliger, R. Kiessling, A. Lundqvist, Inhibition of tumor-derived prostaglandin-e2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity, Clin. Cancer Res. 20 (2014) 4096–4106.
[3] P. Filipazzi, R. Valenti, V. Huber, L. Pilla, P. Canese, M. Iero, C. Castelli, L. Mariani, G. Parmiani, L. Rivoltini, Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine, J. Clin. Oncol. 25 (2007) 2546–2553.
[4] I. Poschke, D. Mougiakakos, J. Hansson, G.V. Masucci, R. Kiessling, Immature immunosuppressive CD14+HLA-DR-/low cells in melanoma patients are Stat3hi and overexpress CD80, CD83, and DC-sign, Cancer Res. 70 (2010) 4335–4345.
[5] B. Weide, A. Martens, H. Zelba, C. Stutz, E. Derhovanessian, A.M. Di Giacomo, M. Maio, A. Sucker, B. Schilling, D. Schadendorf, P. Buttner, C. Garbe, G. Pawelec, Myeloid-derived suppressor cells predict survival of patients with advanced melanoma: comparison with regulatory T cells and NY-ESO-1- or melan-A-specific T cells, Clin. Cancer Res. 20 (2014) 1601–1609.
[6] C. Shipp, L. Speigl, N. Janssen, A. Martens, G. Pawelec, A clinical and biological perspective of human myeloid-derived suppressor cells in cancer, Cell. Mol. Life Sci. (2016).
[7] A. Martens, K. Wistuba-Hamprecht, M. Geukes Foppen, J. Yuan, M.A. Postow, P. Wong, E. Romano, A. Khammari, B. Dreno, M. Capone, P.A. Ascierto, A.M. Di Giacomo, M. Maio, B. Schilling, A. Sucker, D. Schadendorf, J.C. Hassel, T.K. Eigentler, P. Martus, J.D. Wolchok, C. Blank, G. Pawelec, C. Garbe, B. Weide, Baseline peripheral blood biomarkers associated with clinical outcome of advanced melanoma patients treated with ipilimumab, Clin. Cancer Res. 22 (2016) 2908–2918.
[8] A. Martens, H. Zelba, C. Garbe, G. Pawelec, B. Weide, Monocytic myeloid-derived suppressor cells in advanced melanoma patients: indirect impact on prognosis through inhibition of tumor-specific T-cell responses? Oncoimmunology 3 (2014) e27845.
[9] E. Suzuki, V. Kapoor, A.S. Jassar, L.R. Kaiser, S.M. Albelda, Gemcitabine selectively eliminates splenic Gr-1+/CD11b+ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity, Clin. Cancer Res. 11 (2005) 6713–6721.
[10] T. Condamine, D.I. Gabrilovich, Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function, Trends Immunol. 32 (2011) 19–25.
[11] S. Ostrand-Rosenberg, P. Sinha, Myeloid-derived suppressor cells: linking inflammation and cancer, J. Immunol. 182 (2009) 4499–4506.
[12] I. Marigo, L. Dolcetti, P. Serafini, P. Zanovello, V. Bronte, Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells, Immunol. Rev. 222 (2008) 162–179.
[13] S. Kusmartsev, D.I. Gabrilovich, Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer, Cancer Metastasis Rev. 25 (2006) 323–331.
[14] N. Obermajer, R. Muthuswamy, J. Lesnock, R.P. Edwards, P. Kalinski, Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells, Blood 118 (2011) 5498–5505.
[15] L.J. Bayne, G.L. Beatty, N. Jhala, C.E. Clark, A.D. Rhim, B.Z. Stanger, R.H. Vonderheide, Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer, Cancer Cell 21 (2012) 822–835.
[16] M.G. Lechner, D.J. Liebertz, A.L. Epstein, Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells, J. Immunol. 185 (2010) 2273–2284.
[17] S. Solito, E. Falisi, C.M. Diaz-Montero, A. Doni, L. Pinton, A. Rosato, S. Francescato, G. Basso, P. Zanovello, G. Onicescu, E. Garrett-Mayer, A.J. Montero, V. Bronte, S. Mandruzzato, A human promyelocytic-like population is responsible for the immune suppression mediated by myeloid-derived suppressor cells, Blood 118 (2011) 2254–2265.
[18] M.G. Lechner, C. Megiel, S.M. Russell, B. Bingham, N. Arger, T. Woo, A.L. Epstein, Functional characterization of human Cd33+ and Cd11b+ myeloid-derived suppressor cell subsets induced from peripheral blood mononuclear cells co-cultured with a diverse set of human tumor cell lines, J. Transl. Med. 9 (2011) 90.
[19] M. Murphy, J.A. Carlson, M.P. Keough, K.P. Claffey, S. Signoretti, M. Loda, Hypoxia

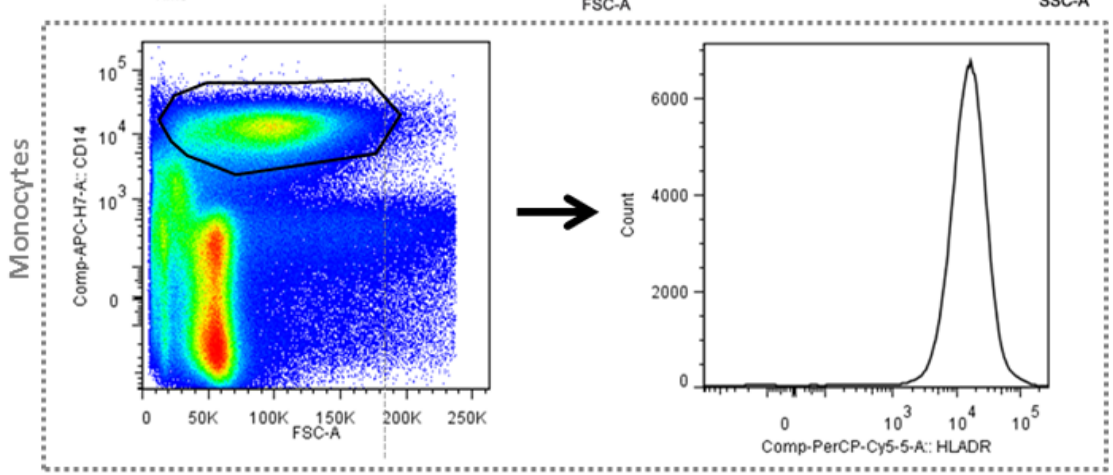
regulation of the cell cycle in malignant melanoma: putative role for the cyclin-dependent kinase inhibitor p27, J. Cutaneous Pathol. 31 (2004) 477–482.
[20] P. Vaupel, L. Harrison, Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response, Oncologist 9 (Suppl. 5) (2004) 4–9.
[21] R.S. Kerbel, Tumor angiogenesis: past, present and the near future, Carcinogenesis 21 (2000) 505–515.
[22] B. Kaur, F.W. Khwaja, E.A. Severson, S.L. Matheny, D.J. Brat, E.G. Van Meir, Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis, Neuro-oncology 7 (2005) 134–153.
[23] C.A. Corzo, T. Condamine, L. Lu, M.J. Cotter, J.I. Youn, P. Cheng, H.I. Cho, E. Celis, D.G. Quiceno, T. Padhya, T.V. McCaffrey, J.C. McCaffrey, D.I. Gabrilovich, HIF-1alpha regulates function and differentiation of myeloid-derived suppressor cells in the tumor microenvironment, J. Exp. Med. 207 (2010) 2439–2453.
[24] J.K. Bailur, B. Gueckel, E. Derhovanessian, G. Pawelec, Presence of circulating Her2-reactive CD8 + T-cells is associated with lower frequencies of myeloid-derived suppressor cells and regulatory T cells, and better survival in older breast cancer patients, Breast Cancer Res. 17 (2015) 34.
[25] M. Weinmann, C. Belka, L. Plasswilm, Tumour hypoxia: impact on biology, prognosis and treatment of solid malignant tumours, Onkologie 27 (2004) 83–90.
[26] J.M. Brown, Tumor hypoxia in cancer therapy, Methods Enzymol. 435 (2007) 297–321.
[27] B. Almand, J.I. Clark, E. Nikitina, J. van Beynen, N.R. English, S.C. Knight, D.P. Carbone, D.I. Gabrilovich, Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer, J. Immunol. 166 (2001) 678–689.
[28] A.H. Zea, P.C. Rodriguez, M.B. Atkins, C. Hernandez, S. Signoretti, J. Zabaleta, D. McDermott, D. Quiceno, A. Youmans, A. O'Neill, J. Mier, A.C. Ochoa, Arginase-producing myeloid suppressor cells in renal cell carcinoma patients: a mechanism of tumor evasion, Cancer Res. 65 (2005) 3044–3048.
[29] C.M. Diaz-Montero, M.L. Salem, M.I. Nishimura, E. Garrett-Mayer, D.J. Cole, A.J. Montero, Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy, Cancer Immunol. Immunother. 58 (2009) 49–59.
[30] S. Nagaraj, D.I. Gabrilovich, Myeloid-derived suppressor cells in human cancer, Cancer J. 16 (2010) 348–353.
[31] C. Shipp, E. Derhovanessian, G. Pawelec, Effect of culture at low oxygen tension on the expression of heat shock proteins in a panel of melanoma cell lines, PLoS One 7 (2012) e37475.
[32] E.A. Nollen, R.I. Morimoto, Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins, J. Cell Sci. 115 (2002) 2809–2816.
[33] Z. Li, P. Srivastava, Heat-shock proteins, Curr. Protocols Immunol. Appendix 1 (2004) Appendix 1T.
[34] L. Whitesell, S.L. Lindquist, HSP90 and the chaperoning of cancer, Nat. Rev. Cancer 5 (2005) 761–772.
[35] L. Bracci, G. Schiavoni, A. Sistigu, F. Belardelli, Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer, Cell Death Diff. 21 (2014) 15–25.
[36] K. Guillemin, M.A. Krasnow, The hypoxic response: huffing and HIFing, Cell 89 (1997) 9–12.
[37] N.A. Baird, D.W. Turnbull, E.A. Johnson, Induction of the heat shock pathway during hypoxia requires regulation of heat shock factor by hypoxia-inducible factor-1, J. Biol. Chem. 281 (2006) 38675–38681.
[38] D. Trisciuoglio, C. Gabellini, M. Desideri, E. Ziparo, G. Zupi, D. Del Bufalo, Bcl-2 regulates HIF-1alpha protein stabilization in hypoxic melanoma cells via the molecular chaperone HSP90, PLoS One 5 (2010) e11772.
[39] C.M. Almgren, L.E. Olson, Moderate hypoxia increases heat shock protein 90 expression in excised rat aorta, J. Vasc. Res. 36 (1999) 363–371.

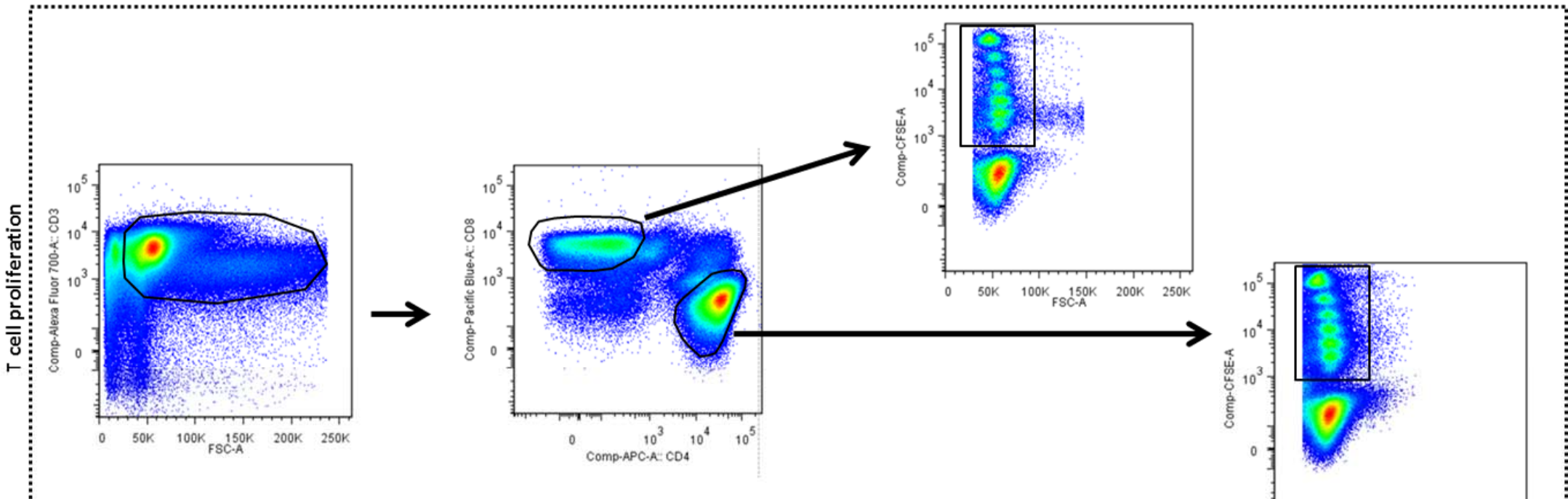
Supplementary Data S1 – Full list of antibodies employed

T cell proliferation	
CD3-A700	BD Biosciences (Franklin Lakes, USA)
CD4-APC	Milteny Biotec (Bergisch Gladbach, Germany)
CD8-PB	BD Biosciences
CD14-APC-H7	BD Biosciences

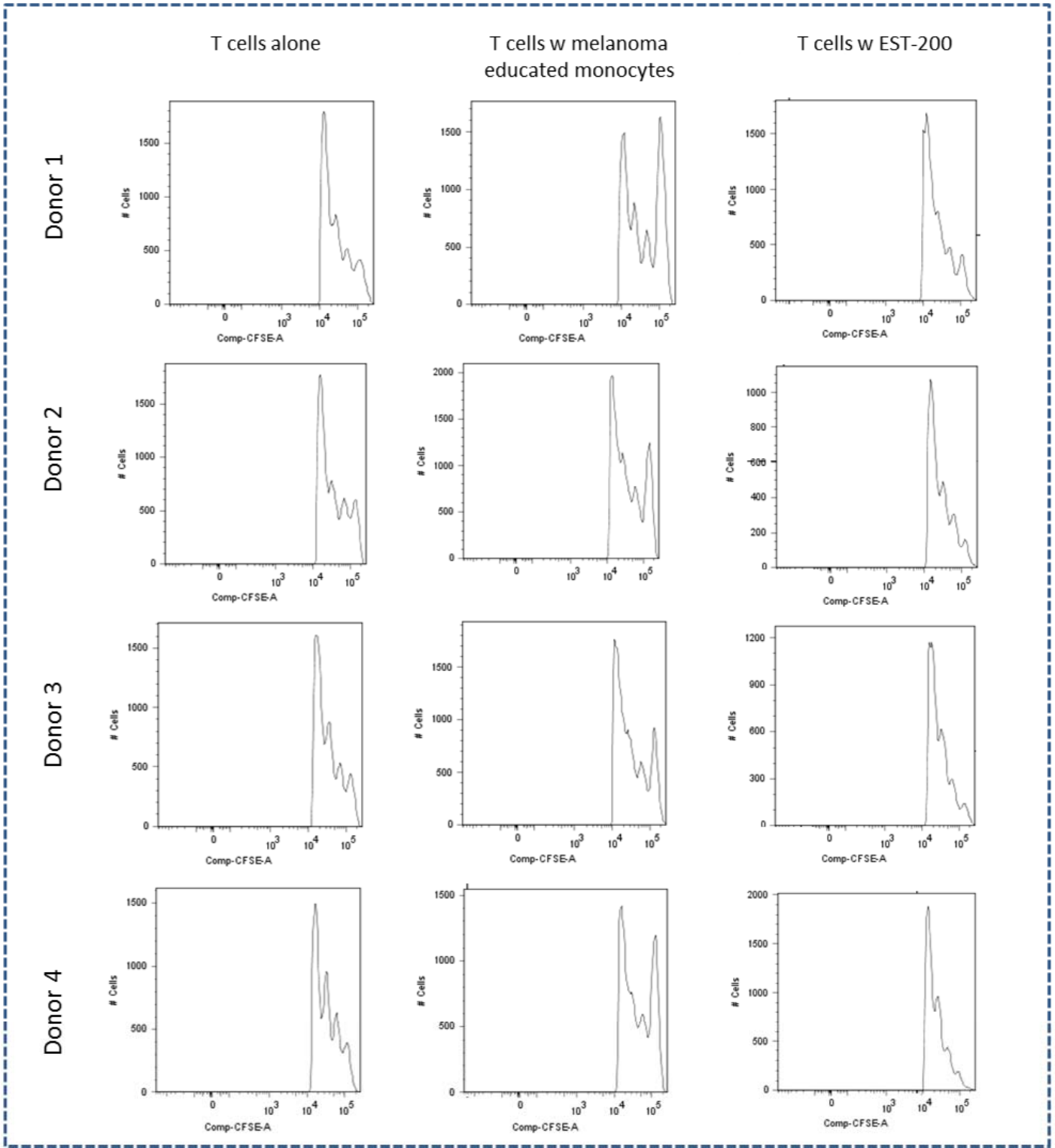
MDSCs	
CD33-PE	eBiosciences (San Diego, USA)
CD14-BV711	Bio Legend (San Diego, USA)
CD15-FITC	BD Biosciences
CD86-PB	Bio Legend
CD34-APC	BD Biosciences
HLA-DR-PerCP-CY5.5	BD Biosciences


SSC-A
Time
time
99,7
FSC-H
FSC-A
Single Cells
94,5
SSC-H
SSC-A
Single Cells
87,8
SSC-A
FSC-A

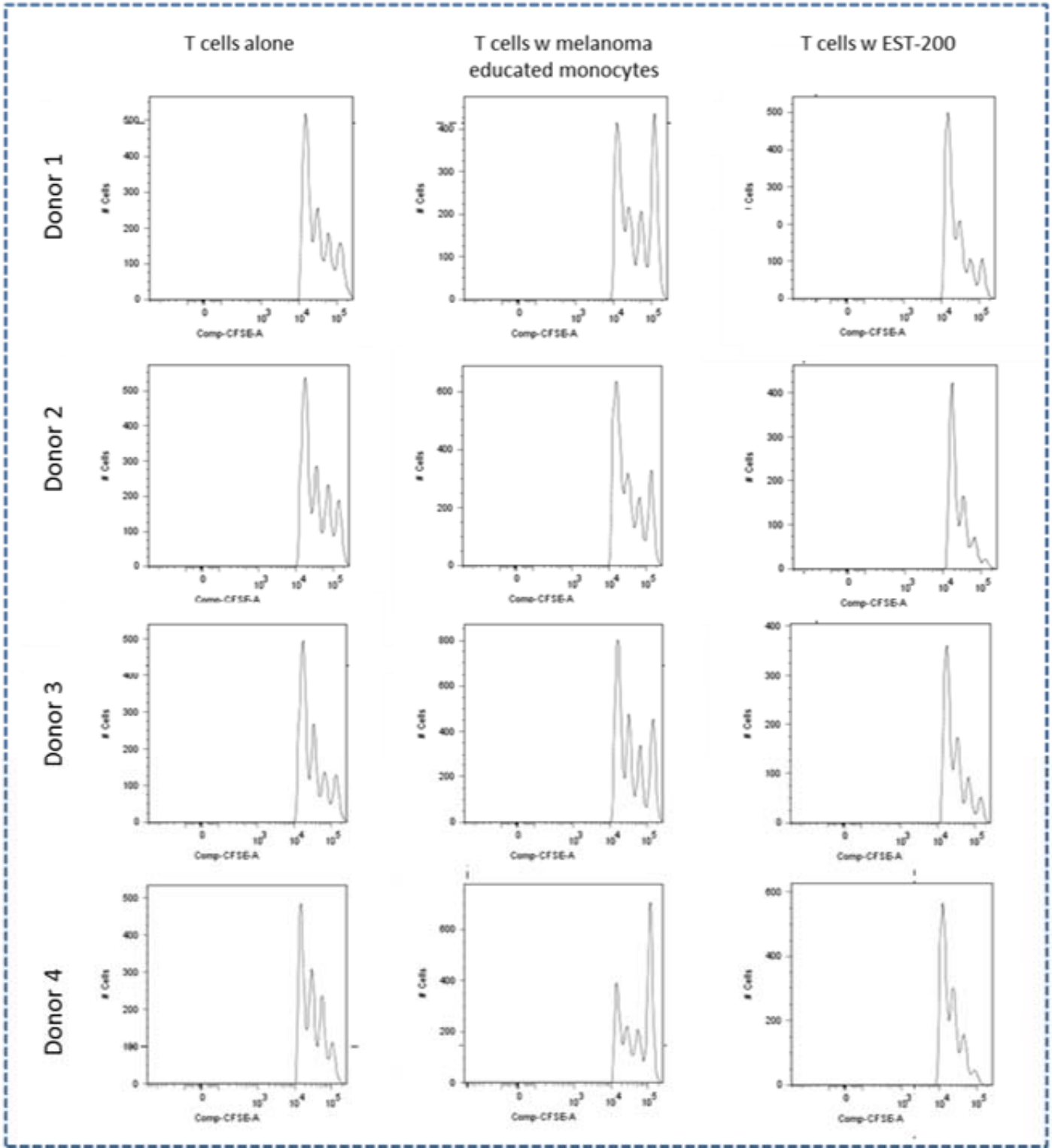

Monocytes
Comp-APC-H7-A:: CD14
FSC-A
Count
Comp-PerCP-Cy5-5-A:: HLADR


T cell proliferation
Comp-Alexa Fluor 700-A:: CD3
FSC-A
Comp-Pacific Blue-A:: CD8
Comp-APC-A:: CD4
Comp-CFSE-A
FSC-A
Comp-CFSE-A

Supplementary Data S3 – CD4 T cell proliferation



Supplementary Data S4 – CD8 T cell proliferation



High levels of blood T cells identify breast cancer patients with HER2, MUC1 and SUR-reactive T cells

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Keywords:	tumour-associated antigen, Breast cancer, HER2, antigen-reactive T cells, Survivin, MUC1

High levels of blood T cells identify breast cancer patients with HER2, MUC1 and SUR-reactive T cells

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High levels of blood T cells identify breast cancer patients with HER2, MUC1 and SUR-reactive T cells

Context:

This study analysed blood leukocytes in order to identify surrogate markers for antigen-reactive T-cells for use in routine patient monitoring.

Material and Methods:

Using flow cytometry we assessed T-cells reactive to the HER2, MUC1 and SUR antigens and determined the levels of T-cells, NK and B-cells in the blood of 50 breast cancer patients.

Results:

High levels of T-cells at various differentiation stages were associated with the presence of antigen-reactive T-cells. Combining leukocyte populations increased sensitivity and specificity up to 100% in identifying patients with antigen-reactive T-cells.

Conclusion:

We identified surrogate markers for antigen-reactive T-cells, which prevent the time-consuming and hard-to-standardise bioassays required to directly measure them.

Keywords: tumour-associated antigen, T cells, NK cells, B cells, breast cancer, HER2, Survivin, MUC1, antigen-reactive T cells

Introduction

The most common treatments for breast cancer such as radio-, chemo- or hormone therapy are often sub-optimal, particularly for late-stage patients. As such, there is a need to optimise the therapeutic management and identify more effective therapies for breast cancer patients. One approach to improve treatment options may be the use of immunotherapies, which utilise the patient's own immune system to control tumour growth. There are different forms of immunotherapy including monoclonal antibodies

such as “checkpoint inhibitors”, cancer vaccines or other methods that aim to harness the immune system. Immunotherapy may be used in combination with chemotherapeutic agents that act directly on tumour cells and which can induce the release of tumour-associated antigens (TAAs). The release of such cancer-associated proteins may allow the immune system to more effectively mount an immune response against tumour cells expressing them. However the efficacy of such approaches relies on the presence of immune cells which can recognise and kill tumour cells expressing relevant tumour antigens.

We have previously shown that breast cancer patients who possess CD8+ T cells reactive to the HER2 TAA experience superior overall survival. This finding closely links TAA-reactive T cells with patient clinical outcome in breast cancer. However not every patient possesses tumour antigen-reactive T cells, making it important to identify these patients in order to optimise their therapeutic management. Lack of antigen-reactive T cells could be due to the state of the immune system which inhibits the presence or function of such reactive T cells. For example, regulatory T, B and Natural Killer (NK) cells as well as myeloid-derived suppressor cells (MDSCs) have been shown to suppress antigen-specific T cell responses (Deniz *et al.*, 2008, Bonertz *et al.*, 2009, van de Veen *et al.*, 2013) and which may limit the potential clinical benefit of antigen-reactive cells to the patient. Given that TAA-reactive T cells are closely associated with a survival benefit for patients (Walter *et al.*, 2012, Bailur *et al.*, 2015, Rittig *et al.*, 2016), understanding the immune contexts that permit the presence of such cells may allow superior prediction of patient survival, better treatment individualisation or the identification of new therapeutic targets.

Our previous study (Janssen *et al.*, 2016) investigated broad immunological features in breast cancer patients including HLA type, serum cytokines, tumour-

infiltrating leukocytes and blood leukocytes, with the aim of identifying the features which most accurately select patients with tumour-antigen reactive T cells. The results from that study showed blood leukocytes to be the most informative parameter for identifying which patients possess antigen reactive T cells, and reciprocally for identifying the patients who lack these cells. Considering our prior studies, here we aimed to establish a more accurate method of identifying patients with TAA-reactive T cells by expanding on the relatively limited set of blood leukocytes previously examined. The identification of easily measurable blood-based surrogate markers that indicate the presence of antigen-reactive T cells may allow the individual prediction of clinical course by avoiding the time- and resource-intensive *in vitro* stimulation procedures required to directly measure TAA-reactive T cells. The protocols required to measure antigen-reactive T cells are complex and require a high degree of expertise; thus finding easily measurable surrogate markers might improve protocol standardisation to facilitate use in less-specialised laboratories. Furthermore, understanding the composition of the immune system that favours the generation of TAA-reactive T cells may uncover new therapeutic targets or allow the selection of patients more likely to benefit from therapies which rely on the presence of tumour antigen-specific T cells such as vaccines or checkpoint inhibitors. As such, this study aimed to take the first step towards the eventual clinical employment of blood leukocytes as markers for antigen-reactive T cells. To this end, we measured a number of B, NK, and T cell populations at different stages of maturation and differentiation in the peripheral blood of 50 non-metastatic breast cancer patients and investigated their association with T cells reactive to the HER2, MUC1 and SUR tumour-associated antigens. Ongoing clinical follow-up will reveal which of the blood leukocyte populations measured here are associated with patient prognosis.

Clinical significance

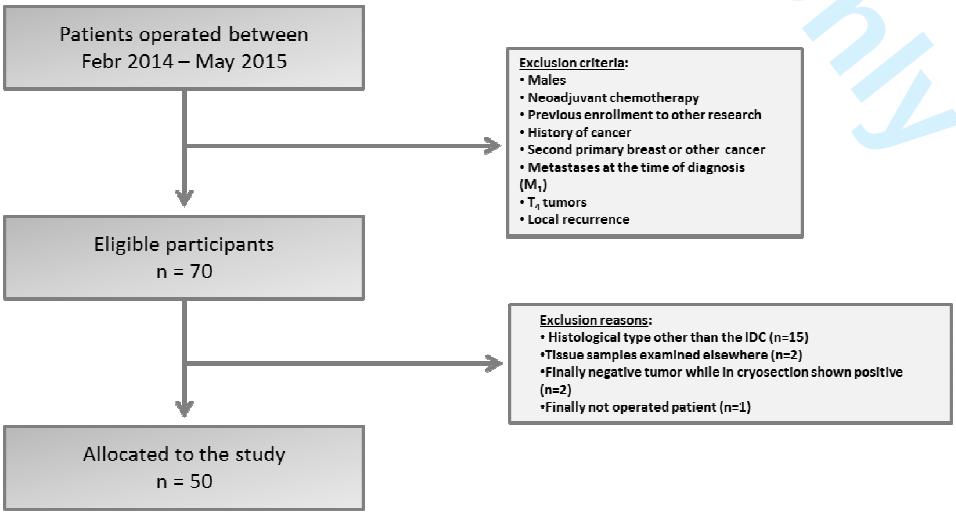
- Understanding the composition of the immune system that favours the generation of TAA-reactive T cells may allow superior prediction of patient survival, better treatment individualisation, the selection of patients more likely to benefit from therapies or the identification of new therapeutic targets
- The identification of easily measurable blood-based surrogate markers that indicate the presence of such cells may allow the use in routine clinical monitoring.

Materials and Methods

Patients

Blood from 50 patients with non-metastatic invasive carcinoma of the breast were recruited at St. Savas Cancer Hospital in Athens between February 2014 and May 2015.

Patient flow diagram



All samples were collected one day prior to surgery. Peripheral blood mononuclear cells were isolated from whole blood using Ficoll-Hypaque gradient and stored in liquid nitrogen before being shipped to the Tübingen University Hospital for analysis. Written informed consent was obtained for all patients, and the study was approved by the ethics committee of St. Savas Cancer Hospital (4-11-2013). Study participants were diagnosed with non-metastatic invasive ductal carcinoma, including patients with tumours of any size but without extension to the chest wall (i.e. patients of AJCC stages 1, 2A, 2B, 3A and 3C (if no extension to the chest wall) were included but AJCC stages 3B and 4 were not). None of these patients were treated with neoadjuvant chemotherapy or were enrolled in other research protocols, nor did they have any history of cancer or other serious health problems. Tumour expression of hormone receptors (progesterone (PR) and oestrogen receptors (ER)), the HER2 oncoprotein and the marker of cell proliferation Ki67 was available for all patients. Expression of MUC1 and SUR were not available. See Table 1 for a detailed description of the patient cohort.

Detection of antigen-reactive T cells

T cell responses to HER2, Mucin1 (MUC1) and Survivin (SUR) were measured after 12 days of *in vitro* culture. Detection of reactive T cells was performed as described in our prior study (Bailur *et al.*, 2015) (see Additional File 1 for a full list of antibodies employed). Cytometer setup and tracking beads (BD Bioscience) were run before and after each sample measurement to ensure consistency in machine performance. The inclusion of a positive biological control (stimulation with influenza peptides,) was used to certify consistency in sample quality and the prevention of false negative results.

Phenotypic analysis of T, B and Natural Killer cells

For characterisation of T, B and NK cells, PBMCs were thawed and stained as

previously described (Bailur *et al.*, 2015) using the antibody panels in Additional file 1. Antibody panels were established using fluorescence minus one controls. Cytometer setup and tracking beads were run before and after each sample measurement to ensure consistency in machine performance. The limited nature of patient material did not permit multiple testing of the same sample, but we performed multiple independent measurement of a healthy control donor (n = 3) in order to ensure consistency in measurement conditions.

Flow cytometry data analysis

Flow cytometry data were analysed by first excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA (ethidium monoazide)-positive events) and cell debris with the use of a morphological gate (see Additional file 2). The assessment of T cell responses to tumour-associated antigens was performed using the same method as in our previous study (Bailur *et al.*, 2015); we compared control (unstimulated) and peptide-stimulated cultures as described in the methods section "Detection of antigen-reactive T cells" and assigned a positive response when the frequency of T cells producing one or more cytokines in the stimulated sample was at least twice that of the control sample (Additional file 3). Additionally, each response was visually assessed to ensure the presence of a clearly distinguishable population of positive events. This method allows the detection of multiple cytokines from each patient, but does not directly assess whether production is from the same or different population of cells. Antibody-stained leukocyte populations, including the assessment of T cell responses to TAAs, were gated according to the approaches shown in Additional file 2-7.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, USA). Two independent groups were compared using the Mann-Whitney U test. Relationships across four grouping variables were assessed with Fisher's exact test. Correlations were assessed 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. For this reason statistical analyses were not corrected using the Bonferroni method, and the results should be interpreted as such.

Results

Peripheral blood leukocytes are associated with clinical parameters of breast cancer patients

We measured subsets of different peripheral blood leukocyte populations in the blood of 50 non-metastatic breast cancer patients and assessed whether T cells (240 different populations assessed, including regulatory T cells), B cells (19 populations), NK cells (70 populations) or myeloid cells (15 populations) were related to clinical features of these breast cancer patients. We observed that all leukocyte types (T cells, B cells, NK cells and myeloid cells) were associated with certain clinical parameters including tumour grade, AJCC stage, Ki67 tumour expression and lymph node infiltration by tumour cells. In order to compare these leukocyte types on their relevance to patient clinical features, we calculated the proportion of identified clinical correlations relative to the number of phenotypes tested. We found that T cells were the most relevant to patient clinical features, followed by B, NK and myeloid cells, respectively (Fig. 1).

Specifically, we found that patients of a higher AJCC stage and/or higher tumour grade

had lower levels of CD4+ T cells (AJCC stage: p = 0.02, tumour grade: p = 0.03) and NK cells (AJCC stage: p = 0.01) compared with patients with less advanced disease (Fig. 2A). Of note is that we could not identify such correlations for Tregs (data not shown), suggesting that these differences indicate a reduction in helper CD4+ T cells with disease progression. Higher tumour grade and/or AJCC stage was additionally found to be associated with higher levels of CD3hiCD56dim cells (tumour grade: p = 0.003, AJCC stage: p = 0.03) and memory B cells (tumour grade: p = 0.02) (Fig. 2B). We also found that patients with a greater number of infiltrated lymph nodes had lower levels of total CD4+ cells (p = 0.02) (Fig. 2C) and CD4+ regulatory T cells (p = 0.04, data not shown). Similar relationships were observed for tumour Ki67 expression and NK cells; patients with a higher percentage of tumour cells expressing Ki67 had lower levels of NK cells (p = 0.04). This stands in contrast to B cells for which we observed an opposite trend with Ki67 expression (p = 0.06) (Fig. 2D). Other leukocyte populations were also observed to correlate with patient clinical parameters (data not shown). We also examined whether relationships between the major populations of T, B, NK or myeloid cells exist. Of these, we observed only one significant relationship; an increasing frequency of CD20+CD40+ B cells was associated with higher levels of CD4+ regulatory T cells (CD4+CD25+FoxP3+) (p = 0.02, Additional file 8). Other leukocyte phenotypes such as MDSCs were not found to correlate with any other leukocyte population.

T cell responses to tumour-associated antigens in breast cancer patients

We detected T cells reactive to HER2, MUC1 or SUR in this cohort of breast cancer patients using *in vitro* peptide stimulation. Our prior study (Bailur *et al.*, 2015) showed that the presence of HER2-reactive T cells in breast cancer patients is associated with a more favourable prognosis. In the present study of prospectively recruited patients, we

have expanded the number of antigens tested to include MUC1 and SUR in addition to HER2. CD4+ or CD8+ T cells were considered reactive to an antigen if they produced any of the 6 cytokines that we measured (IL-2, IL-5, IL-10, IL-17, TNF and IFNγ). Antigen-reactive T cells were found to be common, with a high frequency of patients showing responses by either CD4+ or CD8+ cells to HER2 (96%), MUC1 (80%) and SUR (72%). We found that, in general, CD4+ reactive T cells were more common than CD8+ T cells.

Although tumour antigen-reactive T cells are predictive of patient prognosis, the time- and resource-intensive culture protocols required for their detection limits their use as part of routine clinical monitoring. As previously mentioned, our prior study (Janssen *et al.*, 2016) screened a broad set of patient features with the goal of finding those which are most capable of identifying patients with antigen-reactive T cells. Considering HLA type, serum cytokines, tumour-infiltrating leukocytes and blood leukocytes we found that the latter most accurately identified patients with antigen-reactive T cells. Based on these findings, we sought to extend blood leukocyte phenotyping beyond that previously examined with the goal of investigating if other populations of blood leukocytes can also be used to select patients with TAA-reactive T cells. If so, do they allow a more accurate selection of patients with these reactive T cells than was previously achieved? In the present study we have markedly increased the richness of the phenotyping analysis to encompass a total of 302 different blood leukocyte populations: 213 for T cells, 19 in the case of B cells, and 70 NK cell populations (Additional file 9). Regulatory T cells and myeloid cells including MDSCs were examined in our prior study and were therefore not considered again here.

Blood leukocytes identify patients with tumour antigen-reactive T cells

NK cells are relevant for T cell responses to MUC1 and SUR but not HER2

Assessing antigen-reactive T cells for relationships with NK cells showed that different NK cell populations were associated with T cell responses to MUC1 or SUR but not with HER2 (but note that because the vast majority of patients showed CD4+ HER2 responses it was not possible to test for CD4+ associations to this antigen). We observed different populations of NK cells to be present at either higher or lower frequencies in patients with MUC1- or SUR-reactive T cells than in those without. For example, patients with CD4+ T cells reactive to MUC1 had higher levels of CD27+ NK cells compared to patients who did not possess these reactive T cells ($p = 0.04$) (Fig. 3A). In contrast, another population of NK cells (CD56dimCD16-NKG2D+) was found to be lower in patients with MUC1-responsive T cells ($p = 0.04$) (Fig. 3A). A full set of correlations between NK cells and antigen-reactive T cells can be found in Additional file 10.

High levels of B cells as a marker of patients with HER2-reactive T cells

In contrast to NK cells, which were only associated with T cell responses to MUC1 and SUR, we found that B cells only correlated with HER2-reactive T cells. Of the 19 populations of B cells examined, we found one which was associated with HER2 T cell responses; patients with CD8+ HER2-reactive T cells had higher levels of CD19+CD38-CD27+CD20- B cells ($p = 0.04$) (Fig. 3B). Apart from this, other relationships between B cells and CD4+ or CD8+ T cells reactive to any antigen were not found.

Blood T cells broadly correlate with HER2, MUC1 and SUR-reactive T cells

Compared with NK and B cells, T cells were found to widely identify patients with HER2- MUC1- or SUR-reactive T cells. Of the total number of identified correlations between blood leukocytes and antigen-reactive T cells, 89% of these were with T cell populations, whereas NK cells and B cells accounted for only 10% and 1% of correlations, respectively. Interestingly, we observed that in the vast majority of cases (82/94), patients with TAA-reactive T cells had higher levels of blood T cells than patients without antigen-reactive T cells. This was true for CD4+ and CD8+ T cells of all differentiation stages. For example, high relative levels of CD8+CD27- T cells were associated with SUR-reactive T cells ($p = 0.0007$) (Fig. 3C), while high levels of CD8+TEMRA T cells were associated with MUC1-reactive T cells ($p = 0.01$) (Fig. 3C). In addition, a large number of other correlations between blood T cell populations and antigen-reactive T cells were found and appear in Additional file 11.

A combination of multiple blood leukocyte populations improves the accuracy of predicting patients with TAA-reactive T cells

In our prior study we combined immunological parameters in an attempt to more accurately select patients with TAA-reactive T cells. The results showed that the combination of different types of immunological parameters (such as tumour infiltrating leukocytes and blood leukocytes), but also the combination of multiple different blood leukocyte populations resulted in superior prediction of TAA responses (Janssen *et al.*, 2016). Based on this approach, we sought to determine more accurate markers of TAA-reactive T cells than was previously possible with a relatively limited survey of blood leukocytes. By combining multiple populations of blood leukocytes, we were able to identify groups of patients who all possessed antigen-reactive T cells and therefore yield up to 100% sensitivity and specificity in the selection of patients with antigen-reactive T

cells. For example, a group of patients with above median levels of stem memory T cells (TSCM) and CD3-CD56- cells consisted exclusively of patients who possessed MUC-reactive CD4+ T cells (Fig. 4). Similarly, selecting patients on higher than median levels of NK (CD3-CD56hiCD16-CD27+) and NKT (NKG2D+ CD3+CD56dim) cells identified a group who all possessed CD8+ T cells reactive to SUR (Fig. 4). This approach therefore shows a high degree of accuracy in the selection of patients who possess antigen-reactive T cells.

Discussion

Our prior studies have shown that patients possessing TAA-reactive peripheral CD8+ T cells experience a more favourable prognosis, especially if they have lower than median levels of MDSCs (Bailur *et al.*, 2015). Avoiding the complexity and extended duration of directly testing T cell reactivities and phenotyping MDSCs, assessing blood leukocytes is the most accurate method of identifying patients with these characteristics (Janssen *et al.*, 2016). Considering this, the present investigation was designed to investigate blood leukocytes as markers of TAA-reactive T cells in greater detail. We had two primary aims in performing this study: by conducting an in-depth survey of peripheral immune cells in breast cancer, we sought to identify the leukocyte populations most relevant for clinical outcome, either alone or when combined with information regarding tumour antigen-reactive T cells. We continue to monitor this group of prospectively recruited patients regarding their clinical course, but the information available so far does not yet permit analysis. In addition, we aimed to identify surrogate markers for TAA-reactive T cells that may be used as part of patient clinical monitoring. The establishment of such surrogate markers will serve several purposes. Firstly, the measurement of blood leukocytes could replace the measurement of antigen-reactive T cells, for which a lengthy (12 day), expensive and labour intensive

in vitro stimulation period is required, as well as the additional resources and expertise needed to interpret the results. Furthermore, these culture protocols are complex and demand large numbers of leukocytes, often a limiting factor when dealing with material from cancer patients. Manufacturer- or batch-dependent differences due to the reagents, particularly the TAA peptides, are currently unknown but could likely influence the results. These aspects contribute to the difficulty of standardising the measurement of antigen-reactive T cells, while the considerable costs in time, labour and material have also proven prohibitive for the large-scale implementation of this approach in the clinical setting thus far. Given these limitations and the relative simplicity of directly measuring blood leukocytes, the latter may represent a viable alternative to indirectly allow the clinical monitoring of anti-tumour responses. Secondly, measuring blood leukocytes indirectly selects patients with a more favourable prognosis. Hence identifying these patients as part of routine clinical monitoring may spare them from unnecessary treatment or allow the administration of tailored forms of therapy. The rapid clinical implementation of immunotherapies including immunomodulatory antibodies and cancer vaccines means that optimising the use of these treatments will be increasingly important in future. The efficacy of such approaches is presumably through the stimulation, generation and amplification of tumour antigen-reactive T cells, and the downregulation of suppressive elements such as Tregs and MDSCs. Therefore, identifying patients with TAA-reactive T cells, or patients who show immune profiles favourable to the generation of such immune responses, may have implications for selecting patients more likely to respond to immunotherapy. The findings in this study allow the selection of patients with antigen-reactive T cells, but importantly they also identify patients who lack them. As such these results can be used to pinpoint differences in the immune system that are associated with either the presence or absence

of antigen-reactive T cells. These immune differences may thus represent novel therapeutic targets, whereby the immune systems of patients lacking antigen-reactive T cells can be altered to more closely resemble the immune systems of patients with TAA-reactive T cells. This form of therapy could potentially be used together with immunotherapies to enhance their activity.

We found that peripheral B and NK cells allowed us to select patients with antigen-reactive T cells, but with these leukocyte types we could only identify patients with certain types of T cell responses. In contrast, blood T cells were found to widely correlate with both CD4+ and CD8+ T cells reactive to all antigens tested. Interestingly, the set of T cell phenotypes observed to correlate with antigen-reactive T cells did not share any definable features; we found that these phenotypes covered a range of differentiation stages from naïve to late-differentiated T cells. Despite this, we observed that in the vast majority of cases, patients with TAA-reactive T cells showed higher levels of blood T cells than patients without antigen-reactive cells, implying that the phenotypes found to correlate may directly participate or assist in the generation of antigen-reactive T cells. This is further supported by the observation that blood T cells accounted for 89% of all correlations with antigen-reactive T cells. In contrast B and NK cells together accounted for the remaining 11% of correlations, and unlike T cells, were not relevant to every type of T cell response. These findings suggests that therapies which alter the immune system of breast cancer patients to more closely mirror the immune system of patients with TAA-reactive T cells may result in enhanced anti-tumour responses by T cells. Because we found essentially all T cell phenotypes within both the CD4+ and CD8+ compartments to be higher in patients with TAA-reactive T cells, elevating levels of T lymphocytes in general, such as with gamma-chain cytokines, may result in more favourable conditions for the generation of antigen-

reactive T cells. Underlining the clinical importance of peripheral T cells is that compared with NK and B cells, we found that T cells were most commonly associated with patient clinical parameters, adding further weight to their proposition as therapeutic targets.

Conclusion

The results of this study show that phenotypic assessment of blood leukocytes allows the accurate identification of breast cancer patients with functional TAA-reactive T cells. These findings are intended as the first step towards the possible clinical implementation of measuring blood leukocytes as part of routine monitoring to select patients with antigen-reactive T cells. Ongoing clinical follow-up will reveal which of the peripheral immune populations measured here are informative for patient outcome, and whether they more accurately predict survival when combined with the assessment of antigen-reactive T cells.

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Declaration of interest

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contribution

CH provided samples. NJ, LS, CH, SF, GP and CS contributed to the conception and study design. NJ, LS and CS acquired, analysed and interpreted data. NJ and CS wrote the manuscript. LS, CH, SF and GP edited the manuscript. All authors read and approved the final version of the manuscript.

References

Bailur, J.K., Gueckel, B., Derhovanessian, E. & Pawelec, G., 2015. Presence of circulating Her2-reactive CD8 + T-cells is associated with lower frequencies of myeloid-derived suppressor cells and regulatory T cells, and better survival in older breast cancer patients. *Breast Cancer Res,* 17**,** 34.

Bonertz, A., Weitz, J., Pietsch, D.H., Rahbari, N.N., Schlude, C., Ge, Y., Juenger, S., Vlodavsky, I., Khazaie, K., Jaeger, D., Reissfelder, C., Antolovic, D., Aigner, M., Koch, M. & Beckhove, P., 2009. Antigen-specific Tregs control T cell responses against a limited repertoire of tumor antigens in patients with colorectal carcinoma. *J Clin Invest,* 119**,** 3311-21.

Deniz, G., Erten, G., Kucuksezer, U.C., Kocacik, D., Karagiannidis, C., Aktas, E., Akdis, C.A. & Akdis, M., 2008. Regulatory NK cells suppress antigen-specific T cell responses. *J Immunol,* 180**,** 850-7.

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. & Shipp, C., 2016. Peripheral T cell responses to tumour antigens are associated with molecular, immunogenetic and cellular features of breast cancer patients. *Breast Cancer Res Treat*.

Rittig, S.M., Haentschel, M., Weimer, K.J., Heine, A., Muller, M.R., Brugger, W., Horger, M.S., Maksimovic, O., Stenzl, A., Hoerr, I., Rammensee, H.G., Holderried, T.A., Kanz, L., Pascolo, S. & Brossart, P., 2016. Long-term survival correlates with immunological responses in renal cell carcinoma patients treated with mRNA-based immunotherapy. *Oncoimmunology,* 5**,** e1108511.

Van De Veen, W., Stanic, B., Yaman, G., Wawrzyniak, M., Sollner, S., Akdis, D.G., Ruckert, B., Akdis, C.A. & Akdis, M., 2013. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol,* 131**,** 1204-12.

Walter, S., Weinschenk, T., Stenzl, A., Zdrojowy, R., Pluzanska, A., Szczylik, C., Staehler, M., Brugger, W., Dietrich, P.Y., Mendrzyk, R., Hilf, N., Schoor, O., Fritsche, J., Mahr, A., Maurer, D., Vass, V., Trautwein, C., Lewandrowski, P., Flohr, C., Pohla, H., Stanczak, J.J., Bronte, V., Mandruzzato, S., Biedermann, T., Pawelec, G., Derhovanessian, E., Yamagishi, H., Miki, T., Hongo, F., Takaha, N., Hirakawa, K., Tanaka, H., Stevanovic, S., Frisch, J., Mayer-Mokler, A., Kirner, A., Rammensee, H.G., Reinhardt, C. & Singh-Jasuja, H., 2012. Multipeptide immune response to cancer vaccine IMA901 after single-dose cyclophosphamide associates with longer patient survival. *Nat Med,* 18**,** 1254-61.

Table 1 Characteristics of the breast cancer patients

Patient clinicopathological parameters (n = 50)	
Median Age (range in years)	56 (27-78)
AJCC staging	
1	15
2A	10
2B	12
3A	12
3C	1
Receptor status	
Triple negative	5
Oestrogen receptor +	42
Progesterone receptor+	33
HER2+	15
Ki67	
<10%	7
10% - 20%	21
>20%	22
T stage	
1	21

2	26
3	3
N stage	
0	21
1	17
2	11
3	1
Grade	
1	0
2	26
3	24

Figure 1. Relevance of T, B, NK and myeloid cells to patient clinical parameters.

For each leukocyte type (T, B, NK and myeloid cells) the proportion of identified correlations with patient clinical features was calculated as a percentage relative to the number of phenotypes tested. We observed T cells to be the most relevant leukocyte type to patient clinical features, followed by B, NK and myeloid cells, respectively.

Figure 2. Levels of blood leukocytes are associated with clinical features of breast cancer patients.

T, B, NK and myeloid cells were measured in the blood of 50 breast cancer patients using flow cytometry and were tested for association with patient clinical parameters (Mann-Whitney U test). We found lower levels of CD4+ T cells and NK cells in patients with higher tumour grade or AJCC stage (A). Conversely, patients with a higher grade or AJCC stage showed higher levels of memory B cells or CD3hiCD56dim cells (B). We also found that patients with a greater number of infiltrated lymph nodes had lower CD4+ T cell frequencies (C), while tumour expression of Ki67 was associated with either lower levels of NK cells or showed a trend towards higher levels of memory B cells (D). Phenotypes given in square brackets indicate the reference population used to calculate the frequency of the respective leukocyte population. Bars indicate median values. *, $p < 0.05$; **, $p < 0.01$

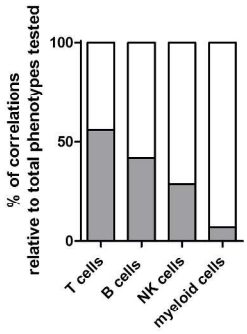
Figure 3. Levels of blood leukocytes differ in patients with HER2, MUC1 and SUR-reactive T cells.

T cell responses to HER2, MUC1 or SUR were measured in 50 breast cancer patients following 12 days of *in vitro* expansion. Levels of blood leukocytes were compared between patients with reactive T cells and those who lacked these cells. T cells were

considered responsive to an antigen if they showed a positive signal for any of the six measured cytokines (Mann-Whitney U test). This showed high or low levels of NK cells to be associated with the presence of MUC1-reactive T cells (A). High levels of B cells were found in patients with CD8+ HER2-reactive T cells (B), while high levels of CD8+CD27- T cells (C, left panel) and CD8+ TEMRA cells (C, right panel) were associated with the presence of SUR- or MUC1-reactive T cells. Phenotypes in square brackets indicate the reference population used to calculate the frequency of each leukocyte population. Bars indicate median values. HER2, Human Epidermal growth factor Receptor 2; MUC1, Mucin 1; NR, Non-Responder; R, Responder; SUR, Survivin; TAA, Tumour-Associated Antigen; TEMRA, terminally differentiated effector memory cells; *, $p < 0.05$; **, $p < 0.01$

Figure 4. Combining blood leukocyte populations allows more accurate prediction of patients with anti-tumour reactive T cells.

Selecting patients with >median levels of NKT and NK cells resulted in sensitivity and specificity of 90% and 100% in identifying patients with CD8+ SUR-reactive T cells. Similarly, the combination of >median levels of CD3-CD56- and TSCM resulted in 75% sensitivity and 100% specificity in selecting patients with CD4+ MUC1-reactive T cells. MUC1, Mucin1; NK cells, Natural Killer cells; NKT cells, Natural killer T cells, SUR, Survivin; TSCM, stem memory T cells



Relevance of T, B, NK and myeloid cells to patient clinical parameters. For each leukocyte type (T, B, NK and myeloid cells) the proportion of identified correlations with patient clinical features was calculated as a percentage relative to the number of phenotypes tested. We observed T cells to be the most relevant leukocyte type to patient clinical features, followed by B, NK and myeloid cells, respectively.

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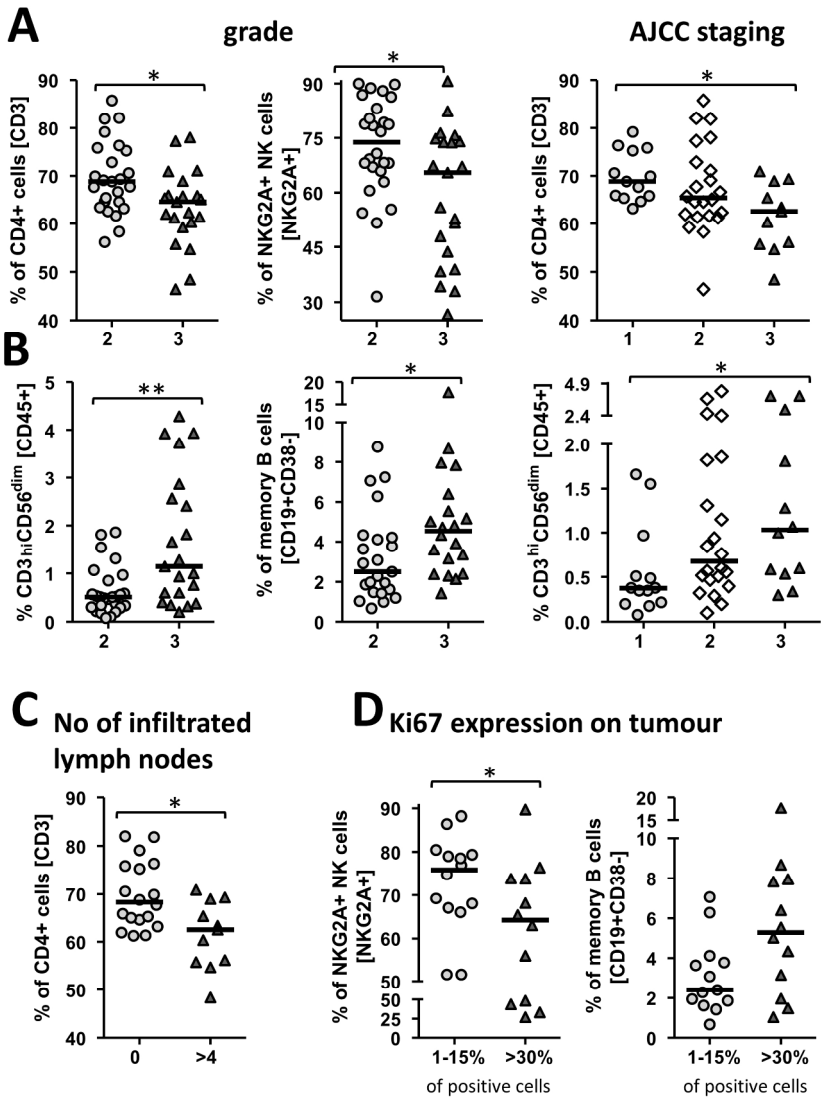

Levels of blood leukocytes are associated with clinical features of breast cancer patients. T, B, NK and myeloid cells were measured in the blood of 50 breast cancer patients using flow cytometry and were tested for association with patient clinical parameters (Mann-Whitney U test). We found lower levels of CD4+ T cells and NK cells in patients with higher tumour grade or AJCC stage (A). Conversely, patients with a higher grade or AJCC stage showed higher levels of memory B cells or CD3hiCD56dim cells (B). We also found that patients with a greater number of infiltrated lymph nodes had lower CD4+ T cell frequencies (C), while tumour expression of Ki67 was associated with either lower levels of NK cells or showed a trend towards higher levels of memory B cells (D). Phenotypes given in square brackets indicate the reference population used to calculate the frequency of the respective leukocyte population. Bars indicate median values. *, $p < 0.05$; **, $p < 0.01$

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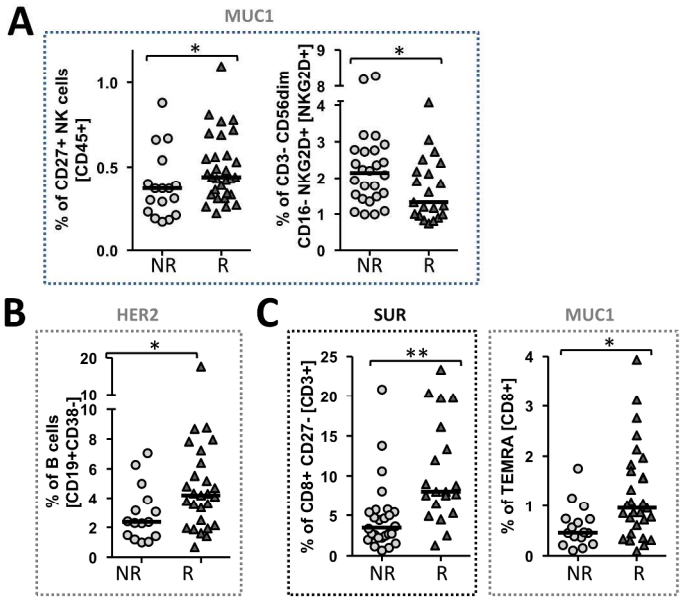

Levels of blood leukocytes differ in patients with HER2, MUC1 and SUR-reactive T cells.
T cell responses to HER2, MUC1 or SUR were measured in 50 breast cancer patients following 12 days of in vitro expansion. Levels of blood leukocytes were compared between patients with reactive T cells and those who lacked these cells. T cells were considered responsive to an antigen if they showed a positive signal for any of the six measured cytokines (Mann-Whitney U test). This showed high or low levels of NK cells to be associated with the presence of MUC1-reactive T cells (A). High levels of B cells were found in patients with CD8+ HER2-reactive T cells (B), while high levels of CD8+CD27- T cells (C, left panel) and CD8+ TEMRA cells (C, right panel) were associated with the presence of SUR- or MUC1-reactive T cells. Phenotypes in square brackets indicate the reference population used to calculate the frequency of each leukocyte population. Bars indicate median values. HER2, Human Epidermal growth factor Receptor 2; MUC1, Mucin 1; NR, Non-Responder; R, Responder; SUR, Survivin; TAA, Tumour-Associated Antigen; TEMRA, terminally differentiated effector memory cells; *, $p < 0.05$; **, $p < 0.01$

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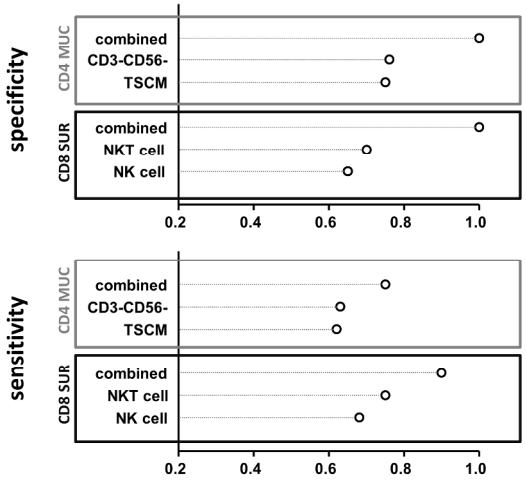


Combining blood leukocyte populations allows more accurate prediction of patients with anti-tumour reactive T cells.
Selecting patients with >median levels of NKT and NK cells resulted in sensitivity and specificity of 90% and 100% in identifying patients with CD8+ SUR-reactive T cells. Similarly, the combination of >median levels of CD3-CD56- and TSCM resulted in 75% sensitivity and 100% specificity in selecting patients with CD4+ MUC1-reactive T cells. MUC1, Mucin1; NK cells, Natural Killer cells; NKT cells, Natural killer T cells, SUR, Survivin; TSCM, stem memory T cells

254x190mm (300 x 300 DPI)

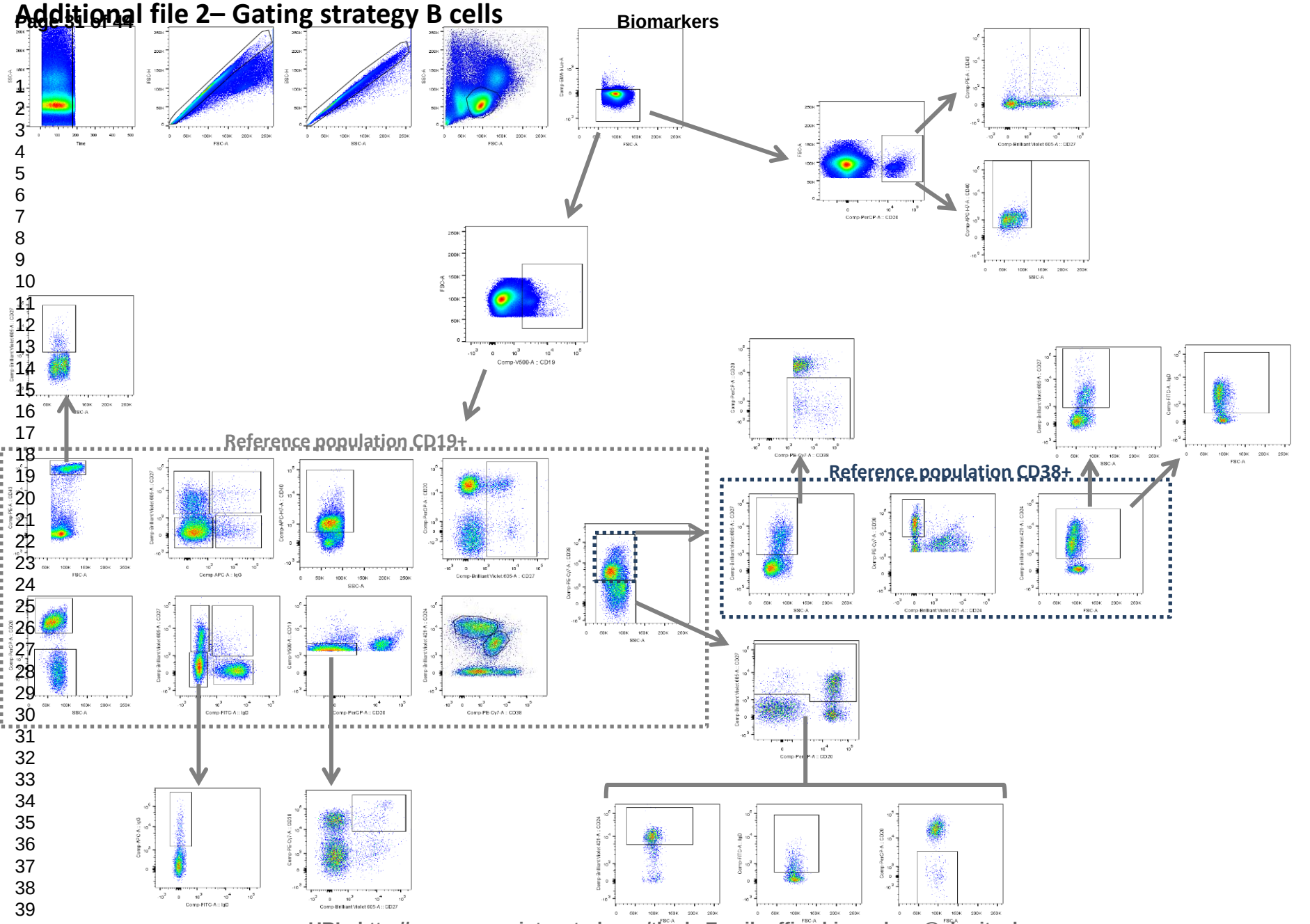
Additional file 1 – Full list of antibodies employed

The following table summarizes a full list of antibodies employed in this study

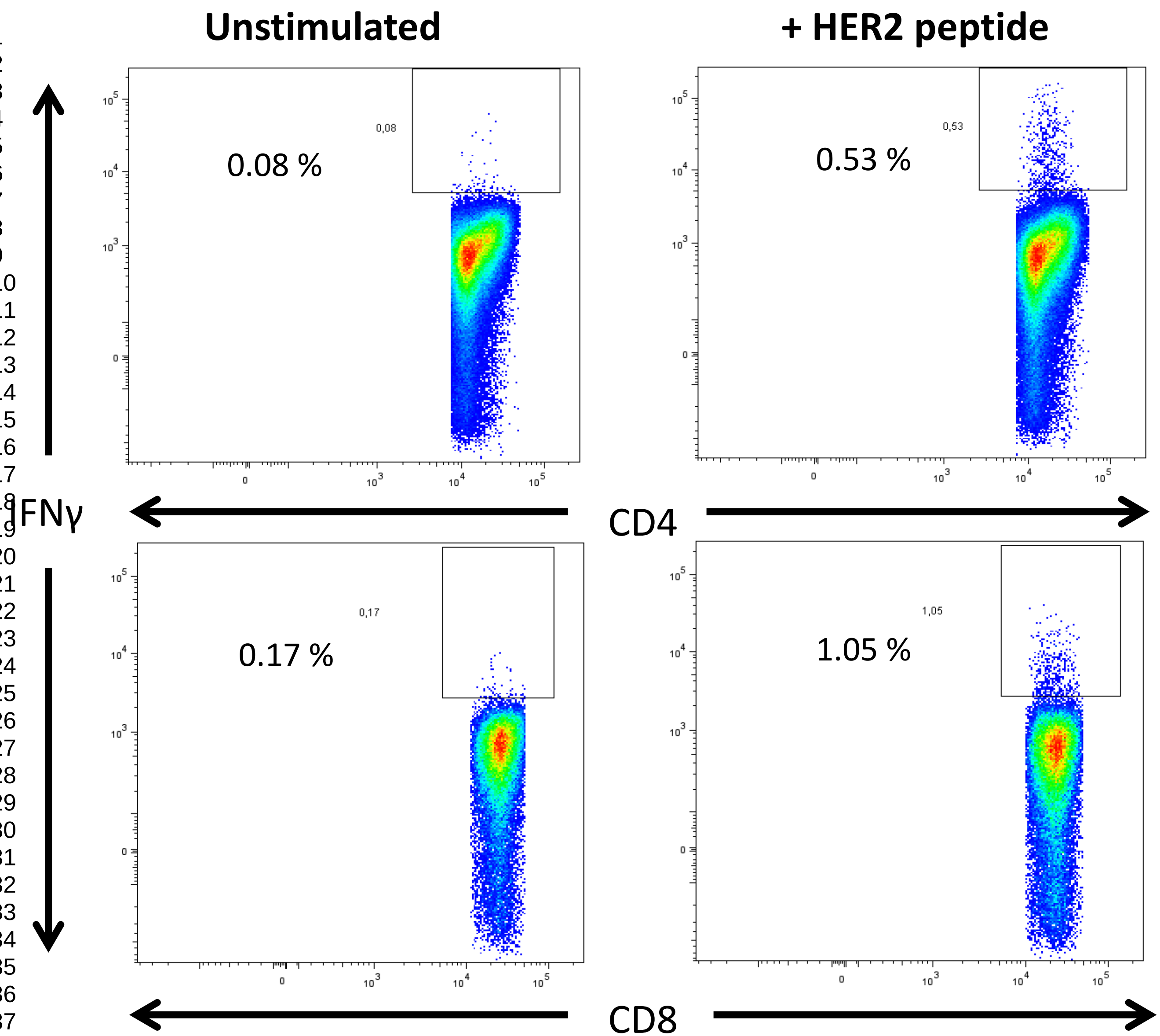
TAA-reactive T cells	
Cell surface	
CD3-BV605	Bio Legend (San Diego, USA)
CD4-Pacific Blue	Bio Legend
CD8-APC-H7	BD Biosciences (Franklin Lakes, USA)
Intracellular	
IL-2-Alexa Fluor 700	Bio Legend
IL-5-PE	Bio Legend
IL-10-APC	Milteny Biotec (Bergisch Gladbach, Germany)
IL-17-PerCP-Cy5.5	eBiosciences (San Diego, USA)
IFNg-PECy7	BD Biosciences
TNF-FITC	Bio Legend
B cells	
CD24-BV421	Bio Legend
CD19-V500	BD Biosciences
IgD-FITC	BD Biosciences
CD43-PE	Bio Legend
CD20-PerCP	Bio Legend
CD38-Pe-Cy7	Bio Legend
IgG-APC	Bio Legend
CD40-APC-H7	BD Biosciences
CD27-BV605	Bio Legend
NK cells	
CD57-PB	Bio Legend
CD45-V500	BD Biosciences
CD159a-PE	Milteny Biotec
CD161-FITC	BD Biosciences
CD314-PerCP-Cy5.5	BD Biosciences
CD159c-PE-Vio 770	Milteny Biotec
CD3-APC	Bio Legend
CD8-APC-H7	BD Biosciences
CD27-A700	Bio Legend
CD56-BV605	BD Biosciences
CD16-BV711	BD Biosciences

T cells	
CD3-A700	BD Biosciences
CD4-PerCP	BD Biosciences
CD8-APC-H7	BD Biosciences
CD27-APC	Bio Legend
CD28-PE	BD Biosciences
CD45RA-V450	BD Biosciences
CD57-FITC	Immunotools (Friesoythe, Germany)
CD95-PeCy7	eBiosciences
CD279-PerCP-Cy5.5	Bio Legend
unconjugated	
CCR7	R&D System (Minneapolis, USA)
PO	Invitrogen (Carlsbad, USA)

Additional file 2– Gating strategy B cells

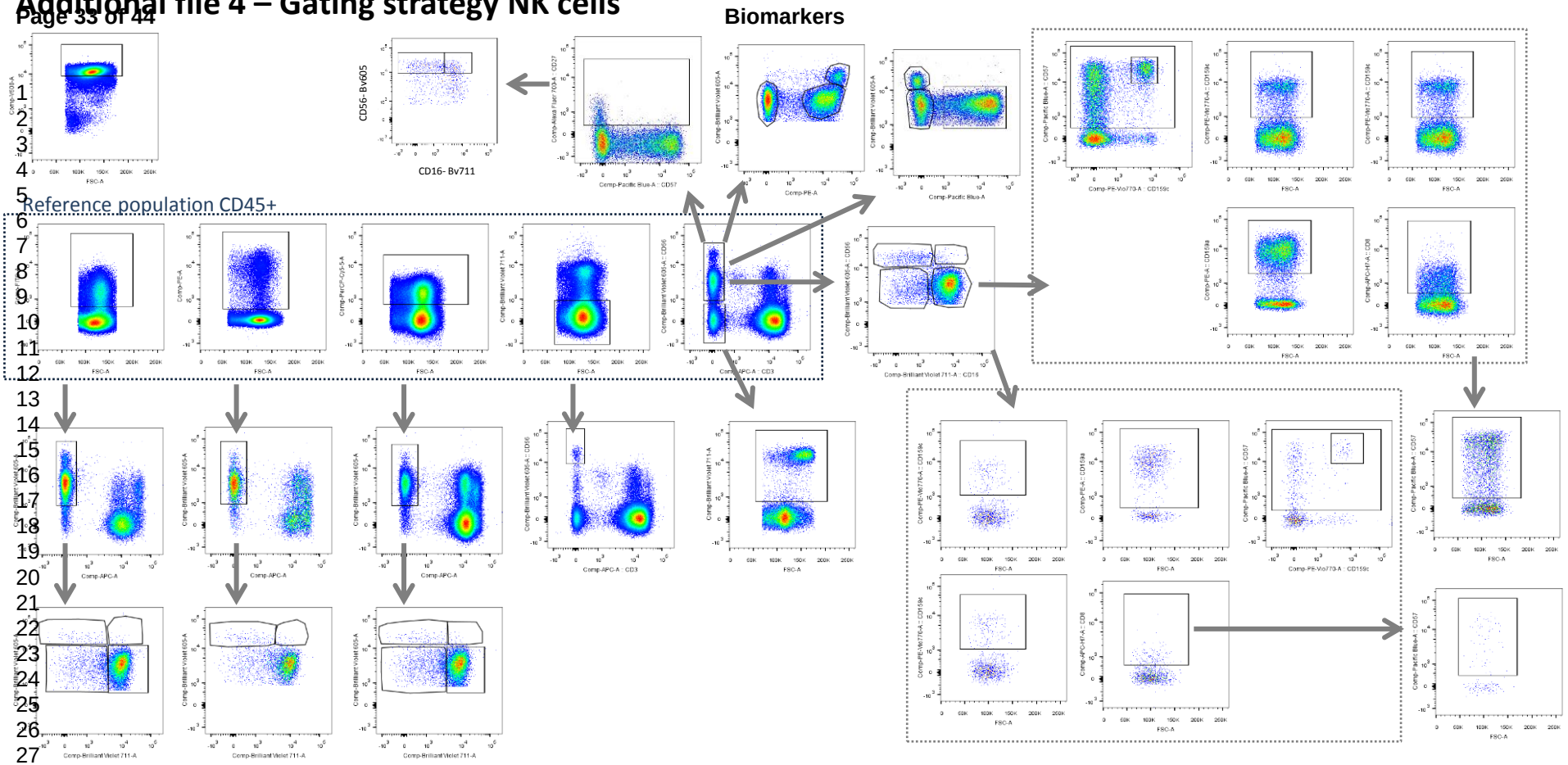


B cell gating strategy. Flow cytometry data were analysed first by excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate. Antibody-stained B cell populations, were gated according to the approaches shown here using the antibody panel summarized in Additional file 1.



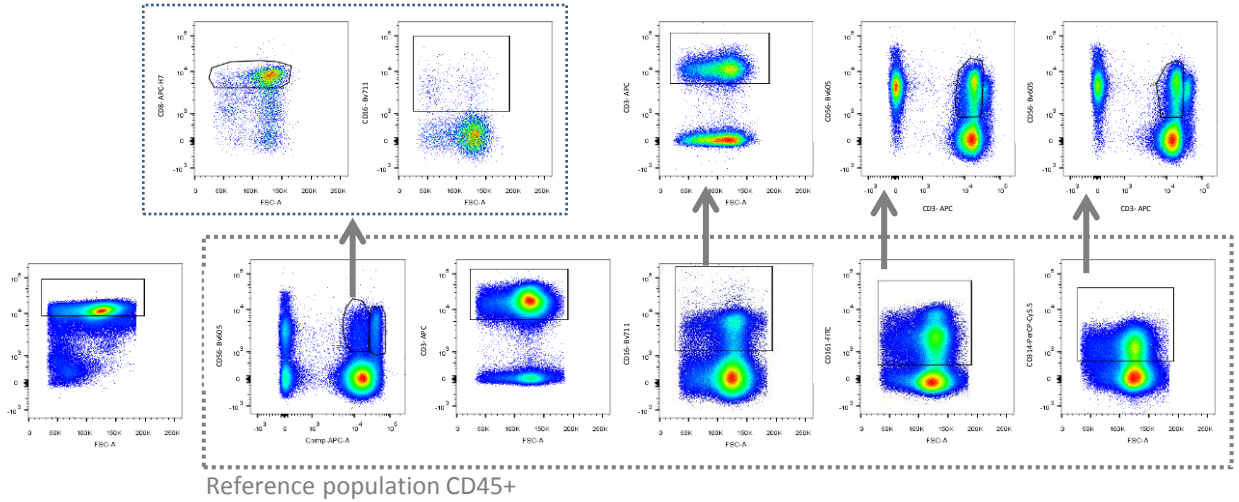
Control (left panels) and stimulated (right panels) CD4 (upper panels) and CD8 (lower panels) example showing a positive response for IFNγ to HER2. We assigned a positive response when the frequency of T cells producing any cytokine in the stimulated sample was at least twice that of the control sample. Additionally, each response was visually assessed to ensure the presence of a clearly distinguishable population of positive events. T cell responses were considered categorically (present or absent) in addition to a

Additional file 4 – Gating strategy NK cells



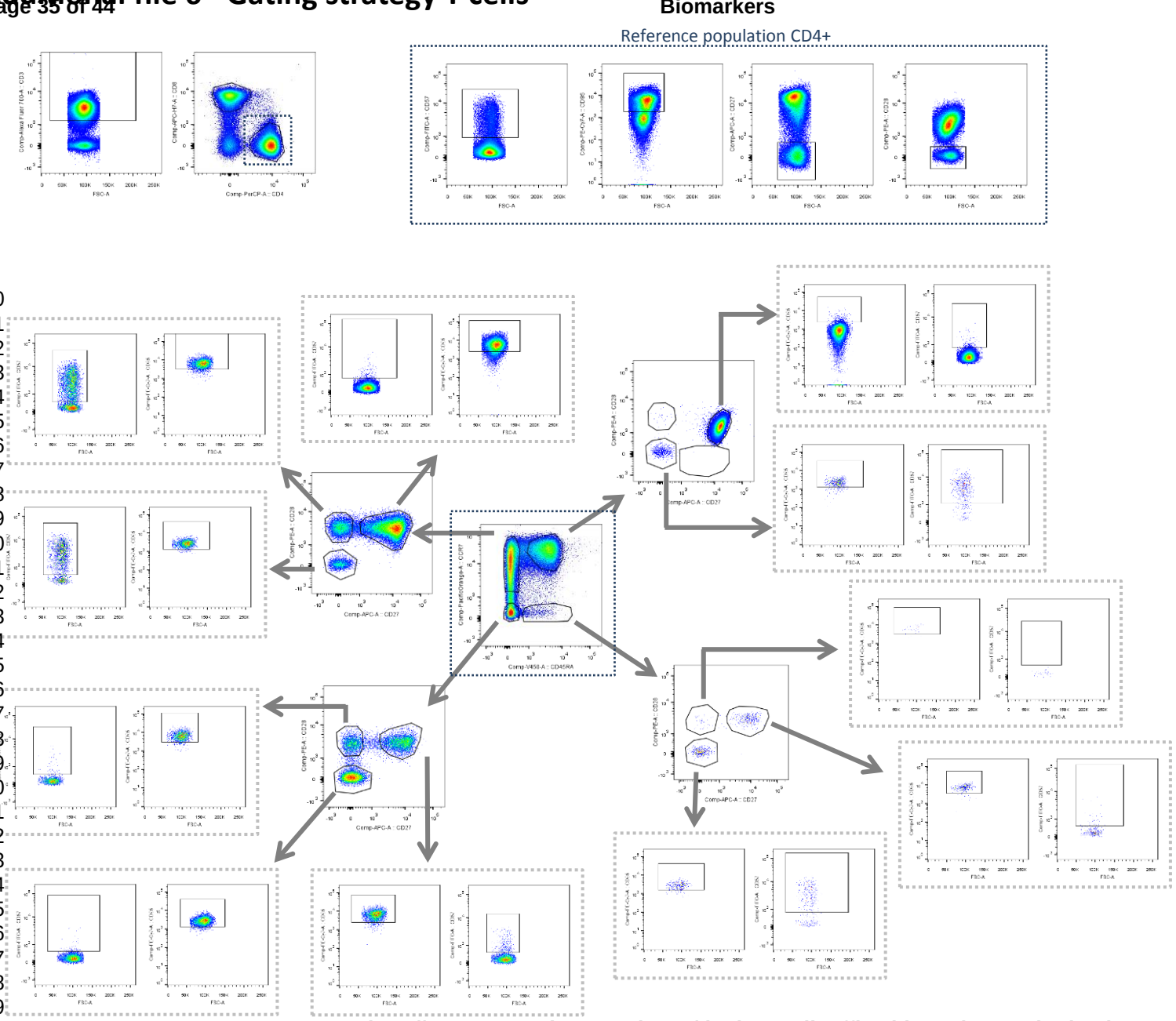
NK cell gating strategy. Flow cytometry data were analysed first by excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate (see Additional file 2). Antibody-stained NK cell populations, were gated according to the approaches shown here using the antibody panel summarized in Additional file 1.

Additional file 5 – Gating strategy NKT cells



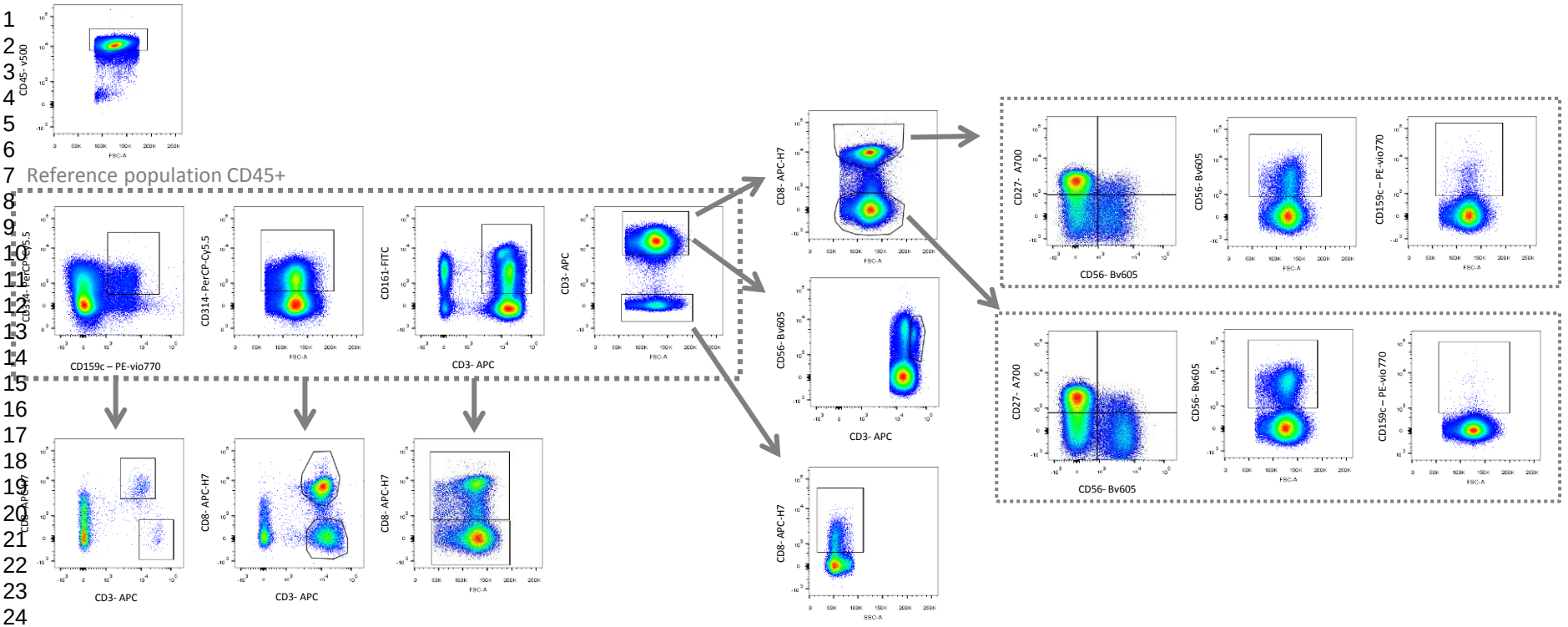
NKT cell gating strategy. Flow cytometry data were analysed first by excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate (see Additional file 2). Antibody-stained NKT cell populations were gated according to the approaches shown here using the antibody panel summarized in Additional file 1.

Additional file 6– Gating strategy T cells

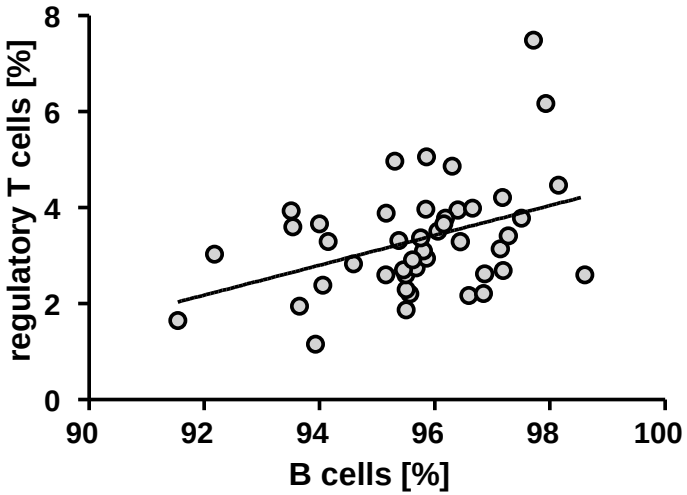


T cell gating strategy. Flow cytometry data were analysed first by excluding events not part of the main acquisition population using a time vs side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate (see Additional file 2). Antibody-stained T cell populations, were gated according to the approaches shown here using the antibody panel summarized in Additional file 1.

Additional file 7 – Gating strategy T cells (continued)



T cell gating strategy. Flow cytometry data were analysed first by excluding events not part of the main acquisition population using a time-vs-side scatter gate. Cell doublets were then removed before the exclusion of dead cells (EMA-positive events) and cell debris with the use of a morphological gate (see Additional file 2). Antibody-stained CD4+ T cell populations, were gated according to the approaches shown here using the antibody panel summarized in Additional file 1.



Frequency of B cells was positively associated with higher levels of CD4+ regulatory T cell.
We examined whether relationships between the major populations of T, B, NK or myeloid cells exist.
Of these, we observed only one significant relationship; an increasing frequency of
CD20+CD40+ B cells was associated with higher levels of CD4+ regulatory T cells (CD4+CD25+FoxP3+) ($p = 0.02$),

Additional file 9 - Total leukocyte populations tested

T cell phenotypes	
CD4+ [CD3+]	CD8+ [CD3+]
CD3+ CD4+ CD27- [CD3+]	CD8+ CD27- [CD3+]
CD4+ CD28- [CD3+]	CD8+ CD28- [CD3+]
CD4+ CD57+ [CD3+]	CD8+ CD57+ CD3+]
CD4+ CD95+ [CD3+]	CD8+ CD95+ [CD3+]
CD4+CD45RA+ CCR7+ (Naive) |[CD3+]	CD8+CD45RA+ CCR7+ (Naive) [CD3+]
CD4+CD45RA+ CCR7+ (Naive)/CD27+ CD28+ [CD3+]	CD8+CD45RA+ CCR7+ (Naive)/CD27+ CD28+ [CD3+]
CD4+CD45RA+ CCR7+ (Naive)/CD27+ CD28+/CD57+ [CD3+]	CD8+CD45RA+CCR7+(Naive)/CD27+CD28+/CD95+(TSCM)[CD3+]
CD4+CD45RA+ CCR7- (TEMRA) [CD3+]	CD8+CD45RA+ CCR7+ (Naive)/CD27- CD28- [CD3+]
CD4+CD45RA- CCR7+ (CM) [CD3+]	CD8+CD45RA+ CCR7+ (Naive)/CD27- CD28-/CD57+ [CD3+]
CD4+CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM) [CD3+]	CD8+CD45RA+ CCR7+ (Naive)/CD27- CD28-/CD95+ [CD3+]
CD4+CD45RA-CCR7+ (CM)/CD27+ CD28+ (CM)/CD57+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA) [CD3+]
CD4+CD45RA- CCR7+(CM)/CD27+ CD28+ (CM)/CD95+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27+ CD28+ [CD3+]
CD4+CD45RA- CCR7+ (CM)/CD27- CD28+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27+ CD28+ /CD95+ [CD3+]
CD4+CD45RA- CCR7+ (CM)/CD27- CD28+/CD95+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27+ CD28- [CD3+]
CD4+CD45RA- CCR7- (EM)[CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27+ CD28-/CD95+ [CD3+]
CD4+CD45RA- CCR7- (EM)/CD27+ CD28+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27- CD28- (E) [CD3+]
CD4+CD45RA- CCR7- (EM)/CD27+ CD28+ /CD95+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27- CD28- (E)/CD57+ [CD3+]
CD4+CD45RA- CCR7- (EM)/CD27- CD28+ [CD3+]	CD8+CD45RA+ CCR7- (TEMRA)/CD27- CD28- (E)/CD95+ [CD3+]
CD4+CD45RA- CCR7- (EM)/CD27- CD28+/CD95+ [CD3+]	CD8+CD45RA- CCR7+ (CM) [CD3+]
CD4+ CD27- [CD4+]	CD8+CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM) [CD3+]
CD4+ CD28- [CD4+]	CD8+CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM)/CD57+ [CD3+]
CD4+ CD57+ [CD4+]	CD8+CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM)/CD95+ [CD3+]
CD4+ CD95+ [CD4+]	CD8+CD45RA- CCR7+ (CM)/CD27+ CD28- [CD3+]
CD45RA+ CCR7+ (Naive) [CD4+]	CD8+CD45RA- CCR7+ (CM)/CD27+ CD28-/CD95+ [CD3+]
CD45RA+ CCR7+ (Naive)/CD27+ CD28+ [CD4+]	CD8+CD45RA- CCR7+ (CM)/CD27- CD28+ [CD3+]
CD45RA+ CCR7+ (Naive)/CD27+ CD28+/CD57+ [CD4]+	CD8+CD45RA- CCR7+ (CM)/CD27- CD28+/CD95+ [CD3+]
CD45RA+ CCR7+ (Naive)/CD27+ CD28+/CD95+ (TSCM) [CD4]	CD8+CD45RA- CCR7+ (CM)/CD27- CD28-/CD57+ [CD3+]
CD45RA+ CCR7- (TEMRA)[CD4+]	CD8+CD45RAint CCR7int [CD3+]
CD45RA- CCR7+ (CM)[CD4+]	CD8+CD45RAint CCR7int/CD27+ CD28+ [CD3+]
CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM) [CD4+]	CD8+CD45RAint CCR7int/CD27+ CD28+ /CD95+ [CD3+]
CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM)/CD57+ [CD4+]	CD8+CD45RAint CCR7int/CD27+ CD28- [CD3+]
CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM)/CD95+ [CD4+]	CD8+CD45RAint CCR7int/CD27+ CD28-/CD57+ [CD3+]
CD45RA- CCR7+ (CM)/CD27- CD28+ [CD4+]	CD8+CD45RAint CCR7int/CD27+ CD28-/CD95+ [CD3+]
CD45RA- CCR7+ (CM)/CD27- CD28+/CD95+ [CD4+]	CD8+CD45RAint CCR7int/CD27- CD28- [CD3+]
CD45RA- CCR7- (EM)/CD27- CD28+ [CD4+]	CD8+CD45RAint CCR7int/CD27- CD28-/CD57+ [CD3+]
CD4+CD27+ CD28+ [CD45RA+ CCR7+ (Naive)]	CD8+CD45RAint CCR7int/CD27- CD28-/CD95+ [CD3+]
CD4CD27+ CD28+/CD57+ [CD45RA+ CCR7+ (Naive)]	CD8+ CD27- [CD8+]
CD4+CD27+ CD28+ (CM) [CD45RA- CCR7+ (CM)]	CD8+ CD28- [CD8+]
CD4+CD27+ CD28+ (CM)/CD95+ [CD45RA- CCR7+ (CM)]	CD8+ CD57+ [CD8+]
CD4+CD27- CD28+ [CD45RA- CCR7+ (CM)]	CD8+ CD95+ [CD8+]
CD4+CD27- CD28+/CD95+ [CD45RA- CCR7+ (CM)]	CD45RA+ CCR7+ (Naive) [CD8+]
CD4+CD27+ CD28+ [CD45RA- CCR7- (EM)]	CD45RA+ CCR7+ (Naive)/CD27+ CD28+ [CD8+]
CD4+CD27+ CD28+ /CD95+ [CD45RA- CCR7- (EM)]	CD45RA+ CCR7+ (Naive)/CD27+ CD28+/CD57+ [CD8+]
CD4+CD27- CD28+ [CD45RA- CCR7- (EM)]	CD45RA+ CCR7+ (Naive)/CD27+ CD28+/CD95+ (TSCM) [CD8+]
CD4+CD27- CD28+/CD95+ [CD45RA- CCR7- (EM)]	CD45RA+ CCR7+ (Naive)/CD27- CD28- [CD8+]
CD4+CD45RA-CCR7+CD57+ [CD27+ CD28+ (CM)]	CD45RA+ CCR7+ (Naive)/CD27- CD28-/CD57+ [CD8+]
CD4+CD45RA-CCR7+CD95+ [CD27+ CD28+ (CM)]	CD45RA+ CCR7+ (Naive)/CD27- CD28-/CD95+ [CD8+]
CD4+CD45RA-CCR7+CD95+ [CD27- CD28+]	CD45RA+ CCR7- (TEMRA) [CD8+]
CD4+CD45RA-CCR7- CD95+ [CD27+ CD28+]	CD45RA+ CCR7- (TEMRA)/CD27+ CD28+ [CD8+]
CD4+CD45RA-CCR7-CD95+ [CD27- CD28+]	CD45RA+ CCR7- (TEMRA)/CD27+ CD28- [CD8+]
CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM) [CD8+]	CD45RA- CCR7+ (CM) [CD8+]
CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM)/CD57+ [CD8+]	CD8+CD45RAint CCR7intCD95+ [CD27- CD28-]
CD45RA- CCR7+ (CM)/CD27+ CD28+ (CM)/CD95+ [CD8+]	CD3+ CD8+ CD57+ [CD3+ CD8+]

T cell phenotypes - continued	
CD45RA- CCR7+ (CM)/CD27- CD28+ [CD8+]	CD3+ CD8+ CD57+ CD27- [CD3+ CD8+]
CD45RA- CCR7+ (CM)/CD27- CD28+/CD95+ [CD8+]	CD3+ CD8+ NKG2C+ [CD3+ CD8+]
CD45RAint CCR7int [CD8+]	CD3+ CD8+/CD56- CD27- [CD3+ CD8+]
CD45RAint CCR7int/CD27+ CD28+ [CD8+]	CD3+ CD8- CD57+ [CD3+ CD8-]
CD45RAint CCR7int/CD27+ CD28+ /CD95+ [CD8+]	CD3+ CD8- CD57+ CD27- [CD3+ CD8-]
CD45RAint CCR7int/CD27+ CD28- [CD8+]	CD3+ CD8- CD57- CD27+ [CD3+ CD8-]
CD45RAint CCR7int/CD27+ CD28-/CD57+ [CD8+]	CD3+ CD8- NKG2C+ [CD3+ CD8-]
CD45RAint CCR7int/CD27+ CD28-/CD95+ [CD8+]	CD3+ CD8-/CD56- CD27- [CD3+ CD8-]
CD45RAint CCR7int/CD27- CD28- [CD8+]	CD3+ [CD45+]
CD45RAint CCR7int/CD27- CD28-/CD57+ [CD8+]	CD3+ CD56dim [CD45+]
CD45RAint CCR7int/CD27- CD28-/CD95+ [CD8+]	CD3+ CD56dim CD8+ [CD45+]
CD8+CD27+ CD28+ [CD45RA+ CCR7+ (Naive)]	CD3+ CD56dim CD8+ [CD3+ CD56dim]
CD8+CD27+ CD28+/CD57+ [CD45RA+ CCR7+ (Naive)]	CD3hi CD56dim [CD45+]
CD8+CD27+ CD28+/CD95+ (TSCM) [CD45RA+ CCR7+ (Naive)]	CD3+ CD16+ [CD45+]
CD8+CD27- CD28- [CD45RA+ CCR7+ (Naive)]	CD3+ CD16+ [CD16+]
CD8+CD27- CD28-/CD95+ [CD45RA+ CCR7+ (Naive)]	CD161+ CD3+ CD56dim [CD45+]
CD8+CD27+ CD28+ [CD45RA+ CCR7- (TEMRA)]	CD161+ CD3+ CD56dim [CD161+]
CD8+CD27+ CD28- [CD45RA+ CCR7- (TEMRA)]	NKG2D+ CD3+ CD56dim [CD45+]
CD8+CD27+ CD28-/CD95+ [CD45RA+ CCR7- (TEMRA)]	NKG2D+ CD3+ CD56dim [NKG2D+]
CD8+CD27- CD28- (E) [CD45RA+ CCR7- (TEMRA)]	CD3+ CD8+ [CD45+]
CD8+CD27- CD28- (E)/CD57+ [CD45RA+ CCR7- (TEMRA)]	CD3+ CD8+ CD57+ [CD45+]
CD8+CD27+ CD28+ (CM) [CD45RA- CCR7+ (CM)]	CD3+ CD8+ CD57+ CD27- [CD45+]
CD8+CD27+ CD28+ (CM)/CD57+ [CD45RA- CCR7+ (CM)]	CD3+ CD8+ CD57- CD27+ [CD45+]
CD8+CD27+ CD28+ (CM)/CD95+ [CD45RA- CCR7+ (CM)]	CD3+ CD8+ /CD56- CD27- [CD45+]
CD8+CD27+ CD28- [CD45RA- CCR7+ (CM)]	CD3+ CD8- [CD45+]
CD8+CD27+ CD28-/CD95+ [CD45RA- CCR7+ (CM)]	CD3+ CD8- CD57+ [CD45+]
CD8+CD27- CD28+ [CD45RA- CCR7+ (CM)]	CD3+ CD8- CD57+ CD27- [CD45+]
CD8+CD27- CD28+/CD95+ [CD45RA- CCR7+ (CM)]	CD3+ CD8- CD57- CD27+ [CD45+]
CD8+CD27- CD28- [CD45RA- CCR7+ (CM)]	CD3+ CD8- NKG2C+ [CD45+]
CD8+CD27- CD28-/CD57+ [CD45RA- CCR7+ (CM)]	CD3+ CD8- / CD56+ CD27+ [CD45+]
CD8+CD27- CD28-/CD95+ [CD45RA- CCR7+ (CM)]	CD3+ CD8- / CD56- CD27- [CD45+]
CD8+CD27+ CD28+ [CD45RA- CCR7- (EM)]	CD3+ /CD3hi CD56lo [CD45+]
CD8+CD27+ CD28+ /CD95+ [CD45RA- CCR7- (EM)]	CD161+ CD3+ [CD45+]
CD8+CD27+ CD28+ /CD95+ [CD45RAint CCR7int]	CD161+ CD3+ CD8+ [CD45+]
CD8+CD27+ CD28-/CD57+ [CD45RAint CCR7int]	CD161+ CD3+ CD8- [CD45+]
CD8+CD27- CD28- [CD45RAint CCR7int]	NKG2D+ CD3+ CD8+ [CD45+]
CD8+CD27- CD28-/CD57+ [CD45RAint CCR7int]	NKG2D+ CD3+ CD8- [CD45+]
CD8+CD27- CD28-/CD95+ [CD45RAint CCR7int]	CD3+ CD8+ [CD3+]
CD8+CD45RA+CCR7+ (naive)CD95+ (TSCM) [CD27+ CD28+]	CD3+ CD8+ CD57+ [CD3+]
CD8+CD45RA+CCR7+ (naive)CD57+ [CD27- CD28-]	CD3+ CD8+ CD57+ CD27- [CD3+]
CD8+CD45RA+CCR7+ (naive)CD95+ [CD27- CD28-)]	CD3+ CD8+ CD57- CD27+ [CD3+]
CD8+ CD45RA+ CCR7- (TEMRA)CD95+ [CD27+ CD28+]	CD3+ CD8+ /CD56- CD27- [CD3+]
CD8+CD45RA+ CCR7- (TEMRA)CD95+ [CD27+ CD28-]	CD3+ CD8- [CD3+]
CD8+CD45RA+ CCR7- (TEMRA)CD57+ [CD27- CD28- (E)]	CD3+ CD8- CD57+ [CD3+]
CD8+CD45RA+ CCR7- (TEMRA)CD95+ [CD27- CD28- (E)]	CD3+ CD8- CD57+ CD27- [CD3+]
CD8+CD45RA-CCR7+ (CM)CD95+ [CD27+ CD28+ (CM)]	CD3+ CD8- CD57- CD27+ [CD3+]
CD8+CD45RA-CCR7+ (CM)CD95+ [CD27+ CD28-]	CD3+ CD8- NKG2C+ [CD3+]
CD8+CD45RA-CCR7+ (CM)CD95+ [CD27- CD28+]	CD3+ CD8- /CD56- CD27- [CD3+]
CD8+CD45RA-CCR7+ (CM)CD57+ [CD27- CD28-]	CD3hi CD56lo [CD3+]
CD8+CD45RA-CCR7+ (CM)CD95+ [CD27- CD28-]	CD161+ CD3+ CD8+ [CD161+ CD3+]
CD8+CD45RA- CCR7- (EM)CD95+ [CD27+ CD28+]	CD161+ CD3+ CD8- [CD161+ CD3+]
CD8+CD45RAint CCR7intCD57+ [CD27+ CD28-]	NKG2D+ CD3+ CD8+ [NKG2D+]
CD8+CD45RAint CCR7intCD95+ [CD27+ CD28-]	
CD8+CD45RAint CCR7intCD57+ [CD27- CD28-]	

B cell phenotypes	
CD19+ CD27+ IgD+ (unswitched (IgM) memory) [ref CD19+]	CD19+ CD38- CD27+ CD24+ [CD19+CD38-CD27+]
CD19+ CD27+ IgD- (switched memory) [CD19+]	CD19+ CD38- CD27+ IgD+ [CD19+]
CD19+ CD38+ CD27+ [CD19+ CD38+]	CD19+ CD38- CD27+ IgD+ [CD19+CD38-CD27+]
CD19+ CD38+ CD27+ CD20- [CD19+]	CD19+CD38-CD27+CD20- [CD19+ CD38-]
CD19+ CD38+ CD27+ CD20- [CD19+ CD38+]	CD19+CD38-CD27+CD20- [CD19+CD38-CD27+]
CD19+ CD38+ CD24+ CD27+ [CD19+CD38+CD24+]	CD19+ CD38- CD24+ [CD19+
CD19+ CD38+ CD24+ IgD [CD19+CD38+CD24+]	CD19+ CD43+ CD27+ [CD19+]
CD19+CD38-CD27+ [CD19+]	CD19lo CD20- CD27hi CD38hi [CD19+]
CD19+ CD38- CD27+ CD24+ [CD19+]	CD20+CD40+ [CD20+]
CD19+ CD38- CD27+ CD24+ [CD19+ CD38-]	
NK cell phenotypes	
CD3- CD56+ [CD45+]	CD3- CD56dim NKG2A- [CD45+]
CD3- CD56+ CD27+ [CD45+]	CD3- CD56dim NKG2A- [CD3- CD56+]
CD3- CD56hi CD16- CD27+ [CD3- CD56+]	CD3- CD56hi CD57- [CD3- CD56+]
CD3- CD56hi CD16- CD27+ [CD3- CD56+ CD27+]	CD3- CD56hi NKG2A+ [CD45+]
CD3- CD56dim CD16+ (mature) [CD45+]	CD3- CD56hi NKG2A+ [CD3- CD56+]
CD3- CD56dim CD16+ (mature) [CD3- CD56+]	CD3- CD56- [CD45+]
CD3- CD56dim CD16+ CD8+ [CD45+]	CD3- CD56- CD16+ (dysfunctional) [CD45+]
CD3- CD56dim CD16+ CD8+ [CD3- CD56+]	CD3- CD56- CD16+ (dysfunctional) [CD3- CD56-]
CD3- CD56dim CD16+ CD8+ [CD3- CD56dim CD16+]	CD161+ CD3- CD56+ [CD45+]
CD3- CD56dim CD16+ CD8+ CD57+ [CD45+]	CD161+ CD3- CD56+ [CD161+]
CD3- CD56dim CD16+ CD8+ CD57+ [CD3- CD56+]	CD161+ CD3- CD56dim CD16- [CD45+]
CD3-CD56dimCD16+CD8+CD57+[CD3- CD56dim CD16+]	CD161+ CD3- CD56dim CD16- [CD161+]
CD3-CD56dimCD16+CD8+CD57+[CD3-CD56dimCD16+CD8+]	CD161+ CD3- CD56dim CD16- [CD161+ CD3- CD56+]
CD3- CD56dim CD16+ CD57+ [CD45+]	CD161+ CD3- CD56hi CD16- [CD45+]
CD3- CD56dim CD16+ CD57+ [CD3- CD56+]	CD161+ CD3- CD56hi CD16- [CD161+]
CD3- CD56dim CD16+ CD57+ [CD3- CD56dim CD16+]	CD161+ CD3- CD56hi CD16- [CD161+ CD3- CD56+]
CD3- CD56dim CD16+ CD57+ NKG2Chi [CD45+]	CD161+ CD3- CD56lo CD16+ [CD45+]
CD3- CD56dim CD16+ CD57+ NKG2Chi [CD3- CD56+]	CD161+ CD3- CD56lo CD16+ [CD161+]
CD3-CD56dimCD16+CD57+NKG2Chi[CD3-CD56dimCD16+]	CD161+ CD3- CD56lo CD16+ [CD161+ CD3- CD56+]
CD3- CD56dim CD16+ NKG2A+ [CD45+]	NKG2A+ CD3- CD56+ [CD45+]
CD3- CD56dim CD16+ NKG2A+ [CD3- CD56+]	NKG2A+ CD3- CD56+ [NKG2A+]
CD3- CD56dim CD16+ NKG2A+ [CD3- CD56dim CD16+]	NKG2A+ CD3- CD56hi CD16- [CD45+]
CD3- CD56dim CD16+ NKG2C+ [CD45+]	NKG2A+ CD3- CD56hi CD16- [NKG2A+]
CD3- CD56dim CD16+ NKG2C+ [CD3- CD56+]	NKG2D+ CD3- CD56+ [CD45+]
CD3- CD56dim CD16+ NKG2C+ [CD3- CD56dim CD16+]	NKG2D+ CD3- CD56+ [NKG2D+]
CD3- CD56dim CD16- [CD3- CD56+]	NKG2D+ CD3- CD56dim CD16+ [CD45+]
CD3- CD56dim CD16- CD8+ [CD45+]	NKG2D+ CD3- CD56dim CD16+ [NKG2D+]
CD3- CD56dim CD16- CD57+ [CD3- CD56+]	NKG2D+ CD3- CD56dim CD16+ [NKG2D+ CD3- CD56+]
CD3- CD56dim CD16- NKG2A+ [CD45+]	NKG2D+ CD3- CD56dim CD16- [CD45+]
CD3- CD56dim CD16- NKG2A+ [CD3- CD56+]	NKG2D+ CD3- CD56dim CD16- [NKG2D+]
CD3- CD56dim CD16- NKG2C+ [CD3- CD56dim CD16-]	NKG2D+ CD3- CD56dim CD16- [NKG2D+ CD3- CD56+]
CD3- CD56dim CD57+ [CD45+]	NKG2D+ CD3- CD56hi CD16- [CD45+]
CD3- CD56dim CD57+ [CD3- CD56+]	NKG2D+ CD3- CD56hi CD16- [NKG2D+ CD3- CD56+]
CD3- CD56dim CD57- [CD45+]	
CD3- CD56dim CD57- [CD3- CD56+]	
CD3- CD56dim NKG2A+ [CD45+]	
CD3- CD56dim NKG2A+ [CD3- CD56+]	

Additional file 10 – Correlations between NK cells and TAA-reactive T cells

		NK cell phenotype	P value	
CD4	MUC	CD3- CD56+ CD27+ [CD45+]	0.039	Higher in Responder
CD8	MUC1	CD3- CD56dim CD16+ CD8+ CD57+ [CD3-CD56dimCD16+CD8+]	0.021	Higher in Responder
		NKG2D+ CD3- CD56dim CD16- [NKG2D+]	0.038	Lower in Responder
CD4	SUR	CD3- CD56dim CD16+ CD57+ NKG2Chi [CD45+]	0.037	Lower in Responder
		CD3- CD56dim CD16+ CD57+ NKG2Chi [CD3- CD56+]	0.025	Lower in Responder
		CD3- CD56dim CD16+ CD57+ NKG2Chi [CD3- CD56dim CD16+]	0.03	Lower in Responder
		CD3- CD56dim CD16- CD8+ CD57+ [CD3- CD56dim CD16-]	0.031	Lower in Responder
		CD3- CD56dim CD16- NKG2C+ [CD3- CD56dim CD16-]	0.013	Lower in Responder
		NKG2A+ CD3- CD56+ [NKG2A+]	0.025	Higher in Responder
CD8	SUR	CD3- CD56hi CD16- CD27+ [CD3- CD56+ CD27+]	0.020	Higher in Responder

Additional file 11 – Correlations between blood T cells and TAA-reactive T cells

	T cell phenotype	P value	
CD4 HER2	CD8posCD45RApos CCR7neg (TEMRA) [CD3pos]	0.035	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27pos CD28neg [CD3pos]	0.045	Higher in Responder
	CD8posCD45RAint CCR7int/CD27pos CD28pos /CD95pos [CD3pos]	0.018	Higher in Responder
	CD45RApos CCR7neg (TEMRA) [CD8pos]	0.042	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27pos CD28neg [CD8pos]	0.041	Higher in Responder
	CD45RAint CCR7int/CD27pos CD28pos /CD95pos [CD8pos]	0.021	Higher in Responder
	CD4CD27pos CD28pos/CD57pos [CD45RApos CCR7pos (Naive)]	0.013	Lower in Responder
	CD4posCD27neg CD28neg (E)/CD57pos [CD45RApos CCR7neg (TEMRA)]	0.038	Higher in Responder
	CD8posCD45RAnegCCR7pos (CM)CD57pos [CD27pos CD28neg]	0.033	Lower in Responder
	CD8posCD45RAnegCCR7pos (CM)CD95pos [CD27neg CD28pos]	0.036	Lower in Responder
CD8 HER2	CD8posCD45RApos CCR7neg (TEMRA)/CD27pos CD28neg [CD3pos]	0.025	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27pos CD28neg/CD95pos [CD3pos]	0.018	Higher in Responder
	CD8posCD45RAposCCR7pos (naive)CD57pos [CD27neg CD28neg]	0.031	Lower in Responder
CD4 MUC1	CD4posCD45RAneg CCR7pos (CM)/CD27neg CD28pos [CD3pos]	0.037	Higher in Responder
	CD4posCD45RAneg CCR7pos (CM)/CD27neg CD28pos/CD95pos [CD3pos]	0.039	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27pos CD28neg [CD3pos]	0.016	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27pos CD28neg/CD95pos [CD3pos]	0.036	Higher in Responder
	CD45RApos CCR7pos (Naive)/CD27pos CD28pos/CD95pos (TSCM) [CD4]	0.046	Higher in Responder
	CD45RAneg CCR7pos (CM)/CD27neg CD28pos [CD4pos]	0.044	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27pos CD28neg [CD8pos]	0.013	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27pos CD28neg/CD57pos [CD8pos]	0.034	Higher in Responder
	CD161pos CD3pos CD8neg [CD45pos]	0.013	Higher in Responder
CD8 MUC1	CD4posCD45RAneg CCR7pos (CM)/CD27neg CD28neg/CD95pos [CD3pos]	0.030	Lower in Responder
	CD45RAneg CCR7pos (CM)/CD27neg CD28neg [CD4pos]	0.030	Lower in Responder
	CD45RAneg CCR7pos (CM)/CD27neg CD28neg/CD95pos [CD4pos]	0.030	Lower in Responder
	CD4posCD45RAnegCCR7neg CD95pos [CD27pos CD28pos]	0.044	Higher in Responder
	CD8posCD45RAneg CCR7neg (EM)/CD95pos [CD27neg CD28pos]	0.050	Higher in Responder
	NKG2Dpos CD3pos CD8pos [CD45pos]	0.029	Higher in Responder
CD4 SUR	CD8posCD45RAneg CCR7pos (CM)/CD27neg CD28pos [CD3pos]	0.027	Higher in Responder
	CD8posCD45RAneg CCR7pos (CM)/CD27neg CD28pos/CD95pos [CD3pos]	0.022	Higher in Responder
	CD8posCD27neg CD28pos [CD45RAneg CCR7pos (CM)]	0.018	Higher in Responder
	CD8posCD27neg CD28pos/CD95pos [CD45RAneg CCR7pos (CM)]	0.019	Higher in Responder
	CD161pos CD3pos CD56dim [CD161pos]	0.037	Higher in Responder
	CD161pos CD3pos CD8pos [CD45pos]	0.009	Higher in Responder
	CD161pos CD3pos CD8pos [CD161pos CD3pos]	0.001	Higher in Responder
	CD161pos CD3pos CD8neg [CD161pos CD3pos]	0.001	Lower in Responder

CD8 SUR	CD4posCD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E) [CD3pos]	0.035	Higher in Responder
	CD4posCD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E)/CD95pos [CD3pos]	0.027	Higher in Responder
	CD8pos CD27neg [CD3pos]	0.001	Higher in Responder
	CD8pos CD28neg [CD3pos]	0.001	Higher in Responder
	CD8pos CD57pos CD3pos]	0.002	Higher in Responder
	CD8pos CD95pos [CD3pos]	0.049	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA) [CD3pos]	0.005	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E) [CD3pos]	0.001	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E)/CD57pos [CD3pos]	0.003	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E)/CD95pos [CD3pos]	0.001	Higher in Responder
	CD8posCD45RAint CCR7int [CD3pos]	0.025	Higher in Responder
	CD8posCD45RAint CCR7int/CD27neg CD28pos/CD95pos [CD3pos]	0.018	Higher in Responder
	CD8posCD45RAint CCR7int/CD27neg CD28neg [CD3pos]	0.006	Higher in Responder
	CD8posCD45RAint CCR7int/CD27neg CD28neg/CD57pos [CD3pos]	0.009	Higher in Responder
	CD8posCD45RAint CCR7int/CD27neg CD28neg/CD95pos [CD3pos]	0.005	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E) [CD4pos]	0.027	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E)/CD57pos [CD4pos]	0.043	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27neg CD28neg (E)/CD95pos [CD4pos]	0.021	Higher in Responder
	CD8pos CD27neg [CD8pos]	0.002	Higher in Responder
	CD8pos CD28neg [CD8pos]	0.003	Higher in Responder
	CD8pos CD57pos [CD8pos]	0.018	Higher in Responder
	CD45RApos CCR7neg (TEMRA) [CD8pos]	0.011	Higher in Responder
	CD45RApos CCR7neg (TEMRA)/CD27neg CD28pos/CD95pos [CD8pos]	0.033	Higher in Responder
	CD45RAint CCR7int/CD27neg CD28pos/CD95pos [CD8pos]	0.048	Higher in Responder
	CD45RAint CCR7int/CD27neg CD28neg [CD8pos]	0.014	Higher in Responder
	CD45RAint CCR7int/CD27neg CD28neg/CD57pos [CD8pos]	0.015	Higher in Responder
	CD45RAint CCR7int/CD27neg CD28neg/CD95pos [CD8pos]	0.013	Higher in Responder
	CD4posCD27pos CD28pos [CD45RAneg CCR7neg (EM)]	0.039	Lower in Responder
	CD8posCD27pos CD28pos [CD45RApos CCR7neg (TEMRA)]	0.001	Lower in Responder
	CD8posCD27neg CD28neg (E) [CD45RApos CCR7neg (TEMRA)]	0.008	Higher in Responder
	CD8posCD27neg CD28neg (E)/CD57pos [CD45RApos CCR7neg (TEMRA)]	0.024	Higher in Responder
	CD8posCD27pos CD28pos /CD95pos [CD45RAint CCR7int]	0.045	Lower in Responder
	CD8posCD27neg CD28neg [CD45RAint CCR7int]	0.009	Higher in Responder
	CD8posCD27neg CD28neg/CD57pos [CD45RAint CCR7int]	0.034	Higher in Responder
	CD8posCD27neg CD28neg/CD95pos [CD45RAint CCR7int]	0.008	Higher in Responder
	CD4posCD45RAnegCCR7posCD95pos [CD27neg CD28neg]	0.050	Higher in Responder
	CD4posCD45RAnegCCR7negCD57pos [CD27pos CD28pos]	0.031	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)CD95pos [CD27pos CD28neg]	0.016	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)CD95pos [CD27neg CD28pos]	0.042	Higher in Responder
	CD8posCD45RApos CCR7neg (TEMRA)CD95pos [CD27neg CD28neg (E)]	0.000	Higher in Responder
	CD8posCD45RAneg CCR7neg (EM)CD95pos [CD27pos CD28neg]	0.037	Higher in Responder
	CD8posCD45RAint CCR7intCD95pos [CD27pos CD28neg]	0.003	Higher in Responder

CD8 SUR	CD8posCD45RAint CCR7intCD95pos [CD27neg CD28neg]	0.012	Higher in Responder
	CD3pos CD56dim [CD45pos]	0.027	Higher in Responder
	CD3pos CD56dim CD8pos [CD45pos]	0.015	Higher in Responder
	NKG2Dpos CD3pos CD56dim [CD45pos]	0.006	Higher in Responder
	NKG2Dpos CD3pos CD56dim [NKG2Dpos]	0.006	Higher in Responder
	CD3pos CD8pos CD57pos [CD45pos]	0.009	Higher in Responder
	CD3pos CD8pos CD57pos CD27neg [CD45pos]	0.010	Higher in Responder
	CD3pos CD8pos /CD56neg. CD27neg [CD45pos]	0.024	Higher in Responder
	CD161pos CD3pos CD8pos [CD45pos]	0.031	Higher in Responder
	CD3pos CD8pos CD57pos [CD3pos]	0.007	Higher in Responder
	CD3pos CD8pos CD57pos CD27neg [CD3pos]	0.006	Higher in Responder
	CD3pos CD8pos /CD56neg. CD27neg [CD3pos]	0.020	Higher in Responder
	CD161pos CD3pos CD8pos [CD161pos CD3pos]	0.023	Higher in Responder
	CD161pos CD3pos CD8neg [CD161pos CD3pos]	0.023	Lower in Responder
	CD3pos CD8pos CD57pos [CD3pos CD8pos]	0.018	Higher in Responder
	CD3pos CD8pos CD57pos CD27neg [CD3pos CD8pos]	0.013	Higher in Responder

Intra-tumoural immune features as prognostic markers in metastatic melanoma

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Abstract:

Introduction:

A number of studies has shown that the “immune context” within the tumour plays a major role in cancer progression and responsiveness to immunotherapies. The efficacy of such immunotherapeutic approaches relies on the presence of immune cells that migrate into the tumour and subsequently kill tumour cells. Thus, a more comprehensive analysis of immune features in the tumour including cellular and soluble features may be required in order to better predict patient outcome or response to therapy.

Methods:

Here, we analysed multifaceted immune parameters in melanoma sections with the goal of identifying the immune factor(s) most relevant for patient outcome. Using fluorescence microscopy, we assessed T cells (CD3+) and myeloid cells (CD15+) in addition to a panel of soluble immune factors (IL-6, GM-CSF, TNF, IL-1Ra, IL-2, IL-10, GDF15, PGE2) as well as the transcription factor STAT3 in metastatic melanoma tissues of 76 patients.

Results:

We observed that tumour-infiltrating immune cells were present in the majority of patient samples, in which high relative levels of CD15+ but not CD3+ cells were found to be a potential prognostic marker for survival. Combining the assessment of CD15+ and CD3+ cells, we found that patients survived longer when their tumours contained high levels of CD3+ cells and low levels of CD15+ cells. No associations between patient clinical parameters and the level of a number of soluble factors were found, apart from high levels of TNF in patients with more advanced disease.

Conclusions:

This pilot study proposes cellular but not soluble features as potential indicators of patient outcome in metastatic melanoma.

Keywords:

TILs, tumour microenvironment, metastatic melanoma, immune context

Introduction

Although malignant melanoma accounts for less than one percent of skin cancer cases (1), it is the most dangerous form, with an ever-increasing incidence worldwide. Despite recent therapeutic advances and sustained efforts at early diagnosis, the majority of metastatic melanoma patients still faces a poor prognosis. The recent introduction of immunomodulatory antibody therapies into the clinic has revolutionised treatment of metastatic melanoma and several other solid cancers. These therapies utilise the patient's own immune system to control or suppress tumour growth. However, the efficacy of such approaches relies on the presence of immune cells which can migrate into the tumour and subsequently kill the cancer cells or otherwise suppress tumour development even when employing intratumoural immunotherapy (2). Supporting this notion, a number of recent studies has shown that the state of the immune system within the tumour plays a major role in cancer progression and patient outcome. The most intensively explored approach is based on investigations on the level of intra-tumoural lymphocytes and other immune cells in colorectal cancer, where the "immune context" had greater prognostic power than the standard staging system for predicting post-operative survival (3-5). Such associations

between the level of tumour-infiltrating lymphocytes and clinical outcome have also been reported in several other cancer types (6-9). As well as providing prognostic information on patient survival, the assessment of the intra-tumoural immune context could also assist in predicting which patients will respond to which forms of therapy, something of great importance in clinical patient management. Supporting this in the context of melanoma, Thume *et al.* has shown that high levels of infiltrating CD8+ cells are associated with responsiveness to therapeutic PD-1 blockade in these patients (10). Associations between the composition of the tumour microenvironment and the clinical efficacy of conventional therapies and immunotherapies have also been documented in several other cancer types (4, 11-18).

However, tumour progression is still frequently seen even in the presence of substantial lymphocytic infiltration, suggesting that either an effective immune response cannot be generated, or that the immune response cannot always control tumour growth in every individual patient. This in turn suggests that a more comprehensive analysis of immune features in the tumour including cellular and soluble factors may be required in order to better predict patient outcome. This proposition is based on the notion that local immune cells are heavily influenced by signals received from soluble immune molecules such as chemokines or cytokines which can promote their migration and stimulate their anti-tumour activity but may also program them to become immunosuppressive. For example, Mlecnik *et al*. reported that the presence of certain chemokines and adhesion molecules is correlated with high levels of T cell infiltration into the tumour and with patient survival (19), while a separate study in uveal melanoma showed that intra-tumoural COX-2 expression was associated with the levels of Tregs and patient survival (20). These observational studies are supported by investigations which reveal interactions between soluble factors and cellular

immune features. For example, high levels of IDO and PGE2 inhibit T cell function, down-regulate NK receptors and increase the frequency of tumour-infiltrating regulatory T cells (21-23). Furthermore, PGE2, IL-6 and a number of other soluble factors has been shown to drive the differentiation of monocytes into immunosuppressive “myeloid-derived suppressor cells” (MDSCs) (24-26). The levels of these molecules and their associated pathways in the tumour have also been shown to correlate with patient survival (27, 28). Collectively, these studies suggest that the soluble milieu in the tumour microenvironment influences the level and behaviour of local immune cells, and that both soluble and cellular immune features are important for patient prognosis.

Considering the role of soluble signalling molecules in generating an efficient anti-tumour immune response, an immunological perspective of the tumour microenvironment would be incomplete without examining these soluble molecules. However, few studies have assessed the clinical importance of both cellular and soluble intra-tumoural immune features, and little is known about the prognostic value of cells infiltrating melanoma lesions. Because soluble factors influence leukocyte behaviour in pleiotropic or context-dependent ways, understanding what constitutes a “favourable” intra-tumoural environment is important for guiding the development of innovative therapies in future, as well as for optimal implementation of existing therapies. To take the first step towards this, the present study was designed to characterise multifaceted immune parameters in melanoma. We assessed T cells and myeloid cells in addition to a panel of soluble immune factors, with the goal of identifying immune factor(s) most relevant for patient outcome.

Materials and Methods

Patients

This study investigated 76 metastatic melanoma patients treated at the Dermatology Department of Tübingen University Hospital. Patients included in this study received diverse forms of anti-cancer therapies. Thus, this pilot study sought informative immune features common to all melanoma patients regardless of the type of treatment they received. The study population consisted of 44 men and 32 women with a median age of 63 (range 35 – 89 years). See Table 1 for a detailed description of the patient cohort. This study was approved by the Ethics Committee of the University of Tübingen (017/2016BO2). Written informed consent was obtained from all patients for the storage and scientific analysis of tissue samples.

Table 1: Description of patient cohort

Patient clinicopathological parameters (n = 76)	
Median Age (range in years)	63 (35-89)
Gender	
Men	44
Women	32
AJCC staging	
3	31
4A	14
4B	9
4C	22

Immunofluorescence

To detect proteins of interest, slide-mounted formalin-fixed paraffin-embedded tissue sections (5 µm thick) were first dewaxed for 15 min in xylene and then rehydrated in a series of graded ethanol solutions (100%, 96% and 70%, 5 min each), followed by incubation in distilled water for 30 min. Antigen retrieval was performed using an alkaline-based solution (1 mM EDTA, 25 mM Tris–HCl, 0.05% SDS, pH 8.5) (29) at 90°C for 45 minutes for all antibodies. Tissues were then rinsed with washing buffer (PBS, 0.025% Tween 20 and 0.005% BSA) for 3 minutes before blocking non-specific binding with a 5% donkey serum solution (diluted in washing buffer) for 30 minutes at room temperature. This was followed by incubation with primary antibody (60 min at room temperature) before washing for 3 x 3 min. After washing, secondary antibody was applied (incubated for 60 min at room temperature) before another round of 3 x 3 min washes. Cell nuclei were subsequently stained with 4',6-diamidino-2-phenylindole (DAPI) (Roche, Mannheim, Germany) (diluted 1:2000 in washing buffer) for 10 minutes before washing (3 x 3 min). Slides were then mounted with a glass coverslip (0.08–0.12 mm) using fluorescence mounting medium (Dako, Santa Clara, US). Stained slides were stored protected from light at 4°C before being measured with a Zeiss Axiophot fluorescence microscope using AxioVision 4.8 software. Soluble molecules were recorded at 20x magnification, whereas infiltrating cells were captured at 40x magnification. The following primary antibodies were used: rabbit polyclonal anti-CD3 (1:30, Abcam, Cambridge, UK), goat polyclonal anti-CD14 (1:40 Novus Biologicals, Abingdon, UK), mouse monoclonal anti-CD14 (clone 5A3B11B5) (1:300 Santa Cruz Biotechnology, Dallas, Texas, USA) rabbit monoclonal anti-CD15 (1:80, Novus Biologicals), rabbit polyclonal anti-GM-CSF (1:20, Bioss, Woburn, Massachusetts, USA), rabbit polyclonal anti-TNF (1:40, Novus Biologicals), rabbit polyclonal anti-PGE2 (1:10,

Biorbyt, Cambridge, UK), rabbit polyclonal anti-GDF15 (1:35, BIOZOL, Eching, Germany), rabbit polyclonal anit-IL10 (1:10, BIOZOL), mouse polyclonal anti- IL-6 (1:20, Bioss), mouse anti- STAT3 (1:8, SantaCruz Biotechnology), mouse monoclonal anti-IL1Ra (clone OTI3B1) (1:45, ORIGENE, Rockville, MD, USA) and mouse monoclonal anti-IL2 (1:25, ACRIS, SanDiego, CA, USA). For the detection of the primary antibody, the following secondary antibodies were used: Alexa Fluor 488 donkey anti-rabbit IgG (H+L) (1:60), Cy3 donkey anti-mouse IgG (H+L) (1:150), Alexa Fluor 488 donkey anti-goat IgG (H+L) (1:60). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Control sections omitting primary antibody were included for each sample. Primary antibodies were individually titrated and tested with different antigen retrieval methods in order to determine optimal staining conditions.

Depending on tumour size, an average of 12 representative images was captured in a non-biased manner covering all areas of the tissue, so that the tumour centre and margin were measured proportional to the tumour size. To determine relative differences in protein expression of different samples, fluorescence intensity was compared between the control (secondary antibody only) and primary antibody-stained tissue sections on the same slide. In order to standardise the measurement of different samples, signal intensity (exposure time) of the control tissue was adjusting in accordance with standard images. After determining comparable signal intensity in accordance with the standard images, an equivalent number of images from control and antibody-stained tissues was recorded. Fluorescence intensity for each image was measured with software designed in-house (created by Christof Zanke, Tübingen University Hospital, Tübingen, Germany) which was used to create a fluorescence index; mean pixel intensity of the antibody-stained tissue divided by mean pixel intensity of images from the control tissue. By including an internal control for each slide, this method

considers any potential differences in sample autofluorescence or in microscope settings or performance that may occur. Results obtained by software were confirmed visually. Tissues were considered to be positive for a protein if they showed fluorescence intensity of at least 50% greater than the control slide (i.e. fluorescence index of 1.5). To quantify numbers of infiltrating cells, images were scored by two independent investigators and results compared to identify potential discrepancies.

Statistical analysis

Two independent groups were compared using the Mann-Whitney U test. Relationships across four grouping variables were assessed with Fisher's exact test. Correlations were assessed using Spearman correlation analysis. Differences in disease-specific survival between two groups were compared using the Kaplan-Meier method with the log-rank test. Surviving patients or deaths not due to melanoma were censored. The patient cohort was dichotomised into two groups for survival analysis using two methods (1) following convention according to median values, or (2) the largest differences in survival between patient groups were determined by comparing all possible combinations of patient groups (group sizes accounted for at least 10% the entire cohort). To account for the additional number of statistical tests that this method requires, we adjusted the significance threshold considering this, whereas all other statistical relationships were considered using a threshold of 5%. Statistical analysis was performed with SPSS 22 (IBM, Ehningen, Germany) and Prism 5 (Graph Pad, La Jolla, USA).

Results

Metastatic melanomas are commonly infiltrated by T cells and granulocytic cells, and possess a diverse milieu of soluble factors

To characterise the tumour microenvironment in melanoma, we assessed soluble factors and cellular features in the tumours of 76 metastatic melanoma patients. This characterisation included T cells, monocytes and granulocytic cells along with the soluble factors IL-6, GM-CSF, TNF, IL-1Ra, IL-2, IL-10, GDF15, PGE2 and the transcription factor STAT3. The presence of soluble molecules was very common, with a large fraction of tumours staining positive for most of the molecules (Fig. 1A). However, they were found to be present at different levels in different tumours, with IL-6, GM-CSF and IL-1Ra showing the greatest range (Fig. 1B). Similar to the soluble molecules, we observed that the majority of melanoma tissues contained CD3+ and CD15+ cells (Fig. 1C), but the latter were much less common (Fig. 1D) with substantially lower abundance compared to CD3+ cells (Fig. 1E). Further, there was no significant difference in the distribution of cells comparing center and margin of the tumour, neither for CD3+ nor for CD15+ cells (Fig. 1E), although there was a tendency towards greater CD15+ cell infiltration into the center of the tumour.

We also attempted to quantify tumour infiltration by CD14+ cells using two commercially-available antibodies (goat polyclonal anti-CD14 from Novus Biologicals and mouse monoclonal anti-CD14 from Santa Cruz (clone 5A3B11B5)). Despite the use of different staining protocols, we were not able to achieve staining quality that allowed quantification of CD14+ positive cells using either antibody.

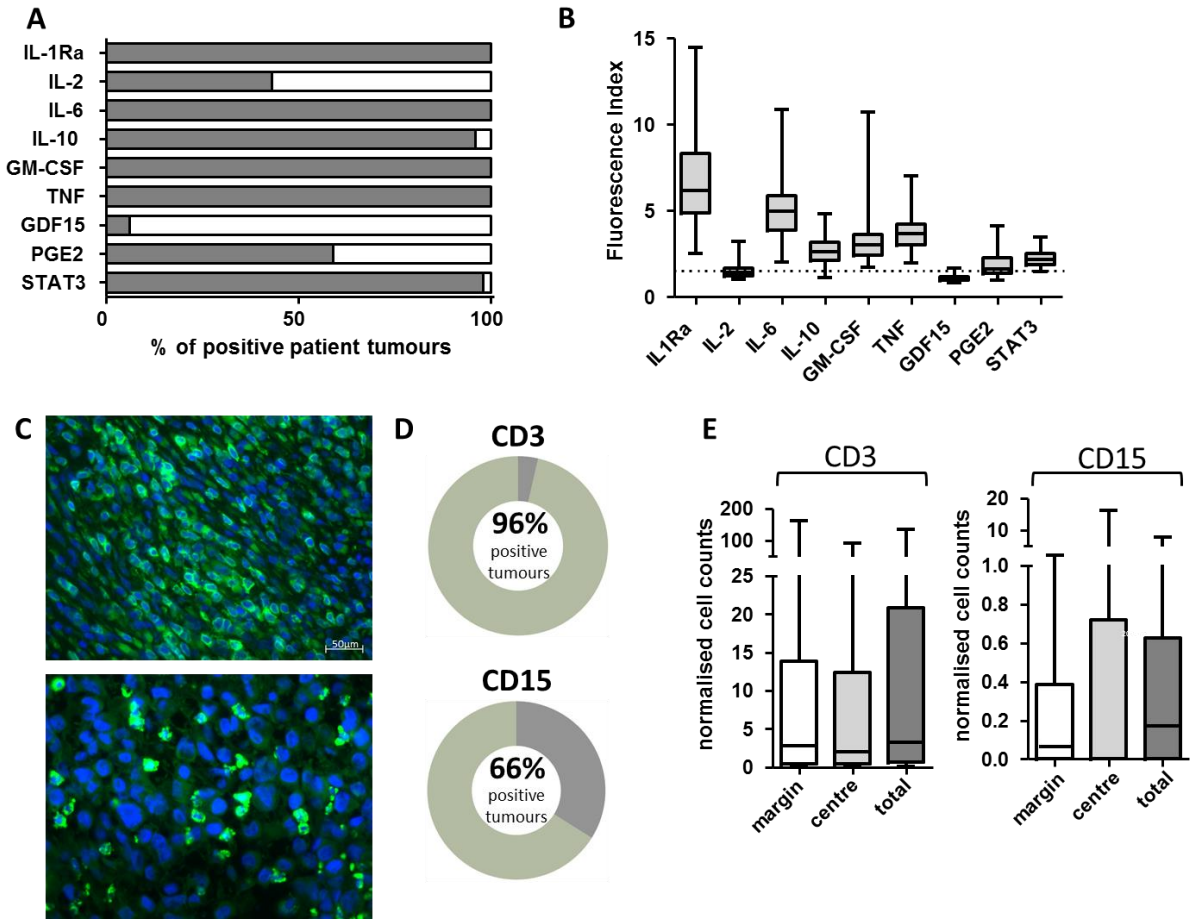

Figure 1. Melanomas are commonly infiltrated by leukocytes and contain a range of different soluble molecules. Tumour infiltration by CD3+ and CD15+ cells (n = 56) and the presence of soluble molecules including IL-1Ra (n = 47), IL-2 (n = 56), IL-6 (n = 62), IL-10 (n = 56), GM-CSF (n = 45), TNF (n = 53), GDF15 (n = 47), PGE2 (n = 39) and STAT3 (n = 53) was determined using fluorescence microscopy. The presence of most soluble molecules was common in melanoma tumours (A), but differed in level (B). The majority of melanoma lesions was infiltrated by CD3+ (C, top image) and CD15+ cells (C, bottom image), with CD3+ cells being more common (D) and occurring at higher levels (E). No significant difference between margin or center was observed for either CD3+ or CD15+ cells (E).

Levels of soluble molecules in the melanoma microenvironment correlate with each other and with infiltration by granulocytic cells

We reasoned that a particular microenvironment might enhance or suppress the infiltration of immune cells into the tumour. We found that tumours more heavily infiltrated by CD15+ cells (which would be expected to suppress immunity) also showed lower expression of the immunosuppressive factor PGE2 ($p = 0.02$) (Fig. 2A). However, no other associations between immune cell infiltration and the level of soluble molecules were observed. Given that many of these soluble signalling molecules are known to act in concert, we next investigated whether the expression of the different molecules investigated here was related. Indeed, several factors were found to be positively associated with the presence of other factors. For example, levels of IL-6 and GM-CSF positively correlated with each other ($p < 0.0001$, $r = 0.7$) (Fig. 2B). Similar relationships were also seen between TNF and STAT3 ($p < 0.0001$, $r = 0.7$), IL-2 and IL-10 ($p < 0.0001$, $r = 0.5$) as well as for other combinations of molecules. See supplementary data 1 for a full set of correlations.

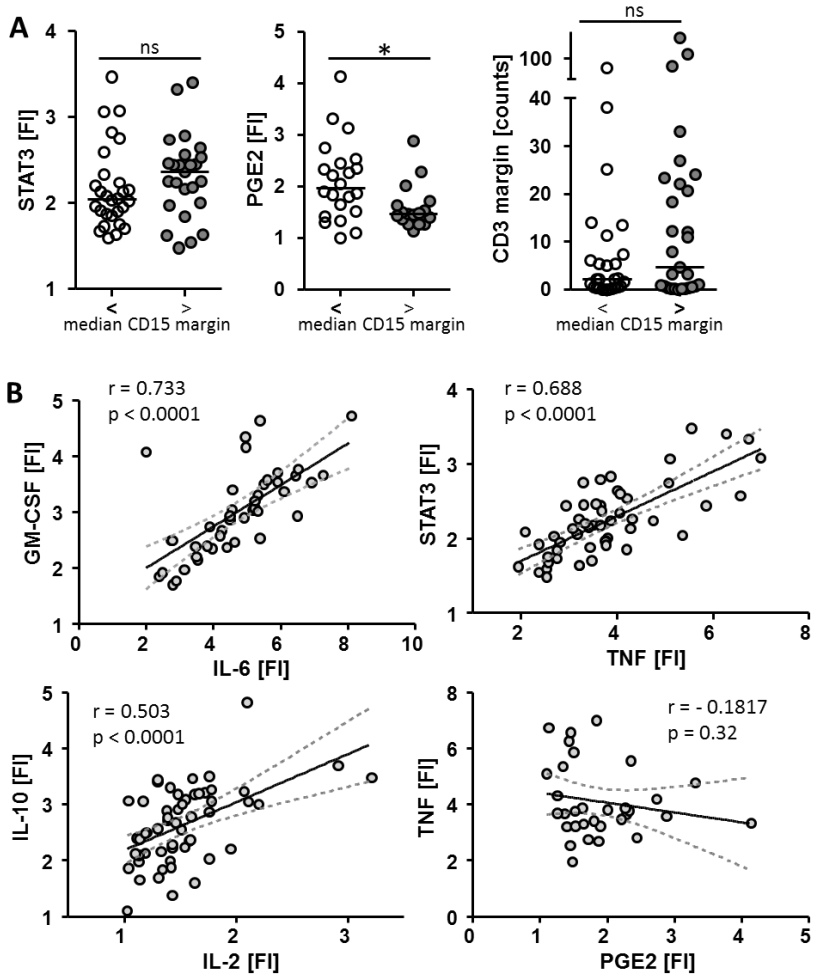

Figure 2. The presence of soluble factors in melanoma tumours is correlated and can be associated with leukocyte infiltration. Intra-tumoural levels of CD3+ and CD15+ cells and the presence of soluble molecules (IL-1Ra, IL-2, IL-6, IL-10, GM-CSF, TNF, GDF15, PGE2, STAT3) was assessed in metastatic melanoma lesions from 76 patients. Patients were grouped according to their median expression level and cell counts, respectively. This revealed that patients with higher than median levels of CD15+ cells in tumours had lower levels of PGE2 (A). We also found that the amount of IL-6 was positively correlated with GM-CSF, with similar relationships observed between STAT3 and TNF and between IL-2 and IL-10 (B). FI, Fluorescence Index

High intra-tumoural levels of TNF are present in patients with more advanced disease

We next investigated whether the presence of soluble factors or tumour infiltration by T cells or granulocytic cells was associated with clinical parameters in these melanoma patients. We investigated whether the immune parameters measured here were associated with clinical features including disease stage, progression time (stage III to stage IV), age and

gender. This analysis showed that patients with stage IV disease had tumours with higher levels of TNF relative to stage III patients (p = 0.02) (Fig. 3A). No other association between stage and any other immune features were found here; neither STAT3 nor CD3+ or CD15+ cells in the margin were associated with tumour stage (Fig. 3A). Older patients were additionally found to have lower numbers of CD15+ cells infiltrating into the tumour margin (p = 0.046) (Fig. 3B), while CD3+ infiltration into the margin, TNF and STAT3 expression where not influenced by age. No other associations between patient clinical parameters and the immune features measured here were found (Fig. 3, selected results shown).

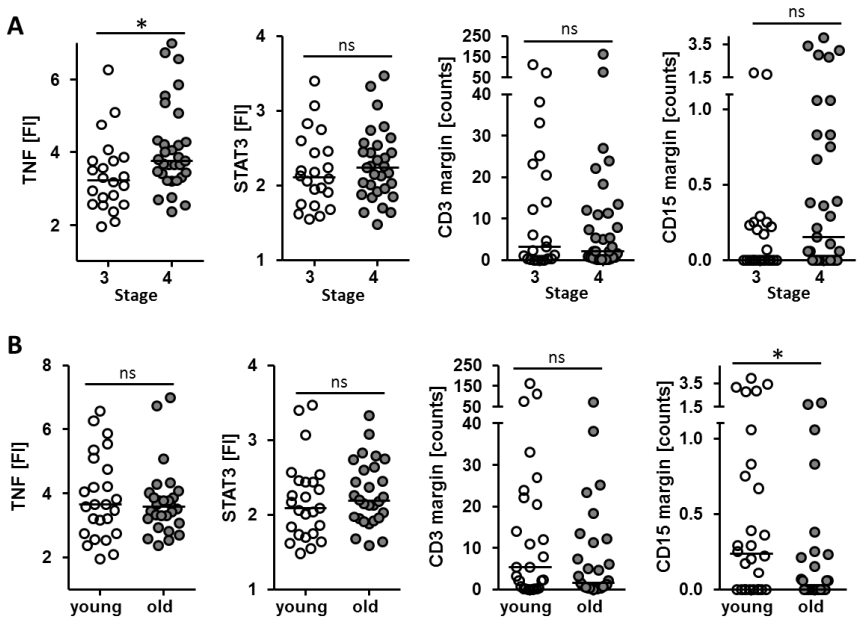

Figure 3. Levels of tumour-infiltrating leukocytes and intra-tumoural soluble factors are associated with some patient clinical parameters. Tumour-infiltrating cells (CD3+ and CD15+) and soluble factors (IL-1Ra, IL-2, IL-6, IL-10, GM-CSF, TNF, GDF15, PGE2 and STAT3) were measured in the tissue of 76 melanoma patients using fluorescence microscopy and tested for association with patient clinical parameters. Patients with more advanced disease had tumours with higher levels of TNF (A). We also found that older patients had lower levels of CD15+ cells infiltrating the tumour margin (young group: median age 53 (range 35 - 63 years); old group: median age 73 (range 64 - 89) (B).

Tumour-infiltrating leukocytes are associated with patient survival

Based on recent findings suggesting that the makeup of the tumour microenvironment can accurately predict patient prognosis in certain types of cancer (6-9), we investigated whether the amounts of soluble factors or the infiltrating immune cells investigated here were associated with patient survival. To achieve this, we first stratified the patients into two groups according to median values and performed Kaplan-Meier survival analysis. Using this approach, we were unable to identify any single soluble or cellular factor which was associated with patient survival (data not shown). Because the aforementioned approach is based on an arbitrary method of dividing a single patient cohort into two groups, we next investigated whether separating the cohort according to different criteria would reveal discrete groups of patients showing differences in survival. To achieve this we compared the survival of two patient groups according to different cut-off values to determine the threshold with the minimum p value and thus the greatest differences in survival. This analysis surprisingly showed that lower intra-tumoural levels of GM-CSF and IL-10, as well as high levels of PGE2, were associated with better patient survival (Fig. 4A, and B). At the same time, patients with higher levels of intra-tumoural T cells or lower levels of granulocytic cells had better survival (Fig. 4C). To account for the additional number of statistical tests that this method requires, we adjusted the significance threshold accordingly. After this adjustment, only the association between survival and CD15+ infiltration into the margin of the tumour remained significant ($p = 0.0006$, adjusted significance threshold $p = 0.0009$) (Fig. 4C). These results therefore identify soluble and cellular factors in the tumour microenvironment as potential prognostic markers, but they require validation in an independent cohort to confirm these initial findings.

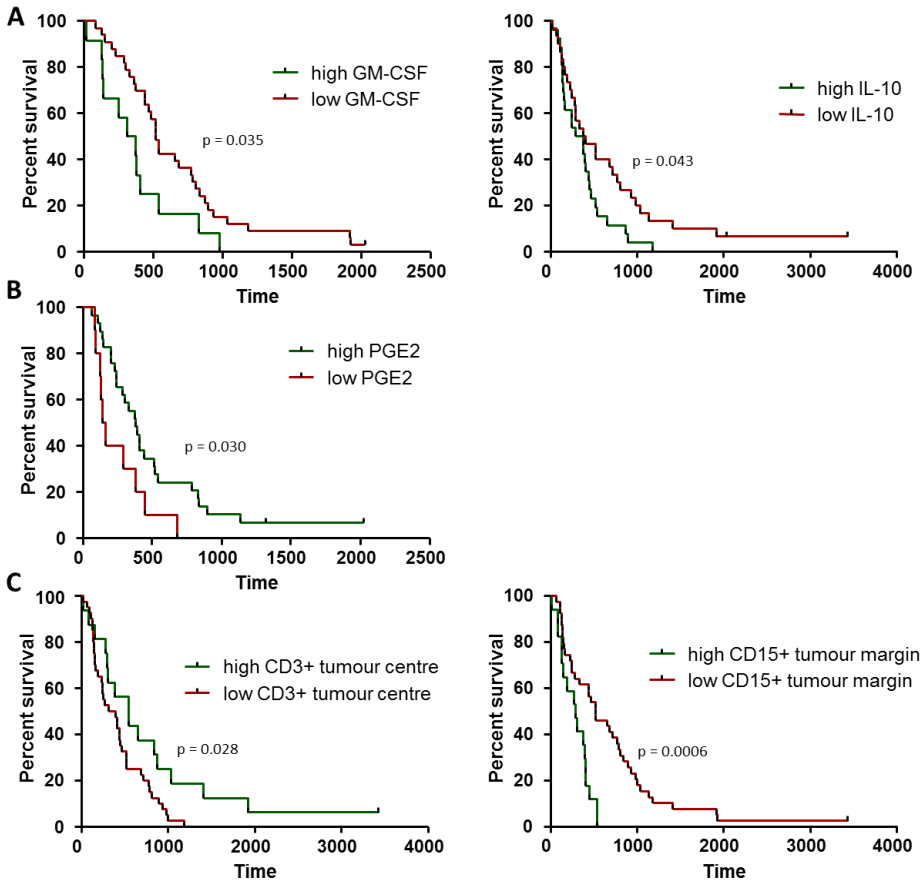

Figure 4. Levels of intra-tumoural leukocytes and soluble molecules are associated with the survival of melanoma patients. We compared the survival of two patient groups according to different cut-off values to determine the largest differences in the survival between patients showing high or low levels of soluble molecules and tumour-infiltrating leukocytes. This showed that low levels of GM-CSF and IL-10 (A) and high levels of PGE2 (B) were associated with longer patient survival. Similarly, patients with high levels of CD3+ or low levels of CD15+ cells in the tumour were found to have a survival benefit (C). When considering the number of statistical tests performed, only intra-tumoural levels of CD15+ cells were found to be associated with survival (adjusted significance threshold p = 0.0009).

GM-CSF (n = 45): 27% of patients in the high group (>), 73% of patients in the low group (<); IL-10 (n = 56): > 46%, < 54%; PGE2 (n = 39): > 74%, < 26%; CD3+ centre (n = 56): > 29%, < 71%; CD15+ margin (n = 56): > 30%, < 70%.

Green lines in survival curves, patients with high levels of indicated feature; red lines, patients with low levels of indicated features; FI, Fluorescence Index

A combination of different types of immune parameters as prognostic markers for metastatic melanoma

Considering our results which suggested that single immune parameters measured in this study might act as prognostic markers, we hypothesised that combinations of different immune features would correlate more closely with clinical outcome. This analysis revealed a survival advantage for patients with high levels of tumour-infiltrating CD3+ cells combined with low levels of CD15+ infiltration, compared to patients with other combinations of these parameters ($p = 0.03$) (Fig. 5A). A trend was observed for patients with high levels of CD3+ cells and low levels of IL-10 ($p = 0.1$) as well as for patients with low levels of both CD15+ cell infiltration and IL-10 ($p = 0.08$) (Fig. 5B). This combinational analysis reduces the number of patients that can be considered, and for this reason these results must be considered preliminary until such time as they are validated.

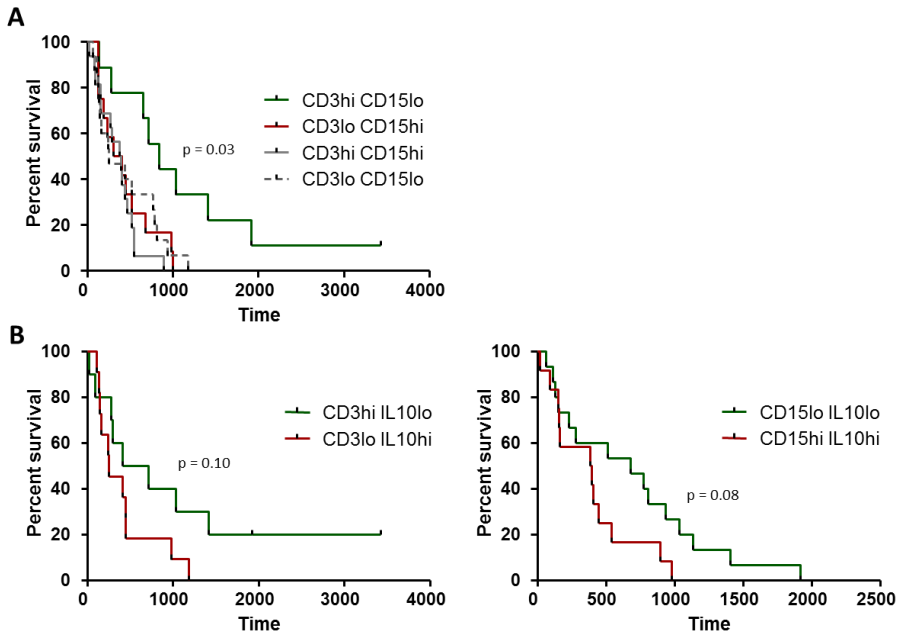

Figure 5 Combinations of multiple immune parameters are more closely associated with survival in melanoma patients. Patients were grouped according to combined immune parameters, and survival between these groups was compared using Kaplan-Meier analysis. This showed that high levels (>median) of tumour-infiltrating CD3+ and low levels (<median) of CD15+ cells were associated with better patient survival (A). Patients with high (> median) levels of tumour-infiltrating CD3+ cells and low (< median) tumour expression of IL-10, or patients with low levels (< median) of tumour-infiltrating CD15+ cells and low (< median) expression of IL-10 were also found to have better survival (B).
CD3hiCD15lo: n = 9, CD3loCD15hi: n = 12, CD3hiCD15hi: n = 16, CD3loCD15lo: n = 15: CD3hiIL-10low: n = 10, CD3lowIL-10hi: n = 11; CD15hiIL-10hi: n = 12, CD15loIL-10lo: n = 15;
Green lines, patients with combined features associated with a better survival; red lines; patients with combined features that are associated with short survival

Discussion

It has been widely reported across different cancer types that high levels of tumour-infiltrating immune cells can be associated with superior patient survival (4, 30). However, tumour progression is frequently seen despite the presence of high levels of such intra-tumoural immune cells, suggesting that there are factors within the tumour microenvironment that impair the function of these cells and/or that these cells lack anti-tumour activity. Therefore, factors providing additional information on the phenotype of

infiltrating cells as well as on the makeup of the tumour microenvironment may be used as biomarkers that can more accurately predict patient survival compared with other approaches that only assess the level of infiltrating immune cells. In support of this, prior investigations have shown that soluble immune molecules in the tumour microenvironment such as PGE2, COX or IL-6, correlate with patient survival (27, 28) and influence the migration, differentiation and functional state of infiltrating immune cells (19, 24, 25) including innate immune cells particularly for TNF (31). These observations support the proposition that the soluble and cellular makeup of the tumour microenvironment plays a fundamental role in the generation of effective anti-tumour immune responses, and thus may more accurately predict patient outcome when compared to the assessment of intra-tumoural leukocytes alone. As the majority of studies on the tumour microenvironment focus on tumour-infiltrating leukocytes, the present study is one of the few to characterise both soluble and cellular intra-tumoural immune features at the same time, with the goal of defining an intra-tumoural immune context linked with favourable patient outcome. To achieve this, we studied tumour-infiltrating T cells (CD3+) and granulocytic cells (CD15+) along with a broad range of soluble molecules (GDF15, PGE2, IL-1Ra, IL-2, IL-6, IL-10, GM-CSF, TNF) and the transcription factor STAT3. Tumour-infiltrating immune cells were present in the majority of patient samples, but CD3+ cells were not associated with any prognostic feature. Only CD15+ cells may be a potential prognostic marker depending on the method of analysis. This result is in contrast to a number of other studies that report an association between CD3+ infiltration and patient prognosis in several other types of cancer (4, 5, 30, 32-38). However, the finding here that intra-tumoural T cells are not related to patient survival adds to the disparate results for the role of tumour-infiltrating T cells in melanoma. Hillen *et al*. reported that high levels of intra-tumoural T cells were associated with poor

survival (39), whereas other studies report the opposite relationship; high levels of intra-tumoural T cells or one study considering peri-tumoural T cell activation markers were found to correlate with longer patient survival (40, 41). Furthermore, a recently published study reported no significant association between the level of intra- or peri-tumoural CD3+ cells and overall survival, in line with the results presented here (42). These discrepancies might be explained by variations in the broad populations of CD3+ T cells, which include helper cells, exhausted or anergic cells, regulatory T cells as well as cytotoxic cells. Thus, CD3 is present on cells with very different functional properties and activation states. Prior studies and the present work suggest that information about the level and distribution of T lymphocytes in the melanoma microenvironment can be associated with patient prognosis, but only in certain contexts. Combining markers providing information on the subsets of infiltrating T cells together with an assessment of the activation state of these cells (eg. measuring markers of exhaustion or activation) may increase the accuracy of this feature to predict patient prognosis. The potential prognostic differences associated with different populations of T cells are highlighted by one study in melanoma which showed an inverse association with patient survival for T lymphocytes, whereas longer survival was found when considering the degree of cells expressing the T cell activation marker CD69 (39). These findings highlight potential limitations in the widespread applicability of studies which propose TILs as markers of patient prognosis. For example, in the “immunoscore” studies performed in colorectal cancer, infiltration by CD3+ T cells alone were sufficient to use as a prognostic marker (8), but despite claims that melanoma is an immunogenic tumour type (43) no such associations were found in the present study.

Many mechanisms inducing T lymphocyte dysfunction have been identified, including suppression by regulatory T cells or through the expression of inhibitory ligands like PD-L1 on tumour cells or APCs (44, 45). These mechanisms have the potential to prevent optimal T cell function within the tumour microenvironment, suggesting that the prognostic value of such TILs is limited by the entire immunological context (both soluble and cellular) which influences the functional state of those cells. In accordance with this, we observed better survival in patients, whose tumours contained high levels of CD3+ cells in addition to low levels of CD15+ cells, suggesting that interactions between intra-tumoural leukocytes are important for their function and activation state. This notion is supported by a study showing that high levels of tumour-infiltrating CD8+ T cells are followed by recruitment of regulatory T cells into the tumour microenvironment, which might serve as a negative feedback mechanism following infiltration by cytotoxic T cells and thus may limit the prognostic accuracy of assessing cytotoxic T cells in isolation (46). In addition to the cellular context, we also observed a trend towards better survival in patients with high levels of CD3+ cells and low expression of the potentially immunosuppressive cytokine IL-10. Considering these data and the increasing clinical use of immunotherapies, more comprehensive analysis of the tumour microenvironment may be required to better predict patient outcome - especially as the clinical efficacy of immunotherapies has been shown to be associated with immune features of the tumour microenvironment in several cancer types (4, 11-18). In the context of immunomodulatory antibodies such as “checkpoint inhibitors”, the level of infiltrating lymphocytes and the expression of the target molecule has been shown to be important. For example Taube *et al*. found an association between the expression of PD-1 on tumour-infiltrating T cells and clinical response to PD-1 antibodies (47), which is in line with the observations by Chen *et al.* who found higher expression of

PD-1 in patients responding to anti-PD-1 therapy as well as higher CD8+ infiltration in responder to CTLA-4 blockade at early on-treatment (48). As such, there is a need to understand the immune context in order to allow better treatment individualization or even the identification of new therapeutic targets.

This was a pilot study investigating soluble and cellular immune parameters as potential combinatorial indicators of patient outcome in metastatic melanoma. The results of this study suggest that certain immune features either alone or in combination may be informative for patient prognosis in this cancer, regardless of the nature of patient pre-treatment. Unlike commonly reported scenarios for other cancer types, T cell infiltration was not strongly associated with patient outcome, whereas granulocytic cells appear to be a more promising factor for the prediction of patient survival in melanoma.

References

1. Society, A. C. 2017. Cancer Facts & Figures 2017.
2. Singh, M., and W. W. Overwijk. 2015. Intratumoral immunotherapy for melanoma. *Cancer immunology, immunotherapy : CII* 64: 911-921.
3. Angel, H., and J. Galon. 2013. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Current opinion in immunology* 25: 261-267.
4. Fridman, W. H., F. Pages, C. Sautes-Fridman, and J. Galon. 2012. The immune contexture in human tumours: impact on clinical outcome. *Nature reviews. Cancer* 12: 298-306.
5. Pages, F., A. Berger, M. Camus, F. Sanchez-Cabo, A. Costes, R. Molidor, B. Mlecnik, A. Kirilovsky, M. Nilsson, D. Damotte, T. Meatchi, P. Bruneval, P. H. Cugnenc, Z. Trajanoski, W. H. Fridman, and J. Galon. 2005. Effector memory T cells, early metastasis, and survival in colorectal cancer. *The New England journal of medicine* 353: 2654-2666.
6. Al-Shibli, K. I., T. Donnem, S. Al-Saad, M. Persson, R. M. Bremnes, and L. T. Busund. 2008. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 14: 5220-5227.
7. DeNardo, D. G., D. J. Brennan, E. Rexhepaj, B. Ruffell, S. L. Shiao, S. F. Madden, W. M. Gallagher, N. Wadhwani, S. D. Keil, S. A. Junaid, H. S. Rugo, E. S. Hwang, K. Jirstrom, B. L. West, and L. M. Coussens. 2011. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer discovery* 1: 54-67.

8. Galon, J., A. Costes, F. Sanchez-Cabo, A. Kirilovsky, B. Mlecnik, C. Lagorce-Pages, M. Tosolini, M. Camus, A. Berger, P. Wind, F. Zinzindohoue, P. Bruneval, P. H. Cugnenc, Z. Trajanoski, W. H. Fridman, and F. Pages. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* 313: 1960-1964.
9. Zhang, L., J. R. Conejo-Garcia, D. Katsaros, P. A. Gimotty, M. Massobrio, G. Regnani, A. Makrigiannakis, H. Gray, K. Schlienger, M. N. Liebman, S. C. Rubin, and G. Coukos. 2003. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *The New England journal of medicine* 348: 203-213.
10. Tumeh, P. C., C. L. Harview, J. H. Yearley, I. P. Shintaku, E. J. Taylor, L. Robert, B. Chmielowski, M. Spasic, G. Henry, V. Ciobanu, A. N. West, M. Carmona, C. Kivork, E. Seja, G. Cherry, A. J. Gutierrez, T. R. Grogan, C. Mateus, G. Tomasic, J. A. Glaspy, R. O. Emerson, H. Robins, R. H. Pierce, D. A. Elashoff, C. Robert, and A. Ribas. 2014. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 515: 568-571.
11. Andre, F., N. Berrada, and C. Desmedt. 2010. Implication of tumor microenvironment in the resistance to chemotherapy in breast cancer patients. *Current opinion in oncology* 22: 547-551.
12. Denkert, C., S. Loibl, A. Noske, M. Roller, B. M. Muller, M. Komor, J. Budczies, S. Darb-Esfahani, R. Kronenwett, C. Hanusch, C. von Torne, W. Weichert, K. Engels, C. Solbach, I. Schrader, M. Dietel, and G. von Minckwitz. 2010. Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 28: 105-113.
13. Gajewski, T. F., J. Louahed, and V. G. Brichard. 2010. Gene signature in melanoma associated with clinical activity: a potential clue to unlock cancer immunotherapy. *Cancer journal* 16: 399-403.
14. Halama, N., S. Michel, M. Kloor, I. Zoernig, A. Benner, A. Spille, T. Pommerencke, D. M. von Knebel, G. Folprecht, B. Luber, N. Feyen, U. M. Martens, P. Beckhove, S. Gnjatic, P. Schirmacher, E. Herpel, J. Weitz, N. Grabe, and D. Jaeger. 2011. Localization and density of immune cells in the invasive margin of human colorectal cancer liver metastases are prognostic for response to chemotherapy. *Cancer research* 71: 5670-5677.
15. Hornychova, H., B. Melichar, M. Tomsova, J. Mergancova, H. Urminska, and A. Ryska. 2008. Tumor-infiltrating lymphocytes predict response to neoadjuvant chemotherapy in patients with breast carcinoma. *Cancer investigation* 26: 1024-1031.
16. Iwamoto, T., G. Bianchini, D. Booser, Y. Qi, C. Coutant, C. Y. Shiang, L. Santarpia, J. Matsuoka, G. N. Hortobagyi, W. F. Symmans, F. A. Holmes, J. O'Shaughnessy, B. Hellerstedt, J. Pippen, F. Andre, R. Simon, and L. Pusztai. 2011. Gene pathways associated with prognosis and chemotherapy sensitivity in molecular subtypes of breast cancer. *Journal of the National Cancer Institute* 103: 264-272.
17. Romano, E., M. Kusio-Kobialka, P. G. Foukas, P. Baumgaertner, C. Meyer, P. Ballabeni, O. Michielin, B. Weide, P. Romero, and D. E. Speiser. 2015. Ipilimumab-dependent cell-mediated cytotoxicity of regulatory T cells ex vivo by nonclassical monocytes in melanoma patients. *Proceedings of the National Academy of Sciences of the United States of America* 112: 6140-6145.
18. Wu, T., and Y. Dai. 2016. Tumor microenvironment and therapeutic response. *Cancer letters*.
19. Mlecnik, B., M. Tosolini, P. Charoentong, A. Kirilovsky, G. Bindea, A. Berger, M. Camus, M. Gillard, P. Bruneval, W. H. Fridman, F. Pages, Z. Trajanoski, and J. Galon. 2010. Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. *Gastroenterology* 138: 1429-1440.
20. Mougiakakos, D., C. C. Johansson, E. Trocme, C. All-Ericsson, M. A. Economou, O. Larsson, S. Seregard, and R. Kiessling. 2010. Intratumoral forkhead box P3-positive regulatory T cells predict poor survival in cyclooxygenase-2-positive uveal melanoma. *Cancer* 116: 2224-2233.

21. Fruci, D., E. Lo Monaco, L. Cifaldi, F. Locatelli, E. Tremante, M. Benevolo, and P. Giacomini. 2013. T and NK cells: two sides of tumor immunoevasion. *Journal of translational medicine* 11: 30.
22. Frumento, G., R. Rotondo, M. Tonetti, G. Damonte, U. Benatti, and G. B. Ferrara. 2002. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *The Journal of experimental medicine* 196: 459-468.
23. Witkiewicz, A., T. K. Williams, J. Cozzitorto, B. Durkan, S. L. Showalter, C. J. Yeo, and J. R. Brody. 2008. Expression of indoleamine 2,3-dioxygenase in metastatic pancreatic ductal adenocarcinoma recruits regulatory T cells to avoid immune detection. *Journal of the American College of Surgeons* 206: 849-854; discussion 854-846.
24. Mao, Y., D. Sarhan, A. Steven, B. Seliger, R. Kiessling, and A. Lundqvist. 2014. Inhibition of tumor-derived prostaglandin-e2 blocks the induction of myeloid-derived suppressor cells and recovers natural killer cell activity. *Clinical cancer research : an official journal of the American Association for Cancer Research* 20: 4096-4106.
25. Obermajer, N., R. Muthuswamy, J. Lesnock, R. P. Edwards, and P. Kalinski. 2011. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood* 118: 5498-5505.
26. Sinha, P., V. K. Clements, A. M. Fulton, and S. Ostrand-Rosenberg. 2007. Prostaglandin E2 promotes tumor progression by inducing myeloid-derived suppressor cells. *Cancer research* 67: 4507-4513.
27. Johansson, C. C., S. Egyhazi, G. Masucci, H. Harlin, D. Mougiakakos, I. Poschke, B. Nilsson, L. Garberg, R. Tuominen, D. Linden, M. F. Stolt, J. Hansson, and R. Kiessling. 2009. Prognostic significance of tumor iNOS and COX-2 in stage III malignant cutaneous melanoma. *Cancer immunology, immunotherapy : CII* 58: 1085-1094.
28. Wouters, M., E. M. Dijkgraaf, M. L. Kuijjer, E. S. Jordanova, H. Hollema, M. Welters, J. van der Hoeven, T. Daemen, J. R. Kroep, H. W. Nijman, and S. H. van der Burg. 2014. Interleukin-6 receptor and its ligand interleukin-6 are opposite markers for survival and infiltration with mature myeloid cells in ovarian cancer. *Oncoimmunology* 3: e962397.
29. Syrbu, S. I., and M. B. Cohen. 2011. An enhanced antigen-retrieval protocol for immunohistochemical staining of formalin-fixed, paraffin-embedded tissues. *Methods in molecular biology* 717: 101-110.
30. Angell, H., and J. Galon. 2013. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Current opinion in immunology* 25: 261-267.
31. Gonzalez-Gugel, E., M. Saxena, and N. Bhardwaj. 2016. Modulation of innate immunity in the tumor microenvironment. *Cancer immunology, immunotherapy : CII* 65: 1261-1268.
32. Al-Attar, A., M. Shehata, L. Durrant, P. Moseley, S. Deen, and S. Chan. 2010. T cell density and location can influence the prognosis of ovarian cancer. *Pathology oncology research : POR* 16: 361-370.
33. Cai, X. Y., Q. Gao, S. J. Qiu, S. L. Ye, Z. Q. Wu, J. Fan, and Z. Y. Tang. 2006. Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. *Journal of cancer research and clinical oncology* 132: 293-301.
34. Nakano, O., M. Sato, Y. Naito, K. Suzuki, S. Orikasa, M. Aizawa, Y. Suzuki, I. Shintaku, H. Nagura, and H. Ohtani. 2001. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer research* 61: 5132-5136.
35. Ropponen, K. M., M. J. Eskelinen, P. K. Lipponen, E. Alhava, and V. M. Kosma. 1997. Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *The Journal of pathology* 182: 318-324.

36. Setala, L. P., V. M. Kosma, S. Marin, P. K. Lipponen, M. J. Eskelinen, K. J. Syrjanen, and E. M. Alhava. 1996. Prognostic factors in gastric cancer: the value of vascular invasion, mitotic rate and lymphoplasmacytic infiltration. *British journal of cancer* 74: 766-772.
37. Simpson, J. A., A. Al-Attar, N. F. Watson, J. H. Scholefield, M. Ilyas, and L. G. Durrant. 2010. Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. *Gut* 59: 926-933.
38. Vesalainen, S., P. Lipponen, M. Talja, and K. Syrjanen. 1994. Histological grade, perineural infiltration, tumour-infiltrating lymphocytes and apoptosis as determinants of long-term prognosis in prostatic adenocarcinoma. *European journal of cancer* 30A: 1797-1803.
39. Hillen, F., C. I. Baeten, A. van de Winkel, D. Creytens, D. W. van der Schaft, V. Winnepenninckx, and A. W. Griffioen. 2008. Leukocyte infiltration and tumor cell plasticity are parameters of aggressiveness in primary cutaneous melanoma. *Cancer immunology, immunotherapy : CII* 57: 97-106.
40. Bogunovic, D., D. W. O'Neill, I. Belitskaya-Levy, V. Vacic, Y. L. Yu, S. Adams, F. Darvishian, R. Berman, R. Shapiro, A. C. Pavlick, S. Lonardi, J. Zavadil, I. Osman, and N. Bhardwaj. 2009. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. *Proceedings of the National Academy of Sciences of the United States of America* 106: 20429-20434.
41. Ladanyi, A., B. Somlai, K. Gilde, Z. Fejos, I. Gaudi, and J. Timar. 2004. T-cell activation marker expression on tumor-infiltrating lymphocytes as prognostic factor in cutaneous malignant melanoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* 10: 521-530.
42. Weiss, S. A., S. W. Han, K. Lui, J. Tchack, R. Shapiro, R. Berman, J. Zhong, M. Krogsgaard, I. Osman, and F. Darvishian. 2016. Immunologic heterogeneity of tumor-infiltrating lymphocyte composition in primary melanoma. *Human pathology* 57: 116-125.
43. Haanen, J. B. 2013. Immunotherapy of melanoma. *EJC supplements : EJC : official journal of EORTC, European Organization for Research and Treatment of Cancer ... [et al.]* 11: 97-105.
44. Gajewski, T. F. 2007. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. *Clinical cancer research : an official journal of the American Association for Cancer Research* 13: 5256-5261.
45. Marincola, F. M., E. M. Jaffee, D. J. Hicklin, and S. Ferrone. 2000. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Advances in immunology* 74: 181-273.
46. Spranger, S., R. M. Spaapen, Y. Zha, J. Williams, Y. Meng, T. T. Ha, and T. F. Gajewski. 2013. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. *Science translational medicine* 5: 200ra116.
47. Taube, J. M., A. Klein, J. R. Brahmer, H. Xu, X. Pan, J. H. Kim, L. Chen, D. M. Pardoll, S. L. Topalian, and R. A. Anders. 2014. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research* 20: 5064-5074.
48. Chen, P. L., W. Roh, A. Reuben, Z. A. Cooper, C. N. Spencer, P. A. Prieto, J. P. Miller, R. L. Bassett, V. Gopalakrishnan, K. Wani, M. P. De Macedo, J. L. Austin-Breneman, H. Jiang, Q. Chang, S. M. Reddy, W. S. Chen, M. T. Tetzlaff, R. J. Broaddus, M. A. Davies, J. E. Gershenwald, L. Haydu, A. J. Lazar, S. P. Patel, P. Hwu, W. J. Hwu, A. Diab, I. C. Glitza, S. E. Woodman, L. M. Vence, Wistuba, II, R. N. Amaria, L. N. Kwong, V. Prieto, R. E. Davis, W. Ma, W. W. Overwijk, A. H. Sharpe, J. Hu, P. A. Futreal, J. Blando, P. Sharma, J. P. Allison, L. Chin, and J. A. Wargo. 2016. Analysis of Immune Signatures in Longitudinal Tumor Samples Yields Insight into Biomarkers of Response and Mechanisms of Resistance to Immune Checkpoint Blockade. *Cancer discovery* 6: 827-837.

Supplementary Data 1: Full set of correlations between soluble, cellular and transcription factors

IL-6	GM-CSF	r=0.733, p=0.000
	TNF	r=0.489, p=0.000
	STAT3	r=0.462, p=0.001
GM-CSF	TNF	r=0.444, p=0.007
	STAT3	r=0.395, p=0.017
	IL-1Ra	r=0.475, p=0.014
PGE2	Ratio CD15 margin/centre	r=-0.408, p=0.031
TNF	STAT3	r=0.688, p=0.000
	CD15 evenness	r=0.32, p=0.034
STAT3	IL-2	r=0.421, p=0.008
	IL-10	r=0.364, p=0.023
	CD15 evenness	r=0.349, p=0.02
GDF15	IL-1Ra	r=0.41, p=0.004
IL-2	IL-10	r=0.503, p=0.000
CD3 margin	CD3 centre	r=0.765, p=0.000
	CD3 total	r= 0.959, p=0.000
	CD3 ratio margin/centre	r=0.362, p=0.026
	CD3 evenness	r=0.913, p=0.000
	CD15 evenness	r=0.296, p=0.033
CD3 centre	CD3 total	r=0.89, p=0.000
	CD3 ratio margin:centre	r=-0.52, p=0.001
	CD3 evenness	r=0.874, p=0.00
	CD15 margin	r=0.312, p=0.025
	CD15 evenness	r=0.293, p=0.035
CD3 total	CD3 evenness	r=0.935, p=0.000
	CD15 margin	r=0.294, p=0.034
	CD15 centre	r=0.276, p=0.047
	CD15 evenness	r=0.308, p=0.026
CD3 evenness	CD15 margin	r=0.284, p=0.041
	CD15 centre	r=0.275, p=0.048
	CD15 evenness	r=0.307, p=0.027
CD15 margin	CD15 centre	r=0.600, p=0.000
	CD15 total	r=0.847, p=0.000
	CD15 ration margin/centre	r=0865, p=0.000
	CD15 evenness	r=0.830, p=0.000
CD15 centre	CD15 total	r=0.880, p=0.000
	CD15 ratio margin/centre	r=0.528, p=0.000
	CD15 evenness	r=0.792, p=0.000
CD15 total	CD15 ratio margin/centre	r=0.740, p=0.000
	CD15 evenness	r=0.914, p=0.000
CD15 ratio margin/centre	CD15 evenness	r=0.729, p=0.000