Aus der Abteilung Mikrovaskuläre Biologie und Pathobiologie
European Center for Angioscience (ECAS)
der Medizinischen Fakultät Mannheim
der Universität Heidelberg

Direktor: Prof. Dr. J. P. Sleeman

TGFβ1-mediated crosstalk in the tumor microenvironment upregulates expression of PAI-1, which predicts poor prognosis in melanoma patients

Inauguraldissertation
zur Erlangung des medizinischen Doktorgrades
der
Medizinischen Fakultät Mannheim
der Ruprecht-Karls-Universität
zu
Heidelberg

vorgelegt von
Kathrin Metzger

aus
St. Ingbert

2020

Dekan: Prof. Dr. Sergij Goerdt
Referent: Prof. Dr. Jonathan. P. Sleeman

TABLE OF CONTENTS

Page

1 LIST OF ABBREVIATIONS

(d)H_2O	(distilled) water
°C	Degrees Celsius
α-SMA	Alpha-smooth muscle actin
µg	Microgram
µl	Microliter
µM	Micromol
4-PL	Four parameter logistic curve fit
AJCC	American Joint Committee of Cancer
AKT	Protein kinase B
AP	Alkaline phosphatase
ATCC	American Type Culture Collection
b-FGF	Basic fibroblast growth factor
BRAF	B-rapidly accelerated fibrosarcoma
BRAFi	BRAF inhibitor
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
CCL	CC-chemokine ligand
CDH2	N-cadherin
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
COL1A1	Collagen Type I alpha 1
CSF	Colony-stimulating factor
CTGF	Connective tissue growth factor
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL	CXC-chemokine ligand
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithelial growth factor
ELISA	Enzyme-linked immunoassay
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal regulated kinase
FAK	Focal adhesion kinase
FAP	Fibroblast activation protein
FBN	Fibronectin
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FPR2	Formyl-peptide receptor 2
FSP1=S100A4	Fibroblast specific protein= S100 calcium-binding protein A4
g	Gravitational acceleration, 9.80665 m/s^2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
h	Hour(s)
H.E.	Hematoxylin and eosin staining
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HDF	Human dermal fibroblast
HRP	Horseradish peroxidase
IL	Interleukin

IFN	Interferon
kb	Kilo bases
LAP	Latency-associated peptide
LDH	Lactate dehydrogenase
LRP1	Low-density lipoprotein receptor-related protein1
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MEK	Mitogen-activated protein kinase kinase
MHC	Major histocompatibility complex
min	Minute
ml	Milliliter
mM	Millimol
MMP	Matrix-metalloproteases
mRNA	Messenger ribonucleic acid
MyD88	Myeloid-differentiation primary response 88
NaCl	Sodium chloride
NF-κB	Nuclear factor “kappa-light-chain-enhancer” of activated B cells
ng	Nanogram
NK cells	Natural killer cells
nm	Nanometer
NRAS	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non-small cell lung carcinoma
OS	Overall survival
OSCC	Oral squamous cell carcinoma
P/S	Penicillin/Streptomycin
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed cell death 1 ligand 1
PFS	Progression-free survival
PI3K	Phosphatidyl-inositol-3-kinase
PMA	Phorbol-12-myristate-13-acetate
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNAse	Ribonuclease
RPLP0	60S acidic ribosomal protein P0
RPMI 1640	Roswell Park memorial institute 1640 medium
RT	Reverse transcriptase
s	Second
S100	S100 calcium-binding protein
SAA	Serum amyloid A 1/2
Smad	Sma/mothers against decapentaplegic
TAE	Tris-acetate EDTA buffer
TAM	Tumor-associated macrophage
Taq Polymerase	Thermus aquaticus polymerase, DNA polymerase I
TBS	Tris-buffered saline
TGFBR	Transforming growth factor beta receptor
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitor of metalloprotease
TLR	Toll-like receptor
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
Tregs	Regulatory T cells
U	Unit
uPA	Urokinase plasminogen activator
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor

2 SUMMARY

Malignant melanoma can be well controlled in early stages, but despite new therapeutic options, the five-year survival rate decreases down to 9 % in metastatic disease. The metastatic propensity of melanoma is a result of the interaction between cancer cells and their microenvironment, in which cancer-associated fibroblasts and tumor-associated macrophages play an important role. Growth factors and cytokines such as transforming growth factor beta 1 and serum amyloid A, secreted by tumor and stromal cells, mediate a dynamic crosstalk between these cellular compartments. This crosstalk stimulates tumor progression through modifying the transcriptional program of those cells. The transcriptional response to this crosstalk by cancer-associated stromal cells represents a promising source of factors with prognostic or theranostic potential, but this currently remains under-investigated in melanoma. This thesis therefore assesses the expression of transforming growth factor beta 1 and serum amyloid A in melanoma, and asks whether the expression of molecules with potential prognostic value might be induced in cancer-associated fibroblasts or macrophages in response to these two factors.

The expression of serum amyloid A and transforming growth factor beta 1 in primary melanoma was investigated by immunohistochemistry and quantitative real-time polymerase chain reaction, which showed that both proteins are expressed by tumor and stromal cells. Next, the transcriptional response of human dermal fibroblasts and monocytic macrophage THP-1 cells to serum amyloid A and transforming growth factor beta 1 was assessed. Serum amyloid A upregulated the expression of factors implicated in melanoma invasion, matrix remodeling, angiogenesis, immune evasion and therapy resistance in THP-1 cells through toll-like receptor 4, including its own expression. Transforming growth factor beta 1 stimulated its own expression in fibroblasts, as well as that of serum amyloid A and the cancer-associated fibroblast marker alpha-smooth muscle actin. Importantly, transforming growth factor beta 1 upregulated plasminogen activator inhibitor 1 in both fibroblasts and THP-1 cells, which was assessed by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay.
Plasminogen activator inhibitor 1 is known as a transforming growth factor beta 1-inducible molecule that can foster tumor progression through several mechanisms, but its potential role as a marker for melanoma has not yet been extensively studied. Therefore, this thesis addresses the question of the prognostic potential of plasminogen activator inhibitor 1 for melanoma. Increased local protein expression of plasminogen activator inhibitor 1 was indeed associated with poor prognosis in melanoma patients. Consistently, the systemic levels of transforming growth factor beta 1 and plasminogen activator inhibitor 1 positively correlated with each other, measured by enzyme-linked immunosorbent assay in the plasma of melanoma

patients. Further work showed that fibroblasts are an important source of plasminogen activator inhibitor 1 in addition to melanoma cells themselves.

Taken together, this thesis shows that melanoma cells condition fibroblasts and monocytic macrophages via transforming growth factor beta 1 and serum amyloid A to create a tumor promoting milieu. Furthermore, fibroblasts stimulated with transforming growth factor beta 1 are an important source of locally overexpressed plasminogen activator inhibitor 1, which predicts poor prognosis in melanoma patients. Therefore, plasminogen activator inhibitor 1 represents a possible new diagnostic tool in melanoma.

3 INTRODUCTION

3.1 Melanoma

Skin cancer is the most common human malignancy. Although melanoma only accounts for 4 % of all skin cancers, it causes the majority of skin cancer deaths[1]. Melanoma is defined as malignancy of the melanocytes, which are neural crest-derived cells located in the basal epidermis, responsible for the production of melanin pigment[2]. The most common form is cutaneous melanoma, but this tumor can also be localized at the mucosa of many organs, the uvea[3] as well as the subungual space[4]. Since these three types of melanoma have different biological and clinical features[5], only melanoma of the skin will be considered here.

The incidence of cutaneous melanoma is rising faster than that of any other solid tumor[6]. From 1999 to 2016, age-standardized incidence rates increased from 12.8 % to 20.1 % per 100 000 inhabitants per year in Germany[7], and are expected to continue rising[8]. Locally limited melanoma can be well controlled by surgical excision, with a five-year survival rate of more than 95 %[9]. Nevertheless, melanoma is an aggressive tumor with high metastatic potential. It is characteristic of melanoma that primary tumors of only a few millimeters thickness are able to spread to distant organs[10]. Once melanoma has metastasized, disease management is a lot more challenging, and the five-year survival rate decreases down to 9 %[9]. The interaction between melanoma cells and stromal cells of the microenvironment is believed to play a crucial role in this metastatic propensity[11].

Exposure to ultraviolet radiation (UVR) is considered the major risk factor for the development of melanomas[12]. It causes oxidative damage to base pairs, proteins and lipids, but can also be responsible for direct deoxyribonucleic acid (DNA) damage[1]. Photoproducts such as pyrimidine dimers can lead to cancer-initiating mutations if they are repaired incorrectly[13]. Additionally, UVR can cause deletions in mitochondrial DNA, leading to mitochondrial dysfunction and cellular energy imbalance, which also contributes to photoaging and tumorigenesis[14]. UVR can also act indirectly to promote tumorigenesis. For example, malfunction or apoptosis of immune cells can be caused by UVR, which disturbs the endogenous antitumor immune control in early melanomagenesis[14]. In line with this, chronic immunosuppression has been found to be a risk factor for the development of melanomas[15].
Several oncogenic mutations are associated with melanoma. Of these, those affecting the mitogen-activated protein kinases (MAPK) pathway play an important role. This pathway regulates cell proliferation, differentiation, migration, senescence and apoptosis in response to stress or growth stimuli[16]. Mutations of the serine/threonine-protein kinase B-rapidly accelerated fibrosarcoma (BRAF), that is part of the MAPK pathway, are considered driver mutations of approximately 50 to

60 % of melanomas[17]. Mutated BRAF leads to constant, ligand-independent activation of the MAPK pathway, resulting in uncontrolled proliferation. After the discovery of this driver mutation, targeted anti-BRAF therapies have become available for BRAF-mutated patients with metastatic disease, leading to better survival. However resistance to treatment often develops, and BRAF inhibitor (BRAFi) resistant tumors pose a clinical challenge[18]. The second most common driver mutation is that of the GTPase Neuroblastoma RAS viral oncogene homolog (NRAS), which is found in approximately 10 to 15 % of all melanomas[17]. NRAS connects the MAPK pathway to the phosphatidyl-inositol-3-kinase/protein kinase B (PI3K/AKT) pathway[19], which regulates survival, metabolism, cell proliferation and angiogenesis. Mutated NRAS is locked in its active form and therefore permanently stimulates both the PI3K/AKT and MAPK pathways[20]. In melanoma, these mutations are associated with more aggressive tumors and poor survival[17]. To date, there are no specific therapeutic approaches against mutated NRAS in melanoma[20].

3.1.1 Prognostic parameters

Currently, melanoma stages are classified based on the eighth version of the American Joint Committee of Cancer (AJCC) staging system[21]. Stages I and II represent the local disease stages with better patient survival. Stage III (lymph node metastases, locoregional disease) and IV (metastatic disease) are associated with worse prognosis. This classification considers tumor thickness, mitotic rate and ulceration of the primary tumor, as well as lymph node status, systemic lactate dehydrogenase (LDH) levels and the presence of distant metastases[21]. The following sections address these parameters and also other histopathological features and laboratory tests that have prognostic value in melanoma.

The histopathological criterion of tumor thickness is measured from the stratum granulosum of the epidermis to the deepest level of tumor invasion, and is also called the Breslow index[22]. However, as there are also thin tumors with high metastatic potential and poor prognosis, or thick but noninvasive tumors with better prognosis, this parameter does not always reflect patient outcome[23]. Additionally, ulceration status of the primary tumor is also linked to a higher risk of melanoma-associated deaths and is part of the current classification system[21]. Another histopathological feature of melanoma (not included in the AJCC staging) is the infiltration of the primary tumor by immune cells. The communication between melanoma and immune cells plays an important role in melanoma initiation and progression[24], but not all mechanisms have been elucidated yet. For instance, the infiltration by lymphocytes has been associated with good prognosis by some researchers, but also with poor outcome by others. This was dependent on the growth phase and the thickness of the tumor[25]. However, the presence of tumor-infiltrating macrophages in melanomas is clearly linked to poor prognosis[26].

Several predictive markers can be determined in the peripheral blood of melanoma patients. Systemic LDH levels are recommended to be assessed in melanoma

patients with suspected or proven metastasis as a part of the staging[21]. This intracellular enzyme can be found in nearly every cell type and catalyzes the conversion of lactate to pyruvic acid (and back) in anaerobic glycolysis[27]. Since LDH levels can indicate enhanced cell turnover[28], systemic LDH levels are used as an indicator for tumor burden in many different cancers[27]. In cutaneous melanoma, this represents a cost-effective parameter to predict worse patient survival in late stages. Additionally, LDH levels can also be used to monitor therapy response[29]. Nevertheless, systemic LDH remains an unspecific parameter, since it is also elevated in other malignancies[23] or in various nonmalignant conditions that result in enhanced cell turnover (e.g. infarction, inflammation, infection or hemolysis[28]). Another biomarker is the S100 calcium-binding protein S100B, which was first detected in melanoma cell lines[30]. Its systemic elevation was found to correlate with advanced clinical stage[31], worse survival and metastasis[28]. However, brain and non-melanoma skin tumors have also been reported to produce S100B[31], and false positive results have been observed in patients with hepatic or renal insufficiency or infectious diseases[28]. Additionally, the predictive value of systemic S100B levels seemed to be restricted to stage III and IV melanoma patients[31], and the measurement of S100B is only conclusive if lymph node or distant metastases are suspected or diagnosed[21].

In conclusion, various parameters are used to predict melanoma patient outcome, focusing on molecules that are produced by tumor cells. However, the tumor microenvironment as a potential source of prognostic molecules has been under-investigated so far.

3.1.2 Treatment

Depending on the stage and the biological features of the tumor, different therapy options are available. At stage I to III, an excision with safety margin is the standard procedure. For *in situ*-melanomas, this margin is 0.5 cm. For tumors with a thickness of up to 2 mm, a margin of 1 cm is required, while for tumors above 2 mm thickness, the margin is 2 cm[21]. Sentinel lymph node biopsy (SLNB) is recommended for primary tumors of 1 mm thickness or above without evidence of lymph node or distant metastases[21]. SLNB is considered to offer accurate information about patient prognosis, but remains controversial since there is little evidence regarding its therapeutic benefit[32].

As 20% of melanoma patients undergoing surgical resection will still develop metastases[33], adjuvant systemic therapy is recommended. At stage I and II, systemic administration of interferons (IFN) can be considered[21]. Interferon-alpha 2b (IFN-α 2b) activates antitumor T cells and natural killer (NK) cells. It also inhibits myeloid-derived suppressor cells and regulatory T cells (Tregs)[18], that dampen the activity of the latter immune cells[25, 34]. Hence, IFN therapy increases the activity of immune cells that are able to eliminate melanoma cells. Frequent systemic side effects are autoimmune reactions, liver toxicity, skin rash, neutropenia and anaemia[18]. Adjuvant

therapy with IFN-α 2b improved survival and decreased the risk of recurrence in a current meta-analysis[35], but only few patients responded to this therapy[36].
At stage III, when lymph node metastases are present, the excision procedure described above followed by a lymph node dissection represent the standard of care. Adjuvant radiation therapy of the suspect lymphatic drainage basin and/or lymph nodes is also recommended when certain criteria are met.

As soon as there are distant metastases (stage IV), surgery should only be realized if a complete resection is achievable, if it will not lead to inacceptable functional deficits and if other therapies were not or might not be successful[21].
Various possibilities for pharmacological intervention are available at advanced melanoma stages. For example, IFNs can be considered. Additionally, from stage IIIB on, it is recommended to test for the occurrence of a driver mutation such as BRAF, that can be subject to a targeted therapy[21]. Approximately 90% of stage IV patients undergoing BRAFi therapy initially responded with tumor regression[37]. The BRAFi vemurafenib improved overall and progression-free survival of melanoma patients compared to the formerly used chemotherapeutic agent dacarbazine[37]. Nevertheless, BRAFi therapy in melanoma is limited by the emergence of therapy resistance caused by different mechanisms[38]. Reasons for BRAFi resistance include the expression of an altered BRAF protein and de novo mutations of other molecules in the MAPK or PI3K/AKT pathways by the malignant cells (e.g. of Mitogen-activated protein kinase kinase (MEK) 1/2, NRAS or AKT1)[18]. Furthermore, stromal cells can also play a role in BRAFi resistance, which will be discussed in more detail later. Thus, a combination of BRAF inhibition together with MEK inhibition is currently recommended for BRAF mutated patients[21].
In addition to targeted therapy, immune checkpoint inhibitors represent a promising therapeutic option for metastatic melanoma patients. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an inhibitory receptor that blocks T cell responses and induces immunotolerance. The blockade of CTLA-4 with ipilimumab resulted in T cell proliferation, cytokine production and infiltration of antitumor immune cells into melanomas[18]. In a clinical trial, approximately 20 % of melanoma patients responded to ipilimumab[39]. However, 10 % suffered from severe adverse effects including colitis, skin reactions and endocrinopathies[40]. Anti-CTLA-4 therapy did not increase overall-survival, but led to better progression-free survival when combined with chemotherapy[41].
Another molecule for immune checkpoint inhibition is programmed cell death protein 1 (PD-1). It is present on the surface of T cells, binds to programmed cell death 1 ligand 1 (PD-L1) on antigen-presenting cells (monocytes/macrophages, B cells and dendritic cells), and acts as a co-inhibitory stimulus for T cells. Additionally, PD-L1 can be expressed by tumor cells, enabling them to inhibit the activity of potential antitumor T cells[42]. PD-1 inhibition with nivolumab or pembrolizumab therefore activates cytotoxic T cells against malignant cells. Response rates to nivolumab in melanoma were approximately 44 %[39], and PD-1 inhibitors showed lower rates of severe adverse effects compared to anti-CTLA-4-antibodies[38]. Additionally, anti-PD-1 therapy was found to increase overall and progression-free

survival compared to chemotherapy in a recent Cochrane review[41]. Since 13 % of patients with PD-L1-negative tumors still profited from PD-1 blockade[43], testing PD-L1 expression is not considered necessary before the application of PD-1 inhibitors[23]. Therefore, as an alternative to the combination of BRAF- and MEK inhibition, anti-PD-1 therapy or a combined blocking of PD-1 and CTLA-4 are recommended[21].

Altogether, activation of the host immune system has led to a variety of new therapy options, especially for metastatic melanoma patients. As a result, the therapeutic landscape of melanoma is becoming more and more complex, creating the need for markers that provide a support for decisions between different clinical strategies on an individualized basis.

3.2 The tumor microenvironment

As mentioned previously, the interaction between cancer cells and their microenvironment plays a major role in melanoma progression and metastasis[11], and might therefore be a promising source of new biomarkers. The microenvironment is defined as the cellular and non-cellular components of the tumor niche[34]. The cellular components include fibroblasts and immune cells (such as T cells, B cells, monocytes/macrophages, NK cells, neutrophils, dendritic cells and tissue-specific immune cells), but also endothelial cells, pericytes and adipocytes. The non-cellular components include extracellular matrix (ECM) components and secreted molecules[44].

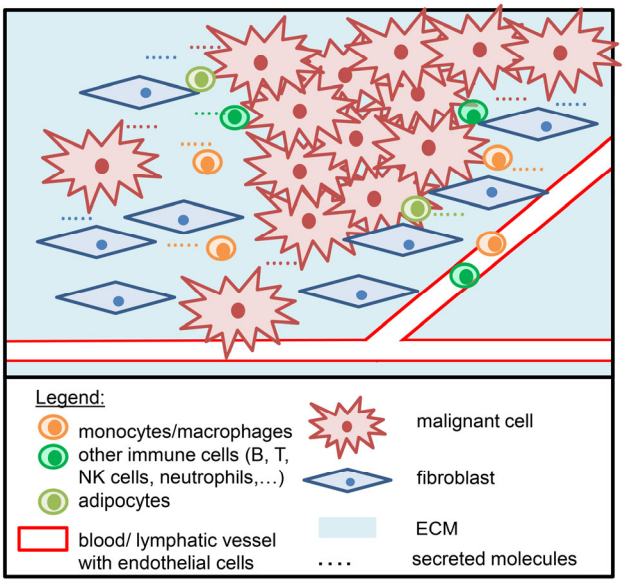

Figure 1: Simplified scheme of the tumor microenvironment. This represents a simplified overview without any claim to completeness. ECM=Extracellular matrix. NK cells=natural killer cells.

Fibroblasts represent the most abundant cell type in the microenvironment of many tumors, besides the tumor cells themselves[45]. These cancer-associated fibroblasts (CAFs) are currently the subject of research in many different tumors, including melanoma[46]. Monocytes/macrophages represent an important first line of defense against pathogens and malignant cells[47], but when they become activated in favor of

the malignant cells, tumor-associated macrophages (TAMs) play a key role in melanoma progression[48]. Therefore, these two stromal cell types will be described in more detail in the next sections.

3.2.1 Cancer-associated fibroblasts

Fibroblasts are spindle-shaped cells of mesenchymal origin that do not express epithelial (cytokeratin, E-cadherin), endothelial (CD31) or immune (CD45) cell markers[49]. Physiologically, they are involved in homeostasis of the ECM by secreting, modifying and degrading its components[50]. Through the production of basement membrane components, fibroblasts control the polarity of epithelial cells[51]. From their quiescent state, they are able to become activated in response to growth stimuli. Then, they proliferate, migrate, change their cytoskeleton architecture to be more contractile, and secrete cytokines, chemokines and ECM components[49]. Hence, fibroblasts are also key players in wound healing, acute and chronic inflammation, fibrosis, cancer and many other processes[50].

As a result of their interaction with malignant cells, fibroblasts can become CAFs, but their phenotype varies in different malignancies[52]. In many tumors including melanoma, the main source of CAFs are resident fibroblasts that become stimulated through tumor-derived transforming growth factor beta 1 (TGFβ1)[46]. In addition, bone marrow-derived mesenchymal stem cells, adipocytes, epithelial, endothelial or smooth muscle cells, pericytes or fibrocytes can also give rise to CAFs[53].

Due to their heterogeneity, CAFs cannot be defined by a single marker, but are characterized by a set of potential markers. For instance, alpha-smooth muscle actin (α-SMA), a TGFβ1-inducible isoform of the cytoskeleton protein actin, is characteristic of CAF populations in melanoma and various other tumors[54]. It is involved in the wound healing-associated formation of stress fibers and is closely linked to myofibroblast differentiation[55]. Additionally, fibroblast activation protein (FAP), fibroblast-specific protein 1 (FSP1, also known as S100 calcium-binding protein A4) and platelet-derived growth factor receptors α and β (PDGFRα and β) can identify CAFs, depending on the tumor[56].

CAFs can foster melanoma progression through various mechanisms. For instance, the direct interaction between CAFs and melanoma cells mediated through CAF surface-bound CD44 promoted melanoma growth and maintained the stemness of tumor-initiating cells[57]. Direct binding of melanoma cells to N-cadherin expressed by dermal CAFs facilitated melanoma invasion through the basal membrane and activated antiapoptotic pathways in melanoma cells[58]. Additionally, factors secreted by CAFs such as interleukin (IL) 6 and 8 were found to promote melanoma invasiveness[59].
A major role of CAFs is the remodeling of the ECM through the production of TGFβ1, matrix components and proteolytic enzymes such as matrix-metalloproteases (MMPs)[60]. This fosters cancer cell invasion and migration in various tumors[61, 62]

including melanoma[63]. Matrix remodeling by CAFs can be mediated through TGFβ1, and was observed to enhance melanoma growth and metastasis *in vivo*[64]. Others found that MMP2[63] and MMP13[65] produced by CAFs stimulated melanoma growth. CAF-derived MMP2 can also cleave activating ligands from the cell surface of NK cells, thereby suppressing their antitumor cytotoxicity[66]. Furthermore, by remodeling the ECM, melanoma-associated fibroblasts exposed to BRAFi can cause increased adhesion of melanoma cells to the ECM via integrin β-1, leading to intracellular activation of focal adhesion kinase (FAK). FAK activity results in MAPK-independent activation of the downstream extracellular signal regulated kinase (ERK), and thus BRAFi resistance[67]. MMP9 secreted by CAFs can cleave PD-L1 from the surface of melanoma cells, leading to anti-PD-1 therapy resistance. Consistent with the importance of TGFβ1 in CAF function, this study also showed that established therapy resistance to anti-PD-1 therapy could be mitigated by TGFβ1 inhibition[68].

In tumors other than melanoma, the histological presence of CAFs has been associated with poor outcome, for example in oral squamous cell carcinoma (OSCC)[69], colorectal carcinoma[70] and breast cancer[71]. Recently, an association between the local expression of connective tissue growth factor by CAFs and poor disease-free survival in a small cohort of 46 melanoma patients nearly reached significance (logrank test: $p=0.06$)[72], suggesting that molecules produced by CAFs in melanoma might have a prognostic potential.

In summary, it is clear that CAFs and the factors they produce can contribute to tumor progression, but an association with patient outcome has not yet been investigated sufficiently in melanoma.

3.2.2 Tumor-associated macrophages

Monocytes are recruited from the blood to several tissues and can be differentiated into distinct types of macrophages. In general, macrophages are responsible for antigen-presentation, as well as recognition and phagocytic elimination of foreign material and apoptotic cells. They play an important role in tissue repair and wound healing as well as inflammatory responses[47].

Due to the plasticity and complexity of macrophage phenotypes, different classification systems with respect to markers, function and polarizing agents of human and murine macrophage subtypes can be found in the literature. Most classification systems for macrophages include at least two activated phenotypes: classically activated M1 macrophages, and alternatively activated M2 macrophages. Generally, M1 and M2 macrophage subtypes are considered to represent the extremes of a continuum. A switch from M1 to M2 or vice-versa might be possible[73].

Polarization of macrophages into the M1 phenotype can be conditioned through IFN-γ, bacterial lipopolysaccharides or toll-like receptor (TLR) agonists. The M1 macrophage subtype fosters inflammatory signaling, has antimicrobial and antitumor

functions, and is characterized by high levels of major histocompatibility complex class II molecules (MHCII), IL1 and IL12, and low levels of IL10[73-75]. M2 macrophages on the other hand promote immune suppression, and display low levels of IL12 and MHCII and high levels of IL10.
Different subtypes of M2 macrophages have been described, whose generation depends on the stimulus to which they have been exposed. IL4 or IL13 stimulation gives rise to “alternatively activated” M2a macrophages, which are responsible for allergic responses and the elimination of parasites and fungi. The M2b phenotype, also referred to as “type 2 macrophage”, is activated by immune complexes and TLR ligands, and negatively regulates other immune cells. Alternatively, glucocorticoids, IL10 or TGFβ1 can polarize monocytes towards “deactivated” M2c macrophages, which regulate immune responses as well as ECM remodeling[73, 75, 76].

TAMs represent a third phenotype that monocytes can adopt when exposed to tumor-derived factors[77]. The main sources of TAMs are monocytes from peripheral blood vessels[78]. Through cancer-derived factors, they are recruited to the primary tumor, where they then further differentiate. These factors depend on the cancer type, and not all conditions that lead to TAM recruitment and polarization have been elucidated yet. In melanoma, it has been reported that tumor-derived CC-chemokine ligand (CCL) 2, vascular endothelial growth factor (VEGF) C[48], VEGFA, IL34, CXC chemokine ligand (CXCL) 12[79] and macrophage colony-stimulating factor (M-CSF)[80] might play a role in the generation of TAMs.
TAMs are believed to resemble M2 macrophages[81], and they are sometimes referred to as “M2d macrophages”[75]. However, other evidence suggests that TAMs can share properties of M1 and M2 macrophages[77]. The surface-bound receptor CD163 is commonly used to identify TAMs *in situ* in several types of cancer[77], including melanoma[82, 83], but it seems to be expressed by certain subsets of M2 macrophages[84]. The following Figure 2 summarizes the most important features and origins of human macrophage subtypes.

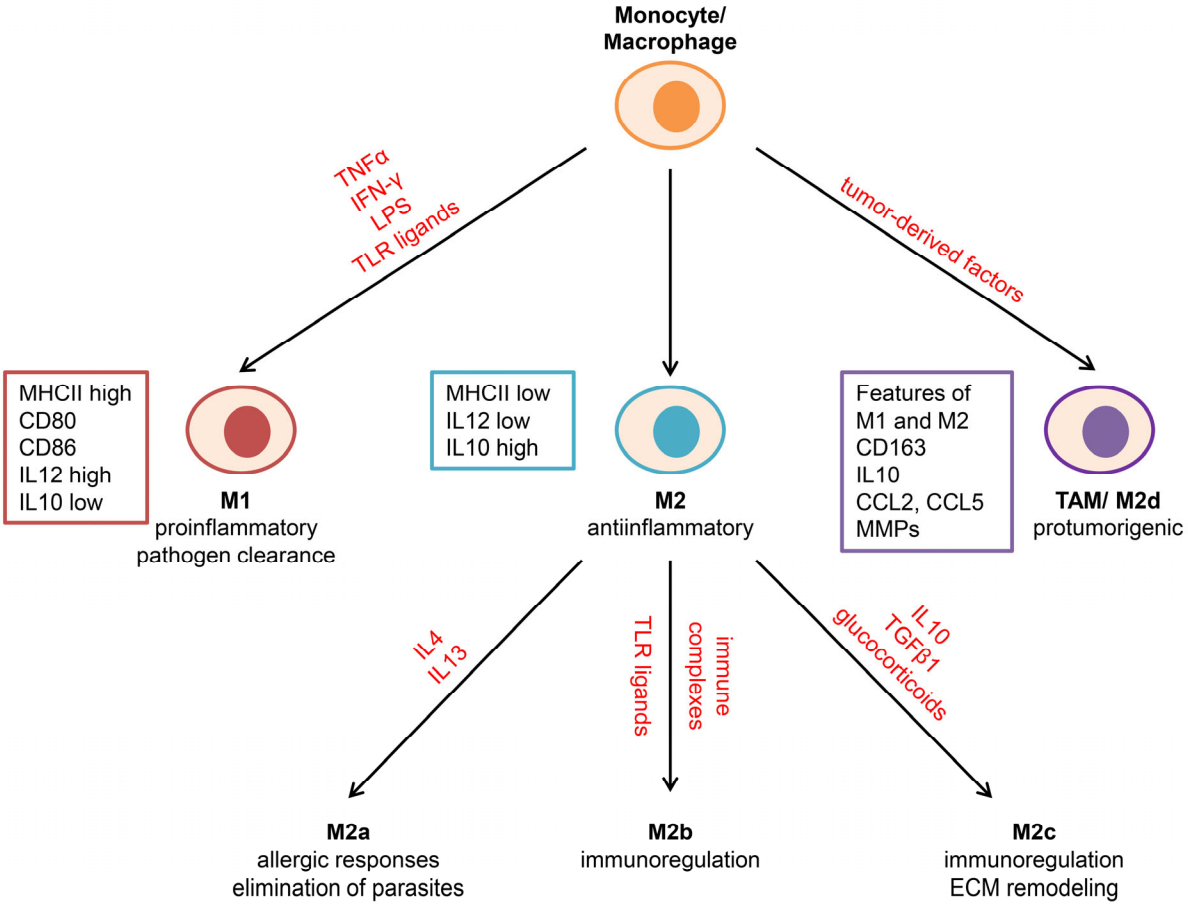

Figure 2: Simplified overview of human macrophage phenotypes (based on references[73-77, 84]). The red color indicates factors polarizing towards a certain phenotype. The molecules in a colored box are produced by the corresponding macrophage subtype (same color as the box). Both represent examples without any claim to completeness. CCL=C-C-chemokine ligand, CD=cluster of differentiation, IL=interleukin, IFN=interferon, LPS=lipopolysaccharide, MHC=major histocompatibility complex, MMP=matrix-metalloproteases, TAM=tumor-associated macrophage, TGFβ=transforming growth factor beta, TLR=toll-like receptor, TNF=tumor necrosis factor.

TAM infiltration is associated with poor prognosis and metastasis in various tumors including gastric cancer[85], OSCC[86], lung adenocarcinoma[87], breast cancer[88] and melanoma[89]. Once recruited, TAMs have been shown to promote melanoma progression in different ways. For example, they can foster melanoma growth through the production of IFN-γ[90], IL1B and other factors[48]. TAMs also endowed melanoma cell lines *in vitro* with resistance to BRAFi, which was mediated by their secretion of MAPK pathway-activating growth factors[91]. Additionally, they increased the survival of cancer initiating cells in melanoma and promoted sphere formation through the TGFβ1- and arginase pathways[92].

In addition to direct effects on melanoma cells, TAMs can also shape a microenvironment that promotes melanoma growth. For example TAMs foster tumor angiogenesis by secreting VEGFs, fibroblast growth factor (FGF), PDGF isoforms and prostaglandin E2, which are known to act as proangiogenic factors[73]. TAMs also produce proteases, including cathepsin B, MMP2, 7 and 9, that cleave the ECM, and thereby facilitate angiogenesis and tumor cell invasion[81]. Furthermore, by secreting immunosuppressive molecules such as IL10 and TGFβ1, TAMs can blunt the antitumor response, since these factors recruit Tregs and dampen the function of antitumor T cells and NK cells[79]. Additionally, TAMs express checkpoint inhibitor

molecules such as PD-L1, which further contributes to the suppression of antitumor immunity[93].

3.2.3 Crosstalk in the tumor microenvironment

The development of a tumor-promoting microenvironment is a dynamic progressive process[94]. The interaction between specific stromal cells with tumor cells has been the focus of much research in recent years. This crosstalk is often mediated through the secretion of growth factors, cytokines and proteases. As outlined before, CAFs and the factors they produce as a result of their interaction with tumor cells can contribute to tumor progression, but correlation of these factors with patient prognosis has not been properly investigated in melanoma. The histological presence of TAMs themselves is associated with poor prognosis in melanoma[89], but it remains unclear how TAMs might produce factors potentially linked to melanoma patient outcome.
The increased expression of growth factors and cytokines involved in the crosstalk within the tumor microenvironment can result in higher levels of such factors being present in the blood of melanoma patients compared to healthy individuals. Transforming growth factor beta 1 (TGFβ1) and serum amyloid A (SAA) play an important role in the communications between stromal and tumor cells, and have been found at increased levels in the blood of melanoma patients[95], [96]. Therefore, a central aim of this thesis was to examine the transcriptional response of fibroblasts and monocytic cells to TGFβ1 and SAA, and to determine its prognostic significance. The role of TGFβ1 and SAA in physiology and cancer (with a focus on melanoma) will now be considered in the following two sections.

3.3 Transforming growth factor beta 1

TGFβ1 is a context-dependent cytokine involved in many physiological and pathological processes. In humans, there are three TGFβ isoforms. The main sources of TGFβ1 are platelets, immune cells including monocytes/macrophages, as well as fibroblasts and epithelial cells. TGFβ2 is physiologically produced by keratinocytes and glial cells, and TGFβ3 can be found in human kidney, liver and spleen and in embryonic heart and lung tissue[97].

TGFβ isoforms are secreted as inactive pre-pro-TGFβ, bound to latency-associated peptide (LAP). In this condition, they can be stored in intracellular granules, at the cell surface or in the ECM. There, the TGFβ-LAP complex binds to latent-TGFβ-binding protein[98]. There are different mechanisms by which active TGFβ can be released from this complex. They include acidic conditions[99], the presence of reactive oxygen species and ionizing radiation, proteolytic cleavage, and proteins that anchor the latent TGFβ-complex to ECM components or the active TGFβ molecule to cell membranes[100].
Upon release, TGFβ isoforms can bind to three different transmembrane receptors: Transforming growth factor beta receptor (TGFBR) type I, II and III. TGFBRIII

facilitates the binding of TGFβ to the other receptors that are involved in intracellular signal transduction. The binding of TGFβ isoforms causes the formation of a hetero-tetrameric complex of TGFBRI and II. Thereafter, the serine/threonine kinase domain of TGFBRII activates TGFBRI, which then initiates intracellular signaling[100]. This is dependent on the sma/mothers against decapentaplegic (Smad) proteins, including Smad2, 3 and 4. Smad2 and 3 are phosphorylated and form oligomeric structures with Smad4. Then, this complex is translocated into the nucleus, where the Smad proteins act as transcription factors. Simultaneously, Smad pathway activation leads to the upregulation of inhibitory Smads such as Smad7, which cause a negative feedback control of the pathway[101]. Besides the Smad-dependent signaling, TGFβ receptors can amongst others also activate the MAPK- or PI3K pathways[100, 102].

Altogether, these pathways regulate the transcription of genes involved in cellular proliferation, apoptosis, differentiation and migration[103]. Therefore, TGFβ plays an important role in injury repair, tissue fibrosis[104], epithelial-mesenchymal transition (EMT)[105], immune modulation, embryonic development and cancer[106].

3.3.1 TGFβ in melanoma and other tumors

In tumors, TGFβ can be produced both by cancer cells, as well as by cells of the microenvironment including fibroblasts, macrophages and platelets[107]. Accordingly, increased systemic levels of TGFβ isoforms can be found in the blood of tumor patients and correlate with poor prognosis and metastasis in many tumors, including glioma[108], colorectal carcinoma[109], pancreatic ductal adenocarcinoma[110], lung adenocarcinoma[111] and melanoma[95, 112].

The roles of TGFβ in melanoma progression are highly context-dependent. In healthy skin and early melanomagenesis, TGFβ1 rather acts as a tumor suppressor. For instance, it upregulates cyclin-dependent kinase-inhibitors such as p21, which leads to cell cycle arrest in the G1 phase[113]. Additionally, TGFβ1 can induce apoptosis through different mechanisms[114], and it can repress proliferative factors including the cellular/cancer-myelocytomatosis oncogene and the inhibitor of differentiation proteins[115]. Melanomas are known to develop resistance to these inhibitory effects of TGFβ1, for example through increased expression of repressors such as Smad7[116].
Malignant melanoma cells secrete more TGFβ1 than healthy cells, and also produce TGFβ2 and 3[117]. In melanoma, TGFβ isoforms additionally promote their own expression[116]. This leads to the death of surrounding nonmalignant melanocytes or tumor cells that have not yet developed resistance to the apoptotic effects of TGFβ[97].
TGFβ1 also stimulates proliferation[118], as well as the acquisition of stem cell-like properties in melanoma cells[100].
TGFβ1 induces EMT in various cell types[105] including melanoma cells[119], and promotes melanoma migration and invasion[119]. In tumor and stromal cells, TGFβ1 was found to mediate the secretion of collagen, fibronectin, and tenascin C and MMPs[102], which contributed to ECM remodeling. Further, TGFβ1 induces

proangiogenic VEGFs and IL6[97] in various cell types, including epithelial cells and fibroblasts[107].
TGFβ1 can inhibit leukocyte proliferation, differentiation and survival[102]. Accordingly, TGFβ1 fosters tumor cell immune evasion by suppressing the cytotoxic gene program in T lymphocytes[104] and inhibiting the activation of NK cells[120]. TGFβ1 also reduces the migration of antigen-presenting cells that recognize malignant cells into the tumor[104]. Furthermore, it promotes the recruitment and differentiation of immunosuppressive Tregs[121], which is associated with melanoma progression[122]. Additionally, TGFβ1 contributes to the polarization of stromal cells towards tumor-promoting phenotypes[100], including TAMs[123] and CAFs[46].

In summary, TGFβ1 plays an important role in melanoma progression and in the communication between tumor and stromal cells.

3.4 Serum amyloid A

The serum amyloid A proteins are a family of four homologous proteins. SAA1 and 2 can be produced by the liver as a response to inflammatory stimuli and host injury[124]. The SAA3 gene is a pseudogene in humans[125], and has similar functions in rodents as human SAA1 and 2[126]. SAA4 is constitutively present in the blood[127], and will therefore not be considered here. As SAA1 and 2 proteins are structurally more than 90% identical[128], they will further be referred to as SAA. SAA plays an important role in diseases that have a significant inflammatory component, such as AA-amyloidosis, rheumatoid arthritis[129], atherosclerosis, obesity and diabetes mellitus type 2[124]. Additionally, it binds to circulating high density lipoprotein[130] and is involved in cholesterol metabolism[129]. Its role in cancer is described in detail in section 3.4.1.

SAA can act through different receptors, which mediate its role in various processes. Most of the functions of SAA involve formyl-peptide receptor 2 (FPR2), TLR2 and TLR4[124], but there are also other receptors for SAA[131], including the receptor for advanced glycation end products that also binds S100 proteins[132].
FPR2 is a G protein-coupled receptor that was found at monocytes, neutrophils, other immune cells[133] and synovial fibroblasts[132]. Upon binding of SAA, FPR2 activates the nuclear factor “kappa-light-chain-enhancer” of activated B cells (NF-κB)[134]. This induced M-CSF, IL8 and CCL2 in monocytes and/or neutrophils[135]. Therefore, SAA plays a role in chemotaxis of immune cells in an indirect way by inducing the above-mentioned promigratory molecules through FPR2. Additionally, SAA can also have direct chemotactic effects on immune cells through FPR2[136]. Besides, SAA participates in ECM remodeling via FPR2 through upregulation of MMP9 in monocytes[137], as well as MMP2 and 3 in fibroblasts[132].
TLR2 and 4 are highly conserved pattern recognition receptors that are expressed in cells such as monocytes and macrophages, synovial and dermal fibroblasts, endothelial cells and chrondrocytes[138, 139]. Upon stimulation of TLR4, NF-κB can be activated by myeloid-differentiation primary response 88 (MyD88), leading to the

induction of proinflammatory molecules. TLR4 can also signal through other pathways independently of MyD88[140]. TLR2 stimulation similarly leads to MyD88-dependent NF-κB signaling, or can additionally involve MAPK activation[141]. Through TLR2 stimulation, SAA has been reported to upregulate various M2-markers in macrophages, including IL10, TGFβ1[142], IL33 and tumor necrosis factor alpha (TNFα)[135]. Additionally, the expression of CCL2, IL1B and IL6 was enhanced in a TLR2/4-dependent manner in monocytic cells and fibroblasts by SAA[135]. This cytokine induction by SAA plays an important role in the pathogenesis of rheumatoid arthritis[143] and atherosclerosis[128], as well as in the progression of various tumors.

3.4.1 SAA in cancer

Besides the hepatic production in response to inflammation, SAA can also be produced by malignant cells including melanoma cells[144] and CAFs[145, 146]. Furthermore, SAA expression was found in monocyte/macrophage cell lines[147], as well as in TAMs of breast cancer[148] and melanoma[149]. Additionally, SAA proteins are associated with enhanced metastatic risk and poor prognosis in tumors of the lung[150], breast[148], ovary[151], uterus[51] and esophagus[152]. Importantly, proteomic profiling has identified SAA as an early blood-borne marker for poor survival in melanoma patients[96].

Despite its association with poor survival, the mechanisms through which SAA worsens patient outcome and promotes metastasis remain poorly investigated. In a variety of cancer types including melanoma, SAA was found to limit the antitumor immune response *in vivo*. For instance, SAA fostered the expansion of myeloid-derived suppressor cells via TLR2 signaling[153]. It induced IL1B and IL6 in monocytes, which promoted the recruitment of immunosuppressive Tregs[154]. SAA has also been reported to upregulate immunosuppressive M2 markers in macrophages[142]. Furthermore, it fosters tumor cell invasion by inducing expression of MMPs[124], and by interacting with adhesion proteins, thereby modulating the affinity of tumor cells for the ECM, which is crucial for the initiation of cell migration[132]. Moreover, the Sleeman lab has previously shown that the metastasis-associated protein S100A4 triggers a potent inflammatory response in breast cancer, in part by stimulating SAA expression. SAA then amplifies its own expression, as well as that of other inflammatory factors. In addition, ectopic expression of SAA in tumor cells was found to initiate metastasis formation in a broad spectrum of organs, which was accompanied by a massive infiltration of immune cells[155].

Taken together, the current evidence suggests that SAA is part of an inflammatory feedforward-loop between tumor and stromal cells, which promotes metastasis.

3.5 Aim of this thesis

Factors produced by tumor-associated stromal cells as a consequence of an intercellular crosstalk represent a promising, but yet under-investigated source of potential prognostic markers in melanoma.
Expression of TGFβ1 and SAA is strongly increased during melanoma progression. Accordingly, their expression in primary melanoma lesions as well as in the blood of melanoma patients is associated with poor prognosis[96, 112, 144]. As outlined before, both proteins are substantially involved in the crosstalk between melanoma cells and stromal cells of the cancer microenvironment: TGFβ1 activates cancer-associated fibroblasts and plays an important role in proliferation, invasion and antitumor immune escape of melanoma cells. SAA is a key molecule in cytokine induction and chemotaxis of immune cells, especially monocytic cells, and plays a role in metastasis.

Based on these observations, I addressed three major questions during my doctoral thesis work:

1. In melanoma, do malignant cells, stromal cells (such as fibroblasts and macrophages), or both contribute to the increased expression of TGFβ1 and SAA?

2. Do SAA and TGFβ1 stimulate the expression of cancer-relevant genes in fibroblasts or macrophages?

3. Is there a correlation between patient prognosis and any of the cancer-relevant genes whose expression is induced by stimulation of fibroblasts or macrophages with SAA or TGFβ1?

4 MATERIAL AND METHODS

4.1 Material

4.1.1 Cell culture

Table 1: Reagents used for cell culture

Component	Distributor
0.05% Trypsin/EDTA (1x), phenol red	Thermo Fisher Scientific, Schwerte, Germany
Advanced DMEM/F12	Thermo Fisher Scientific, Schwerte, Germany
Advanced RPMI 1640 Medium	Thermo Fisher Scientific, Schwerte, Germany
Aqua Ad Inject Miniplasco connect (sterile H_2O)	B. Braun Melsungen AG, Melsungen, Germany
B-27 supplement	Gibco, Invitrogen, Karlsruhe, Germany
b-FGF	Peprotech, Rocky Hill, CT, US
DMEM	Thermo Fisher Scientific, Schwerte, Germany
DMSO	Merck KGaA, Darmstadt, Germany
EGF	Sigma-Aldrich/ Merck, Darmstadt, Germany
FCS	Thermo Fisher Scientific, Schwerte, Germany
Heparin	Sigma-Aldrich/ Merck, Darmstadt, Germany
L-Glutamin	Gibco, Invitrogen, Karlsruhe, Germany
P/S	Thermo Fisher Scientific, Schwerte, Germany
PBS	Thermo Fisher Scientific, Schwerte, Germany
RPMI 1640 Medium	Thermo Fisher Scientific, Schwerte, Germany
Trypan blue	Merck KGaA, Darmstadt, Germany

Table 2: Reagents used for cell stimulation

Reagents for stimulation	Catalogue number	Distributor
CLI-095=TAK242	243984-11-4	Cayman, Ann Arbor, MI, USA
Human recombinant Apo-SAA (E. coli-derived)	300-13	Peprotech, Rocky Hill, CT, USA
Human recombinant TGFβ1 (HEK293-derived)	100-21	Peprotech, Rocky Hill, CT, USA

4.1.2 Nucleic acid analysis

Table 3: Reagents used for nucleic acid analysis

Component	**Distributor**
Biozym LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Chloroform:isoamyl alcohol (24:1)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
DEPC	Merck KGaA, Darmstadt, Germany
DNAse I	Thermo Fisher Scientific, Schwerte, Germany
DNAse I buffer (10x)	Thermo Fisher Scientific, Schwerte, Germany
dNTPs	Thermo Fisher Scientific, Schwerte, Germany
DreamTaq green buffer (10x)	Thermo Fisher Scientific, Schwerte, Germany
DreamTaq polymerase (5 U/µL)	Thermo Fisher Scientific, Schwerte, Germany
EDTA 50 mM	Thermo Fisher Scientific, Schwerte, Germany
Ethanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ethidiumbromide	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Gene ruler 1 kb plus DNA ladder	Thermo Fisher Scientific, Schwerte, Germany
Isopropanol	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Power SYBR Green qPCR Master Mix	Thermo Fisher Scientific, Schwerte, Germany
Random Hexamer Primer	Thermo Fisher Scientific, Schwerte, Germany
RevertAid H Minus Reverse Transcriptase	Thermo Fisher Scientific, Schwerte, Germany
RevertAid H Minus Reverse Transcriptase Buffer	Thermo Fisher Scientific, Schwerte, Germany
RNAse Exitus spray	AppliChem, Darmstadt, Germany
TRIzol reagent	Thermo Fisher Scientific, Schwerte, Germany

Primers were purchased from Metabion International AG, Planegg, Germany, and were used at a concentration of 0.4 µM for polymerase chain reaction (PCR) and 0.5 µM for quantitative real-time PCR (qPCR).

Table 4: Primer pair sequences and annealing temperatures for polymerase chain reaction (PCR) and quantitative real-time polymerase chain-reaction (qPCR)

Primer name	Sequence (5' → 3')	Annealing temperature (°C)
hu_ASMA=ACTA2 for1	TGGGTGACGAAGCACAGAGC	55
hu_ASMA=ACTA2 rev1	CTTCAGGGGCAACACGAAGC	55
hu_CCL2=MCP1 for	GGGCTGAGACTAACCCAGAAACATCC	55
hu_CCL2=MCP1 rev	TGGGTTGTGGAGTGAGTGTTCAAGT	55
hu_CCL4 for2	CCAAACCAAAAGAAGCAAGC	55
hu_CCL4 rev2	ACAGTGGACCATCCCCATAG	55
hu_CCL5=RANTES for	AACCCAGCAGTCGTCTTTGT	55
hu_CCL5=RANTES rev	TTCAAGGACTCTCCATCCTAGC	55
hu_CD163 for	TTGCCAGCAGTTAAATGTG	55
hu_CD163 rev	AGGACAGTGTTTGGGACTGG	55
hu_CDH2 for1	CGTCCACCTTGAAATCTGCT	55
hu_CDH2 rev1	TTACAGCGCAGTCTTACCGA	55
hu_COL1A1 for1	CACACGTCTCGGTCATGGTA	55
hu_COL1A1 rev1	AAGAGGAAGGCCAAGTCGAG	55
hu_CSF1 for	ATGACAGACAGGTGGAACTGCCAG	55
hu_CSF1 rev	TCACACAACTTCAGTAGGTTCAGG	55
hu_CTGF for	CAGCATGGACGTTCGTCTG	55
hu_CTGF rev	CCAACCACGGTTTGGTCCTT	55
hu_CXCL12=SDF1 for	ATGAACGCCAAGGTCGTGGTC	55
hu_CXCL12=SDF1 rev	CTTGTTTAAAGCTTTCTCCAGGTACT	55
hu_CXCR4 for	GCATGACGGACAAGTACAGGCT	55
hu_CXCR4 rev	AAAGTACCAGTTTGCCACGGC	55
hu_FAP for1	TCAGTGTGAGTGCTCTCATTGTAT	55
hu_FAP rev1	GCTGTGCTTGCCTTATTGGT	55
hu_FBN1 for	AAACATGGGCCTGTCCTGTA	55
hu_FBN1 rev	TCAATGGAGGAAGGTGTGTG	55
hu_FGF1 for	GAAGCCCAAACTCCTCTACTGTAG	55
hu_FGF1 rev	TGTTGTAATGGTTCTCCTCCAGC	55

hu_FGF2 for	CCTCTCTCTTCTGCTTGAAGTTG	55
hu_FGF2 rev	AGCGGCTGTACTGCAAAAAC	55
hu_FPR2 for	GGATTTGCACCCACTGCATTT	55
hu_FPR2 rev	ATCCAAGGTCCGAGATCAC	55
hu_GAPDH_for qPCR	CGACCACTTTGTCAAGCTCA	55
hu_GAPDH_rev qPCR	AGGGGTCTACATGGCAACTG	55
hu_G-CSF for	GCTGTGCCACCCCGAGG	55
hu_G-CSF rev	TGCAGGAGCCCCTGGTAGAGG	55
hu_GM-CSF=CSF2 for	GTCTCCTGAACCTGAGTAGAGACA	55
hu_GM-CSF=CSF2 rev	AAGGGGATGACAAGCAGAAAGTCC	55
hu_IL10 for2	GCCTAACATGCTTCGAGATC	55
hu_IL10 rev2	TGATGTCTGGGTCTTGGTTC	55
hu_IL12A for	CCTTGCACTTCTGAAGAGATTGA	55
hu_IL12A rev	ACAGGGCCATCATAAAAGAGGT	55
hu_IL1A_for	TGTATGTGACTGCCCAAGATGAAG	55
hu_IL1A_rev	AGAGGAGGTTGGTCTCACTACC	55
hu_IL1B_for	CCACAGACCTTCCAGGAGAATG	55
hu_IL1B_rev	GTGCAGTTCAGTGATCGTACAGG	55
hu_IL6_for	AGACAGCCACTCACCTCTTCAG	55
hu_IL6_rev	TTCTGCCAGTGCCTCTTTGCTG	55
hu_IL8 for qPCR	AAGAGAGCTCTGTCTGGACC	55
hu_IL8 for TAQ	TGGGTGCAGAGGGTTGTG	55
hu_IL8 rev qPCR	GATATTCTCTTGGCCCTTGG	55
hu_IL8 rev TAQ	CAGACTAGGGTTGCCAGATTTA	55
hu_MMP13 for 2	AACATCCAAAAACGCCAGAC	55
hu_MMP13 rev2	GGAAGTTCTGGCCAAAATGA	55
hu_MMP2 for	CCACTGCCTTCGATACAC	55
hu_MMP2 rev	GAGCCACTCTCTGGAATCTTAAA	55
hu_MMP3 for	CACTCACAGACCTGACTCGGTT	55
hu_MMP3 rev	AAGCAGGATCACAGTTGGCTGG	55

hu_MMP9 for 2	GCACTGCAGGATGTCATAGG	55
hu_MMP9 rev 2	ACGACGTCTTCCAGTACCGA	55
hu_PDGFA for	CACACCTCCTCGCTGTAGTATTTA	55
hu_PDGFA rev	GTTATCGGTGTAAATGTCATCCAA	55
hu_PDGFB for	TCCCGAGGAGCTTTATGAGA	55
hu_PDGFB rev	ACTGCACGTTGCGGTTGT	55
hu_PDGFRA for1	AGGTGGTTGACCTTCAATGG	55
hu_PDGFRA rev 1	TTTGATTTCTTCCAGCATTGTG	55
hu_PDGFRB for1	CAGGAGAGACAGCAACAGCA	55
hu_PDGFRB rev1	AACTGTGCCCACACCAGAAG	55
hu_PD-L1 for 3	ATTTGGAGGATGTGCCAGAG	55
hu_PD-L1 rev 3	CCAGCACACTGCGAATCAACA	55
hu_RPLP0 for	AGACAATGTGGGCTCCAAGCAGAT	55
hu_RPLP0 rev	GCATCATGGTGTTCTTGCCCATCA	55
hu_S100A4 for 2	CCGGATCCATGGCGTGCCCTCTGG	55
hu_S100A4 rev 2	CGAAGCTTTCATTTCTTCCTGGGCTG	55
hu_S100A8 for	GAATTTCCATGCCGTCTACAGG	55
hu_S100A8 rev	CCACGCCCATCTTTATCACCAG	55
hu_S100A9 for	AAAAGGTCATAGAACACATCATGG	55
hu_S100A9 rev	GAAGCTCAGCTGCTTGTCTG	55
hu_SAA for1 *	GCCGATGTAATTGGCTTCTC	55
hu_SAA rev1 *	AGCCGAAGCTTCTTTTCGTT	55
hu_Serpine1=PAI-1 for	GGCCATTACTACGACATCCTG	55
hu_Serpine1=PAI-1 rev	GGTCATGTTGCCTTTCCAGT	55
hu_Smad7 for 2	CCTTAGCCGACTCTGCGAACTA	55
hu_Smad7 rev 2	CCAGATAATTCGTTCCCCCTGT	55
hu_TGFB1 for 2	TGAACCGGCCTTTCCTGCTTCTCATG	55
hu_TGFB1 rev 2	GCGGAAGTCAATGTACAGCTGCCGC	55
hu_TGFB2 for2	CTCCATTGCTGAGACGTCAA	55
hu_TGFB2 rev2	CGACGAAGAGTACTACGCCA	55

hu_TGFB3 for 2	CTGGATTGTGGTTCCATGCA	55
hu_TGFB3 rev2	TCCCCGAATGCCTCACAT	55
hu_TGFBR II for 2	CAGTTGCTCATGCAGGATTT	60
hu_TGFBR II rev 2	GCACGTTCAGAAGTCGGTTA	60
hu_TGFBR III for 2	TCGGAGCACTCCTGACGGGG	60
hu_TGFBR III rev 2	TGGGCTGCGCTGCTGTTCTC	60
hu_TIMP-1 for	GGGACACCAGAAGTCAACCA	55
hu_TIMP-1 for	GGCTTGGAACCCTTTATACATC	55
hu_TLR2 for	TTTCACTGCTTTCAACTGGTA	55
hu_TLR2 rev	TGGAGAGGCTGATGATGAC	55
hu_TLR4 for	CAGAGTTGCTTTCAATGGCATC	55
hu_TLR4 rev	AGACTGTAATCAAGAACCTGGAGG	55
hu_TNFa for	GGCTCCAGGCGGTGCTTGTTC	55
hu_TNFa rev	AGACGGCGATGCGGCTGATG	55
hu_uPA for	CGCCACACACTGCTTCATTG	55
hu_uPA rev	CCCCTTGCGTGTTGGAGTT	55

*the SAA primer pair amplifies both SAA1 and SAA2.

4.1.3 Protein analysis

Table 5: Reagents used for protein analysis

Component	**Distributor**
BSA (fraction V), biotin-free	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Dako fat pen	Dako, Hamburg, Germany
Eosin	Merck KGaA, Darmstadt, Germany
Glacial acetic acid	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
H_2O_2 30 %	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Hematoxylin	Merck KGaA, Darmstadt, Germany
Limonene mounting medium	Abcam, Cambridge, UK
Mayer’s hemalum solution	Merck KGaA, Darmstadt, Germany
NaCl	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Polysorbate 20 (Tween20)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Rabbit serum	Thermo Fisher Scientific, Schwerte, Germany
Roti-Histol for Histology	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
StayGreen AP substrate Kit	Abcam, Cambridge, UK
Target Retrieval Solution Citrate pH 6.1	Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany
Tris-HCl	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Vectastain Elite ABC AP Kit	Vector laboratories, Burlingame, CA, US
Vectastain Elite ABC HRP Kit	Vector laboratories, Burlingame, CA, US
Vector NovaRed HRP Substrate Kit	Vector laboratories, Burlingame, CA, US

4.1.4 Buffers

Table 6: Composition of buffers

Buffer	**Components**
TBS	50 mM Tris-HCl (pH 7.5), 150 mM NaCl, pH adjusted to 7.5
TAE (50x)	50 mM EDTA, 2 M Tris-HCl, 1 M glacial acetic acid

4.1.5 Antibodies

Table 7: Antibodies used for immunohistochemistry

Antibody (species)	**Clone/ Catalogue number**	**Distributor**
Anti-CD163 (mouse)	Clone 3B4 ab192666	Abcam, Cambridge, UK
Anti-goat (rabbit)	BA-5000	Vector laboratories, Burlingame, CA, USA
Anti-mouse (horse)	BA-2001	Vector laboratories, Burlingame, CA, USA
Anti-mouse (rabbit)	EO413	Dako, Glostrup, Denmark
Anti-PAI-1 (goat)	AF1786	R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany
Anti-rabbit (goat)	BA-1000	Vector laboratories, Burlingame, CA, USA
Anti-S100B (rabbit)	Z0311	Dako, Glostrup, Danmark
Anti-SAA * (mouse)	Clone 115 ab687	Abcam, Cambridge, UK
Anti-TGFβ1,2,3 (anti-TGFβ) (mouse)	Clone 1D11 MAB1835	R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany

*the anti-SAA antibody detects both SAA1 and 2.

4.1.6 Enzyme-linked immunoassay (ELISA) Kits

Table 8: Enzyme-linked immunoassay (ELISA) Kits

Target protein	Catalogue number	Distributor
Human active TGFβ1	BMS 249-4	Thermo Fisher Scientific, Schwerte, Germany
Human total PAI-1	DTSE100	R&D Systems GmbH, Wiesbaden, Germany

4.1.7 Consumables

Table 9: Consumables

Consumable	Distributor
8-Microstrip classic (200 µl PCR tubes)	Biovendis Ltd., Mannheim, Germany
Aluminium foil	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Autoclavable waste bags	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Carpex Mouth guard	BSN medical, Hamburg, Germany
Cell culture dishes: 6 cm and 10 cm	SARSTEDT AG & Co, Nümbrecht, Germany
Cell culture flasks: 25 cm^2, 75 cm^2,	SARSTEDT AG & Co, Nümbrecht, Germany
Centrifuge tubes: 15 ml, 50 ml	Nerbe Plus, Winsen/Luhe, Germany
Greiner Cellstar serological pipettes (5 ml, 10 ml and 25 ml)	Greiner Bio-One, Kremsmünster, Austria
Handystep (Multistep pipette)	Merck KGaA, Darmstadt, Germany
Microscope coverslips: 24 mm x 60 mm	Langenbrinck, Emmendingen, Germany
Nitril gloves S powder-free, non-sterile	neoLab Migge GmbH, Heidelberg, Germany
Optical seal film for qPCR plates	Biozym, Hessisch Oldendorf, Germany
Parafilm	Bemis Company, Inc., Neenah, WI, USA
Pipette Tips: 20 µl, 200 µl, 1000 µl with and without filters	Nerbe plus, Winsen/Luhe, Germany
Pipettes: 2 µl to 20 µl, 20 µl to 200 µl, 100 µl to 1000 µl	Gilson, Middleton, WI, USA
Precision wipes tissue wipers	Kimberly clark professional, Roswell, USA
qPCR plate	Biozym, Hessisch Oldendorf, Germany
Seal Film BZO (Optical adhesive film)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Sterile Filters for syringes	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
SuperFrost Ultra Plus microscope slides	Langenbrinck, Emmendingen, Germany
Syringes: 10 ml, 20 ml	BD Biosciences, Heidelberg, Germany

Tips for multistep pipettes: 5 ml, 10 ml	Eppendorf AG, Hamburg, Germany
Transferpette S (Multichannel pipette): 30 µl to 300 µl	Brand GmbH& Co. KG Heidelberg, Germany
Tubes: 1.5 ml, 2 ml	Eppendorf AG, Hamburg, Germany

4.1.8 Devices

Table 10: Devices

Device	**Distributor**
Calculator	Soennecken, Overath, Germany
Centrifuge Heraeus Multifuge 1S-R	Heraeus Deutschland GmbH & Co. KG, Hanau, Germany
Centrifuge Heraeus pico 17	Thermo Fisher Scientific, Schwerte, Germany
Cooling centrifuge Heraeus Fresco 17	Thermo Fisher Scientific, Schwerte, Germany
Gel electrophoresis cell Mini Protean Tetra Cell	Bio-Rad Laboratories GmbH, Rüdigheim, Germany
Hera cell 150i CO_2 incubator	Thermo Fisher Scientific, Schwerte, Germany
Hera cell safety bench	Thermo Fisher Scientific, Schwerte, Germany
Magnetic stirrer	Heidolph Instruments, Schwabach, Germany
Microscope axiovert 40CFL	Zeiss, Oberkochen, Germany
Microscope Zeiss Imager Z1 with AxioCam HRc	Zeiss, Oberkochen, Germany
Microwave oven NN E245WB	Panasonic, Kadoma, Japan
Mini-centrifuge	neoLab Migge GmbH, Heidelberg, Germany
PCR cycler Flexcycler Twin-Block 48	Analytik Jena, Jena, Germany
PCR cycler PTC200	MJ Research, St. Bruno, Canada
pH Meter 766 Calimatic	Knick Elektronische Messgeräte GmbH & Co. KG, Berlin Germany
Pipette controller PIPETBOY acu 2	INTEGRA Biosciences Deutschland GmbH, Biebertal, Germany
Plate reader Multiskan Ascent 96/384	Thermo Fisher Scientific, Schwerte, Germany
Power Supply EV 231	Peqlab Biotechnologie, Erlangen, Germany
qPCR Machine Stratagene Mx3005P	Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany
Rotary microtome SLEE CUT 4060	SLEE medical, Mainz, Germany
Rotating mixer RM5	Ingenieurbüro CAT, M. Zipperer GmbH
Shaker Rotamax 120	Heidolph Instruments GmbH& Co. KG, Schwabach, Germany

Spectrophotometer NanoDrop 2000	NanoDrop Technologies, Wilmington, USA
Steam cooker Multigourmet	Braun, Kronberg im Taunus, Germany
Thermomixer compact	Eppendorf AG, Hamburg, Germany
Timer	Neo Lab, Heidelberg, Germany
Tube Rotator SB2	Stuart Equipment, Staffordshire, UK
Vacuum pump MD4CNT+AK+EK	VACUUBRAND GMBH + CO KG, Wertheim, Germany
Vortex Mixer	VWR International GmbH, Darmstadt, Germany
Waterbath B-480 for paraffin block slices	Büchi, Flawil, Switzerland
Waterbath WB22 for cell culture	Memmert GmbH + Co. KG, Büchenbach, Germany

4.1.9 Software

Table 11: Software

Software	**Distributor**
Adobe Creative Suite 4	Adobe Systems Corporation, San José, CA, USA
Elisaanalysis.com	Elisakit.com
Graph Pad Prism 7	GraphPad Software, Inc., la Jolla, CA, USA
Microsoft Office	Microsoft Germany GmbH, Schwabing, Germany
AxioVision Rel. 4.8	Carl Zeiss MicroImageing GmbH, Jena, Germany
MxPro MX3005P (v4.10) qPCR Software	Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany

4.1.10 Patient-derived samples

Formalin-fixed, paraffin-embedded tumor samples derived from patients with malignant melanoma at different stages were kindly provided by Prof. Dr. Utikal (Universitätsmedizin Mannheim/DKFZ cooperation unit dermatooncology).
Human dermal fibroblasts GS3, GS4, GS5 and GGN77 were a gift from PD Dr. Herskind. They were derived from skin explants of post mastectomy patients as described previously[156].
Plasma samples of melanoma patients were a kind gift from Dr. Quagliata (Universitätsspital Basel/ Institut für Medizinische Genetik und Pathologie). The plasma was isolated from blood samples that were taken at the University of Zürich Hospital, funded by the University Research Priority Program. All patients have signed a patient release form, which has been approved by an ethics committee and assigned the numbers EK 647 and EK 800.

4.2 Methods

4.2.1 Cell culture and stimulations

The human melanoma cell lines A375, G361, Mewo and SKMel23 were a kind gift from Prof. Dr. Utikal, originally bought from the American Type Culture Collection (ATCC). The human melanoma cell lines CRL1, C32, HT144 and SKMel28 were purchased from ATCC. The human monocytic macrophage cell line THP-1 was kindly provided by Prof. Dr. Kzhyshkovska, purchased from ATCC, and was used as a model for naïve monocytic macrophages in the primary melanoma niche. The human melanoma cell lines as well as the THP-1 cells were used for a maximum of 24 passages in total. Heat-inactivated fetal calf serum (FCS) was used for the culture of THP-1 cells in order to inactivate complement factors contained in FCS. Heat-inactivation was performed in a pre-heated water bath at 56 °C for 30 min. Patient-derived primary human dermal fibroblast (HDF) lines GS3, GS4, GS5 and GGN77 were used as a model for naïve fibroblasts in the primary melanoma niche. They were used for a maximum of four passages.
An overview of the cell's origins and cell culture maintenance media is given in the following Table 12. Table 13 summarizes the media used during experiments. Melanoma cell lines as well as THP-1 cells were maintained at 37 °C, 5 % CO_2, atmospheric oxygen and maximal humidity in a cell culture incubator. HDF were cultured at 7 % CO_2 as previously described[156].

Table 12: Characteristics and maintenance culture media of cell lines and primary cells

Cell type	Origin	First description	Maintenance culture medium
A375 (adherent)	Primary tumor	Giard et al.[157]	DMEM + 10 % FCS + 1 % P/S
C32 (adherent)	Primary tumor	Chen et al.[158]	RPMI 1640 + 10 % FCS + 1 % P/S
CRL1=WM2664 (adherent)	Metastatic site (skin)	Herlyn et al.[159]	RPMI 1640 + 10 % FCS + 1 % P/S
G361 (adherent)	Primary tumor	Deschodt-Lanckmann et al.[160]	RPMI 1640 + 10 % FCS + 1 % P/S
GS3, GS4, GS5, GGN77 (adherent)	Patient-derived primary HDF	Herskind et al.[156]	DMEM + 10 % FCS + 1 % P/S
HT144 (adherent)	Metastatic site (subcutaneous)	Fogh et al.[161]	RPMI 1640 + 10 % FCS + 1 % P/S
Mewo (adherent)	Lymph node metastasis	Fogh et al.[161]	DMEM + 10 % FCS + 1 % P/S
SKMel 23 (adherent)	Primary tumor	Fogh et al.[161]	DMEM + 10 % FCS + 1 % P/S
SKMel28 (adherent)	Primary tumor	Fogh et al.[161]	RPMI 1640 + 10 % FCS + 1 % P/S
THP-1 (suspension/ adherent)	Human acute monocytic leukemia cells	Tsuchiya et al.[162]	RPMI 1640 + 10 % heat-inactivated FCS + 1 % P/S

Table 13: Media during experiments for different cells

Media during experiments	Components
HDF serum starvation medium	Advanced DMEM/F12 + 1 % P/S
THP-1 serum starvation medium	RPMI 1640 + 1 % P/S
Melanoma cell serum starvation medium: Melanosphere assay medium (MAM)	Advanced RPMI or advanced DMEM/F12 + 2 % B27 + EGF (20 ng/ml) + b-FGF (20 ng/ml) + Heparin (4 µg/ml)+ 1 % L-Glutamine + 1 % P/S

For freezing, cells were centrifuged at 300 g for 3 to 5 min and re-suspended in a mixture of 90 % FCS and 10 % dimethyl sulfoxide (DMSO), then placed in isopropanol boxes at -80 °C. Thawing was performed by leaving the vial for 1 to 2 min in a water bath at 37 °C and then carefully re-suspending the cells in pre-warmed culture medium. All cells except HDF were then again centrifuged at 300 g for 3 to 5 min and re-suspended in fresh pre-warmed medium before their placement into the desired cell culture dish or flask.

Human recombinant TGFβ1 (HEK293-derived) and SAA (E.coli-derived) were reconstituted according to the manufacturer's recommendations as follows. TGFβ1 was reconstituted in 1 % bovine serum albumin (BSA) with 10 mM citric acid in sterile distilled water (dH_2O) to ensure the activation of latent TGFβ1[99]. SAA was reconstituted in 1 % BSA in dH_2O. The endotoxin levels of recombinant SAA were lower than 0.1 ng/µg, according to the manufacturer and as confirmed by others[163].
The TLR4 inhibitor CLI-095 (=TAK242) was reconstituted in DMSO according to the manufacturer's protocol to a concentration of 2.5 µM.
For TGFβ1 and SAA stimulation, a pilot concentration titration was performed to determine the optimal concentration for induction of a set of marker genes. An overview of stimulation conditions resulting from these preliminary tests is given in Table 14.

Table 14: Concentrations of reagents used for stimulation

Reagent	HDF	THP-1 cells
CLI-095=TAK242		5 µM 6 h prior to SAA stimulation
SAA	100 ng/ml 24 h	100 ng/ml 24 h
TGFβ1	2.5 ng/ml 24 h or 48 h	10 ng/ml 24 h or 48 h

Expression of Smad7 was used as a response gene to monitor TGFβ1 stimulation of HDF and THP-1 cells[101]. For SAA-stimulated THP-1 cells, CCL2[164] was used as response gene. Response markers for SAA-stimulated HDF were IL6[165] and SAA[166]. Furthermore, for stimulated THP-1 cells, CD163 expression was tested as marker for

TAMs[77]. IL10 and IL12 expression was used to investigate M2/ M1 activation respectively[76]. Similarly, stimulated HDF were tested for α-SMA (ACTA2) expression as a marker for CAFs[54]. An overview of the different response markers for each condition is presented in Table 15. Based on a preliminary screen by PCR for each individual stimulation and cell type, expression of further genes was then investigated by qPCR.
In these preliminary experiments, SAA stimulation upregulated expression of the corresponding markers in THP-1 cells but not in HDF. Therefore, the TLR4 inhibitor CLI-095 was exclusively used in SAA-stimulated THP-1 cells in order to test an involvement of TLR4 in the SAA-stimulated transcriptional response.

Table 15: Response and activation markers for different cell types and different stimulations

Stimulated cells	Response marker(s)	Activation marker(s)
TGFβ1-stimulated THP-1	Smad 7[101]	CD163 (TAMs); IL10 (M2); IL12 (M1)
SAA-stimulated THP-1	CCL2[164]	
TGFβ1-stimulated HDF	Smad 7[101]	α-SMA (ACTA2) (CAFs)
SAA-stimulated HDF	IL6[165]; SAA[166]	

For messenger ribonucleic acid (mRNA) expression analysis and cell culture media isolation, THP-1 cells were seeded in serum-free Roswell Park Memorial Institute (RPMI) 1640 medium at a density of 400 000 cells/ml in a 12 well format in 1 ml of serum starvation medium. They were left to rest for 24 h before the stimulation with SAA or TGFβ1. After 24 h, supernatant and cells were harvested.
For mRNA preparation, HDF were seeded in their usual culture medium mentioned above (150 000 cells in a 6 well format or 65 000 cells in a 12 well format). To harvest cell culture medium, 800 000 HDF were seeded in a 10 cm dish. In both assays, a medium change to serum-free advanced Dulbecco's modified eagle medium (DMEM)/F12 was performed 24 h later, and again 24 h later. The cells were stimulated as indicated in Table 14.
For the generation of melanoma cell conditioned medium and mRNA, 450 000 melanoma cells were seeded into a T75 cell culture flask in their normal culture medium mentioned above. After washing with phosphate-buffered saline (PBS), a medium change to melanosphere assay medium (MAM) with a total volume of 8 ml was performed 24 h later. After an additional 72 h, supernatant and mRNA were harvested.
The media during experiments in Table 13 were chosen out of a preliminary test with different cell culture media. The aim was to use a medium without FCS, containing relatively low levels of TGFβ1, and resulting in normal cell morphology and growth. Cell-free conditioned media were obtained by centrifuging the harvested conditioned medium at 18 000 g for 3 min, and then taking the liquid phase. Supernatants were frozen at -80 °C.

4.2.2 Nucleic acid analysis

4.2.2.1 Ribonucleic acid isolation

For Ribonucleic acid (RNA) isolation, the TRIzol reagent phase separation method was used. For 12 wells and smaller formats, cells were re-suspended in 0.5 ml of TRIzol reagent; for 6 wells or larger formats, 1 ml of TRIzol reagent was used. All following volumes take reference to 1 ml of TRIzol reagent. THP-1 cells in suspension were centrifuged at 300 g for 5 min, supernatant was transferred into a fresh tube and the remaining cells were re-suspended in TRIzol and pooled with the adherent THP-1 cell population on the cell culture plate. Before adding 200 µl of chloroform:isoamyl alcohol (24:1) per 1 ml of TRIzol, the cell-TRIzol-suspension was incubated for 5 min at room temperature. Then, the mixture was vortexed for 15 s and again incubated for 3 min at room temperature. Afterwards, the vials were centrifuged at 4 °C and 16 000 g for 15 min. Then, three phases were visible. The upper aqueous phase was pipetted into a fresh tube containing 500 µl of isopropanol. After mixing by multiple inversions, RNA was precipitated for 10 min at room temperature. Then, samples were centrifuged for 20 min at 16 000 g and 4 °C. Afterwards, a white RNA pellet was visible at the bottom of the tube. This was washed twice with 750 µl of 75 % ethanol by centrifuging at 7 500 g and 4 °C for 5 min. After decanting the ethanol, the RNA pellet was air-dried and then dissolved in 20 µl of diethylpyrocarbonate (DEPC)-treated H_2O using a pre-heated thermoblock at 55 °C for 10 min. Total RNA concentration was measured with a NanoDrop spectrophotometer. To remove any genomic DNA potentially carried over the RNA samples, 2 µg of RNA was treated with deoxyribonuclease (DNase) I for 30 min at 37 °C using the PCR Cycler PTC200 or Flexcycler Twin-Block 48.

Table 16: Deoxyribonucelase (DNAse) I digestion ingredients

Ingredient	Volume per reaction (µl)
10 x DNase buffer	4
DEPC-treated H_2O	3
DNase I	4

The reaction was stopped by adding 3 µl of ethylenediaminetetraacetic acid (EDTA) to each well and then heating the samples to 65 °C for 10 min.

4.2.2.2 Reverse transcription

For complementary deoxyribonucleic acid (cDNA) synthesis, 1 µl of random primer was added to the DNase I-treated RNA and incubated at 65 °C for 5 min. Samples were cooled on ice. They were split into a reverse transcriptase-positive (RT+) and negative (RT-) sample. Reverse transcriptase (RT) was added to the RT+ sample to transcribe RNA into cDNA, and DEPC-treated H_2O was added to the RT- sample

respectively. RT buffer as well as deoxyribonucleotide triphosphates (dNTPs) were added to both samples. Table 17 summarizes the ingredients for reverse transcription:

Table 17: Reverse transcription (RT) ingredients

Ingredient	Volume per reaction (µl)
10x RT buffer	5
dNTPs	2
Reverse transcriptase (RT+) or DEPC-treated H_2O (RT-)	1

The reverse transcription was performed through the following incubation steps, using the PCR Cycler PTC200 or Flexcycler Twin-Block 48:

Table 18: Reverse transcription (RT) program

Step	Temperature	Time
Annealing of random primers	25 °C	10 min
Reverse transcription	42 °C	60 min
Inactivation	72 °C	10 min
Cooling	4 °C	Hold

Subsequently, the obtained cDNA was diluted 1:20 in DEPC-treated H_2O.

4.2.2.3 Polymerase chain reaction and quantitative real-time polymerase chain reaction

PCR analysis of the RT+ and RT- samples was performed to analyze efficient removal of genomic DNA and afterwards to test primer specificity. To investigate transcriptional changes introduced by SAA- or TGFβ1 stimulation, a first screen of potential target genes was performed by PCR. Amplification of cDNA was performed using the PCR Cycler PTC200 or Flexcycler Twin-Block 48. The housekeeping gene in this case for melanoma cells and human fibroblasts was the 60S acidic ribosomal protein P0 (RPLP0), and for THP-1 cells glyceraldehyde 3-phosphate dehydrogenase (GAPDH) respectively. The mastermix for PCR is described in the following table:

Table 19: Polymerase chain reaction (PCR) mastermix

Ingredient	Volume per reaction (µl)
10x DreamTaq buffer	2.5

DEPC-treated H_2O	14.8
dNTPs	0.5
DreamTaq	0.2
Forward/reverse primer mix	2
cDNA sample	5

For PCR analysis, the following thermal profile was used. Steps 2) to 4) are the amplification steps and were repeated 32 to 40 times:

Table 20: Polymerase chain reaction (PCR) program

Step	Temperature	Time
1) Initialization	95 °C	3 min
2) Denaturation of double stranded DNA	95 °C	30 s
3) Primer annealing	55 °C or 60 °C *	45 s
4) Elongation by DreamTaq	72 °C	10 s
5) Cooling	4 °C	Hold

*annealing temperature depending on the primer: see Table 4

The differentially regulated genes in this preliminary screen were then determined quantitatively by qPCR using a Stratagene Mx3005P device. This was performed with SYBR Green dye for detection. The mastermix for qPCR is described in the following table:

Table 21: Quantitative real-time PCR (qPCR) ingredients

Ingredient	Volume per reaction (µl)
DEPC-treated H_2O	3
Forward and reverse primer mix	2
cDNA sample	5
Power SYBR Green PCR Master Mix	10

For qPCR analysis, the following thermal profile was used. Steps 2) to 4) are the amplification steps and were repeated 40 times.

Table 22: Quantitative real-time PCR (qPCR) program

Step	Temperature	Time
1) Initialization	95 °C	10 min
2) Denaturation of double stranded DNA	95 °C	30 s
3) Primer annealing	55 °C or 60 °C *	1 min
4) Elongation	72 °C	1 min
5) Cooling	4 °C	Hold

*annealing temperature depending on the primer, see Table 4

4.2.2.4 Analysis of qPCR data

For expression analysis, MxPro MX3005P (v4.10) qPCR Software was applied to set a threshold in the linear phase of the amplification curves resulting in Ct-values (i.e. the number of cycles, by which the amplification curve has crossed the threshold).
The same threshold value has been applied for the housekeeping gene and the gene of interest if they were analyzed in different qPCRs. Ct-values obtained for a housekeeping gene were subtracted from the Ct-value of the target gene (ΔCt).

$$\Delta Ct = Ct\ (target\ gene) - Ct\ (housekeeping\ gene)$$

The housekeeping gene in the case of qPCR was RPLP0 for melanoma cells and human fibroblasts, and GAPDH for THP-1 cells. To compare the expression of TGFβ1, 2, 3, SAA and Plasminogen activator inhibitor 1 (PAI-1) between melanoma cell lines, HDF and THP-1 cells, RPLP0 was used as a housekeeping gene for all cell types.
The ΔCt model assumes that each cycle results in a doubling of DNA. Relative expression of the target gene compared to the housekeeping gene is therefore calculated as follows:

$$Relative\ expression\ of\ target\ gene = 2^{-\Delta Ct}$$

To compare the relative expressions of treated vs untreated samples, the arithmetic mean of the $2^{-\Delta Ct}$-values of the replicates of the unstimulated cells was calculated, and the $2^{-\Delta Ct}$-values of the treated cells were divided by this mean for normalization. The normalization of endogenous expression of TGFβ1, 2, 3, SAA and PAI-1 of each cell type to the cell line G361 was carried out in an analogous way.

All data for the human melanoma cell lines and THP-1 cells were generated in three biological replicates. For human primary fibroblasts, four independent healthy donors represent the replicates. All results are expressed as mean + standard error of the mean (SEM).

4.2.3 Protein analysis

4.2.3.1 Immunohistochemistry

Single stainings with S100B (marker for malignant melanoma cells[30]), CD163 (marker for tumor-associated macrophages[167]) and the three molecules of interest were established for human melanoma patient samples. The anti-TGFβ- and anti-SAA antibodies recognize all TGFβ and SAA isoforms respectively, as specified in the datasheets. Costainings were also performed for S100B together with each of the three molecules of interests, and for PAI-1 with CD163. To this end, 5 µm sections were cut with a rotary microtome and placed on microscope slides. Tissue sections on slides were dried at 37 °C overnight. For deparaffinization, tissue sections were incubated three times for 5 min in Roti-Histol, then for 3 min in 100 %, 96 %, 80 % and 70 % ethanol and for 3 min in dH_2O. Antigen retrieval was then performed by boiling the sections for 30 min in a steam cooker, using a citrate-based target retrieval solution. For horseradish peroxidase (HRP)-based detection, endogenous peroxidase activity was quenched with a 3 % hydrogen peroxide (H_2O_2) solution for 5 min, followed by washing in tris-buffered saline (TBS) for 5 min. Blocking was performed with 1 % biotin-free BSA in TBS for 1 h before primary antibody application. For the anti-CD163 antibody, 10 % rabbit serum in 1 % BSA was used for blocking. All primary antibodies were incubated over night at 4 °C. The following Table 23 indicates the concentration for HRP-based and alkaline phosphatase (AP)-based detection.

Table 23: Concentrations of primary antibodies for different immunohistochemistry detection methods

Primary antibody	Concentration for HRP detection	Concentration for AP detection
Anti-SAA	20 µg/ml	
Anti-PAI-1	10 µg/ml	15 µg/ml
Anti-S100B		1:600 *
Anti-CD163	10 µg/ml	
Anti-TGFβ1,2,3 (anti-TGFβ)	16 µg/ml	

*for the anti-S100B antibody, concentrations were not available from the provider

After primary antibody incubation, slides were washed for 5 min with TBS. Next, biotinylated secondary antibodies were incubated for 30 min at room temperature. After washing with TBS, the HRP reagent was incubated for 30 min or the AP reagent was incubated for 45 min, respectively. After another wash step as indicated above, enzyme substrates were added. The reaction was stopped with dH_2O for 5 min. In these experiments, the NovaRed HRP substrate produced a red-brown staining. The color resulting from the enzymatic degradation of the StayGreen AP substrate was blue-green. The incubation times of secondary antibodies as well as of the enzyme substrates are represented in the following table.

Table 24: Concentrations of secondary antibodies and incubation times of enzyme substrates

Primary antibody (species	Secondary antibody (Cat No., Company)	Concentration Of secondary antibody	NovaRed (HRP substrate)	StayGreen (AP substrate)
Anti-SAA (mouse)	Anti-mouse made in horse (BA-2001,Vector laboratories)	7.5 µg/ml	5 min	
Anti-PAI-1 (goat)	Anti-goat made in rabbit (BA-5000, Vector laboratories)	7.5 µg/ml	5 min	20 min
Anti-S100B (rabbit)	Anti-rabbit made in goat (BA-1000, Vector laboratories)	7.5 µg/ml		20 min
Anti-CD163 (mouse)	Anti-mouse made in rabbit (EO413,Dako)	3.95 µg/ml	3 min	
Anti-TGFβ1,2,3 (anti-TGFβ) (mouse)	Anti-mouse made in horse (BA-2001, Vector laboratories)	7.5 µg/ml	8 min	

Costainings were performed using a sequential procedure, with the primary antibodies raised in different species. The counterstaining was omitted and a hematoxylin and eosin (H.E.) stained section of the patient was included into the analysis. The following table summarizes the corresponding antibody pairs, species and detection methods for costainings:

Table 25: Sequence of antibodies and corresponding detection methods for costainings

First primary antibody (species)	Detection method: enzyme (substrate)	Second primary antibody (species)	Detection method: enzyme (substrate)
Anti-S100B (rabbit)	AP (StayGreen)	Anti-SAA (mouse)	HRP (NovaRed)
Anti-S100B (rabbit)	AP (StayGreen)	Anti-TGFβ1,2,3 (anti-TGFβ) (mouse)	HRP (NovaRed)
Anti-S100B (rabbit)	AP (StayGreen)	Anti-PAI-1 (goat)	HRP (NovaRed)
Anti-CD163 (rabbit)	HRP (NovaRed)	Anti-PAI-1 (goat)	AP (StayGreen)

Sections were dehydrated (3 min incubation in 70 %, 80 %, 96 % and 100 % ethanol) and then incubated three times for 5 min in Roti-Histol. Finally, they were mounted with Limonene mounting medium and dried at room temperature before the analysis with the Axio Imager Z1 microscope.

4.2.3.2 Enzyme-linked immunosorbent assays

To assess the levels of active total PAI-1 and TGFβ1 in the blood of melanoma patients, plasma samples were analyzed via enzyme-linked immunosorbent assay (ELISA). Additionally, culture medium from melanoma cells, THP-1 cells and HDF was analyzed for PAI-1 using ELISAs. Additionally, the amount of PAI-1 in cell

culture conditioned medium was determined in stimulated and unstimulated THP-1 cells and HDF by this ELISA.
The following table shows the different dilutions for melanoma patient plasma as well as conditioned medium. Reagents were diluted with the recommended sample dilution reagent provided in every ELISA Kit.

Table 26: Dilutions for biological material undergoing the different enzyme-linked immunosorbent assays (ELISAs)

Sample	ELISA target protein	Dilution of samples
Patient plasma	TGFβ1	1:30
Patient plasma	PAI-1	1:10
Conditioned medium from HDF	PAI-1	1:30
Conditioned medium from THP-1 cells	PAI-1	none
Conditioned medium from melanoma cell lines	PAI-1	none

ELISAs were carried out according to the respective manufacturer protocols. The optical density of each well was determined using the microplate reader Multiskan Ascent 96/384 set to 450 nm wavelength. Another measurement at 540 nm was carried out and then subtracted from the optical density measured at 450 nm. For the calculation of the concentrations, the free web software elisaanalysis.com (https://elisaanalysis.com/) was used, performing a four parameter logistic (4-PL) curve-fit. Then, the concentration was multiplied by the dilution factor.

All measurements were carried out in two technical replicates. The ELISAs with HDF were carried out with four biological replicates. ELISAs with two biological replicates of TGFβ1-stimulated THP-1 cells were performed. The results show the means + standard errors of the mean in the indicated concentrations.

4.2.4 Statistical analysis

The number of technical replicates is indicated in the description of each experimental procedure. Statistical analysis was performed using the Graph Pad Prism software. All error bars refer to the standard error of the mean. For comparisons between stimulated and unstimulated HDF, the paired student's t-test was used to match the corresponding conditions of each donor-derived fibroblast line. The unpaired t-test was used to compare unstimulated and TGFβ1-stimulated THP-1 cells. The one-way ANOVA was used to compare the three different stimulation conditions for SAA-stimulated THP-1 cells with Sidak's post hoc tests for multiple comparisons, in the following manner: Unstimulated THP-1 cells were compared to SAA-stimulated cells, and THP-1 cells incubated with SAA alone were compared to THP-1 cells incubated with the TLR4 inhibitor CLI-095 and then treated with SAA.

For survival analysis of melanoma patients with different levels of PAI-1 protein expression, z-scores measured by reverse-phase protein array and clinical data of the TCGA skin melanoma cohort (TCGA Provisional) were downloaded from the cBio portal[168, 169]. This cohort includes 479 samples from 471 patients. Concerning the overall survival (OS) analysis, there were clinical data and PAI-1 protein expression data available for 348 patients. Concerning the progression-free survival (PFS) analysis, there were 308 patients for which both clinical data and PAI-1 protein expression data were available. Next, samples were sorted by z-scores and the cutoff between low and high PAI-1 protein expression was set at the upper tertile. To calculate the statistical difference between Kaplan-Meier survival curves, the logrank test was used. In all experiments, the level of significance was $p<0.05$ (using the abbreviations "*" or “#” for $p<0.05$, “**” or “##” for $p<0.01$ and “***” or “###” for $p<0.001$ as indicated in each legend).

5 RESULTS

5.1 Identification of cells that express TGFβ and SAA in melanoma

To determine which cells express TGFβ and SAA in melanoma, I took two approaches. First, immunohistochemistry was used to analyze sections of human melanoma. Then, I examined the expression of SAA and TGFβ1 in cultured human melanoma cell lines, in human dermal fibroblasts (HDF), and in the human monocytic macrophage cell line THP-1.

5.1.1 Immunohistochemistry

To assess the expression of TGFβ and SAA in human melanoma, consecutive sections from a melanoma patient (Clark level III, local tumor stage pT3) were stained with antibodies against S100B, as well as with TGFβ and SAA antibodies alone, or in the indicated combinations as sequential costainings. In addition, staining with hematoxylin and eosin (H.E.) was performed.

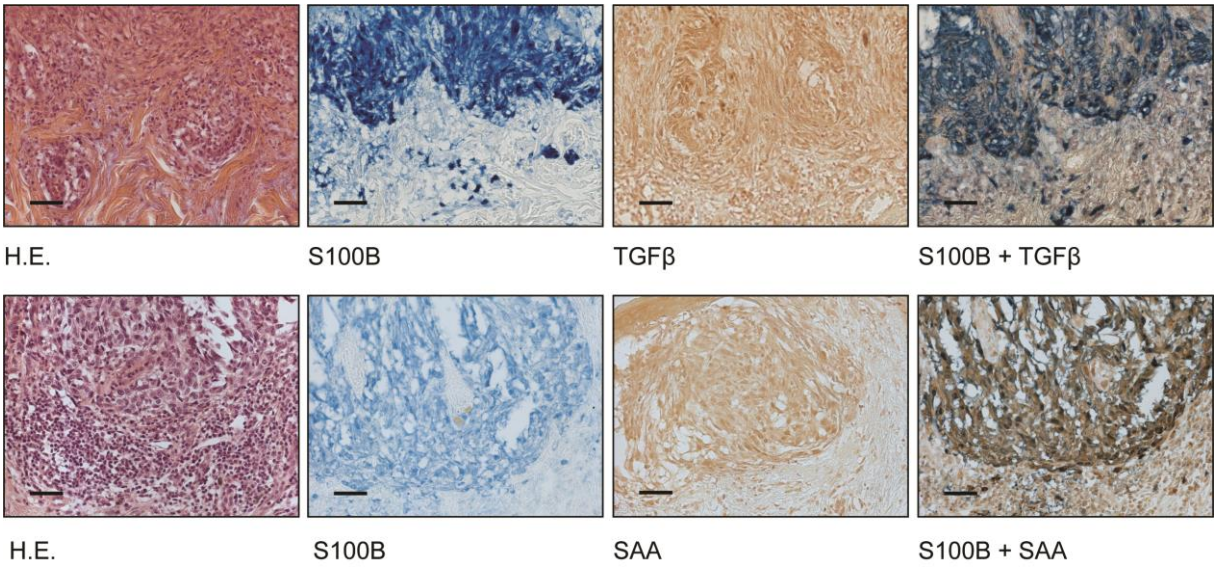

Figure 3: Localization of TGFβ and SAA in human melanoma. Immunohistochemical stainings for S100B (blue) and TGFβ (brown) or SAA (brown) respectively in human melanoma sections. H.E=hematoxylin and eosin staining. The scale bar represents 50 µm.

TGFβ was strongly expressed in both S100B-positive melanoma cells as well as in S100B-negative cells. SAA was expressed in S100B-positive cells, and also in some S100B-negative stromal cells. In conclusion, TGFβ and SAA isoforms are expressed in primary tumor niches of human melanomas, especially by but not restricted to tumor cells.

5.1.2 Expression of TGFβ and SAA in human melanoma cells, THP-1 cells and HDF

In order to investigate the mRNA expression of the three TGFβ isoforms and SAA, eight human melanoma cell lines as well as the unstimulated monocytic macrophage

cell line THP-1 were analyzed by qPCR in three biological replicates. In addition, four different donor-derived HDF were included in the analysis. Expression was normalized to the melanoma cell line G361, which showed a relatively low overall expression of these molecules.

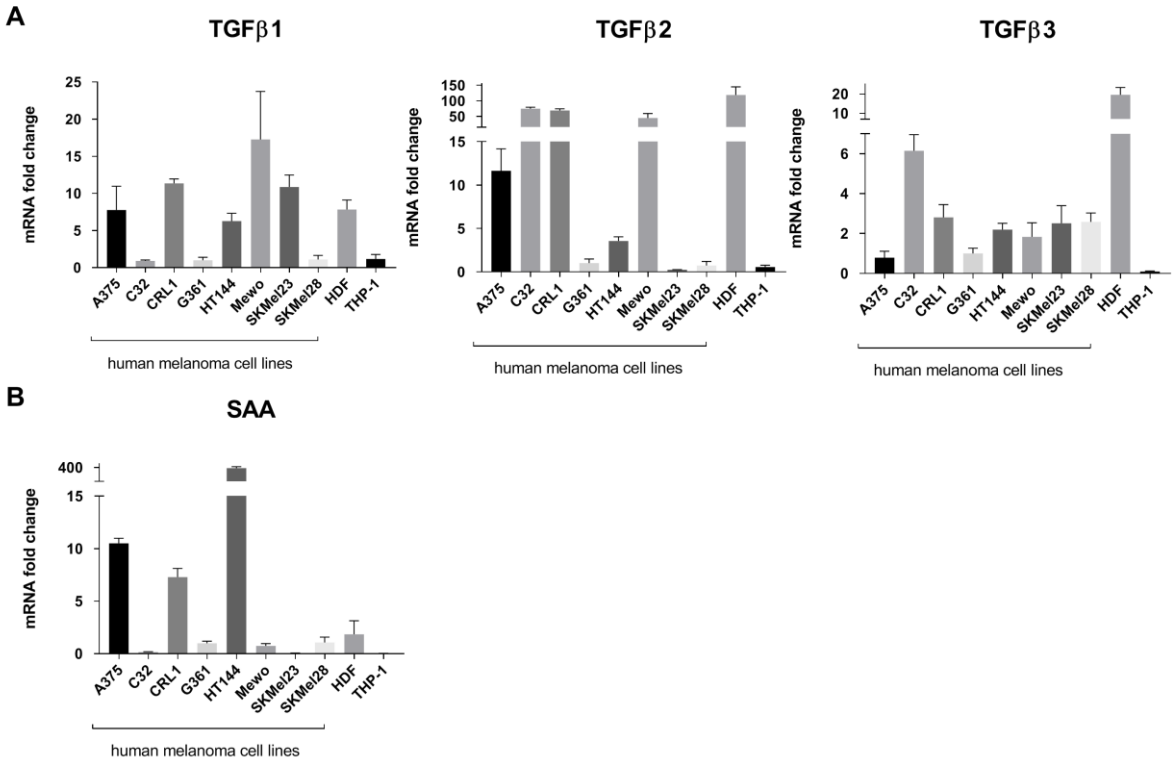

Figure 4: mRNA expression levels of TGFβ and SAA isoforms in different cell types. TGFβ isoforms (A) as well as SAA (B) are expressed in eight human melanoma cell lines (n=3) and also in unstimulated HDF (n=4) and THP-1 cells (n=3), represented at the x-axis. The ordinate represents the relative mRNA expression normalized to the melanoma cell line G361. The error bars indicate standard errors of the mean.

The melanoma cell lines had different mRNA expression profiles for TGFβ1, 2 and 3. Altogether, TGFβ1 mRNA levels were higher in most of the melanoma cell lines compared to THP-1 cells, whereas HDF showed intermediate levels. Additionally, HDF expressed relatively high levels of TGFβ2 and 3. These results are consistent with the staining of the anti-TGFβ-antibody (which recognizes all three TGFβ isoforms) in S100B-negative stromal cells in Figure 3. Additionally, SAA mRNA was expressed in most melanoma cell lines, and in lower amounts in HDF and unstimulated THP-1 cells.

In conclusion, most melanoma cell lines produced relatively high amounts of SAA and TGFβ1 in comparison to HDF or THP-1 cells. These data, together with the immunohistochemistry data, suggest that in this context, melanoma cells are the main source of SAA and TGFβ1. In the next experiments, I therefore set out to investigate whether SAA and TGFβ1 produced by melanoma cells induce expression of cancer-promoting genes in HDF and THP-1 cells.

5.2 SAA induces a protumorigenic mRNA expression signature in THP-1 cells

To test the impact of SAA stimulation on the transcription of cancer-relevant genes in monocytes/macrophages, THP-1 cells were stimulated with 100 ng/ml SAA for 24 h. The expression of a panel of genes associated with tumor and/or stromal progression was tested by PCR. This panel is listed in Table 4 with reference to the cancer-relevance of these genes. On the basis of the PCR results, the most highly up- or downregulated genes were then also analyzed by qPCR. In the case of SAA-stimulated THP-1 cells, the expression of CD163 (as a marker for TAMs[77]), IL10 and IL12A (as markers for macrophage polarization[76]) and CCL2 (as a positive control for SAA stimulation[164]) were also assessed by qPCR (also see Table 15).
To investigate a potential involvement of TLR4 in mediating the effects of SAA, cells were also incubated in the presence or absence of 5 µM of the TLR4 inhibitor CLI-095 starting 6 h prior to SAA stimulation.

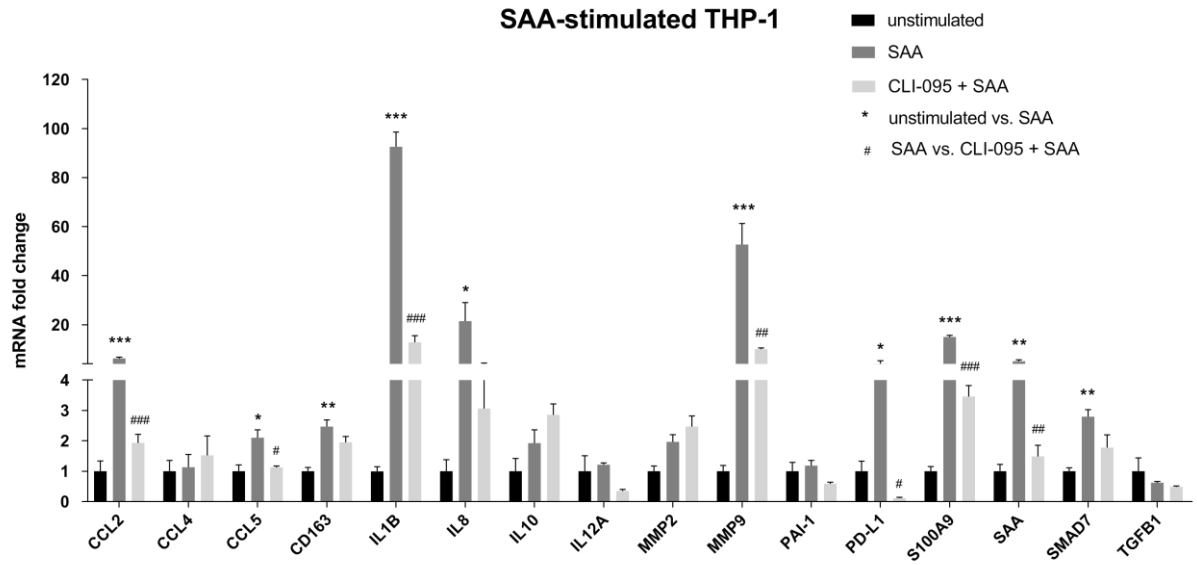

Figure 5: SAA upregulates various factors in a TLR4-dependent way in THP-1 cells. The ordinate displays the mRNA fold change relative to the levels of the unstimulated cells. The error bars indicate standard errors of the mean (n=3, except for CCL4: n=2). To compare the different stimulation conditions, a one-way ANOVA with Sidak's post hoc tests for multiple comparisons was used. "*", "**" and "***" indicate statistically significant differences between unstimulated and stimulated cells; "#", "##" and "###" indicate statistically significant differences between cells incubated with SAA alone vs. SAA-treatment after pre-incubation with the TLR4 inhibitor CLI-095 ("*" or "#" for 0.01<p<0.05, "**" or "##" for 0.001<p<0.01 and "***" or "###" for p<0.001).

SAA was able to induce its own expression as well as the expression of CCL2, CCL5, IL1B, MMP9, PD-L1 and S100A9, which were TLR4-dependent. A TLR4-independent upregulation of CD163, IL8 and Smad7 was also observed. As explained later, these SAA-induced factors participate in melanoma progression and metastasis.

To investigate the impact of SAA stimulation on fibroblasts in the cancer microenvironment, the four donor-derived HDF were also stimulated with 100 ng/ml SAA for 24 h. Again, a preliminary screen including genes linked to CAFs and tumor progression by PCR was carried out (Table 4). Here, HDF did not significantly change mRNA expression of the analyzed genes upon SAA stimulation. Therefore, cells were not pre-incubated with CLI-095.
For quantitative analysis of response and activation markers in SAA-stimulated HDF, transcription of the corresponding genes mentioned in Table 15 was determined by qPCR. Additionally, transcription of PAI-1 and TGFβ1 was tested, as their expression has been previously described in CAFs (PAI-1[170], TGFβ1[61]).

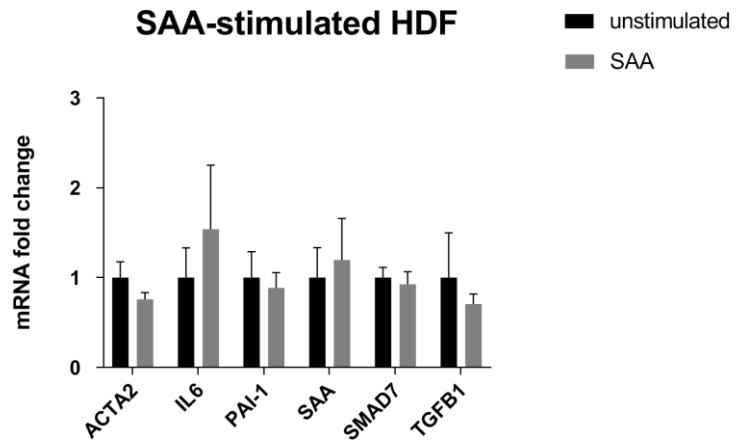

Figure 6: SAA-stimulated HDF do not show significant changes in transcription of the analyzed genes. The ordinate indicates the mRNA fold change relative to the unstimulated control, error bars display standard errors of the mean (n=4). The paired t-test was used to compare the stimulated condition with the unstimulated for each HDF line.

These results indicate that SAA did not induce changes in expression of the investigated genes in HDF.

5.3 TGFβ1 induces expression of protumorigenic factors in THP-1 cells and HDF

To analyze the transcriptional response of THP-1 cells to TGFβ1, the cells were incubated with 10 ng/ml TGFβ1 for 24 h. As before, the expression of a panel of genes linked to tumor progression and functions of TAMs were assessed by PCR (Table 4), out of which the most up-/downregulated genes were then also quantified by qPCR. In addition, expression of marker genes for TGFβ1 stimulation (Table 15) was also assessed in THP-1 cells by qPCR as a positive control.

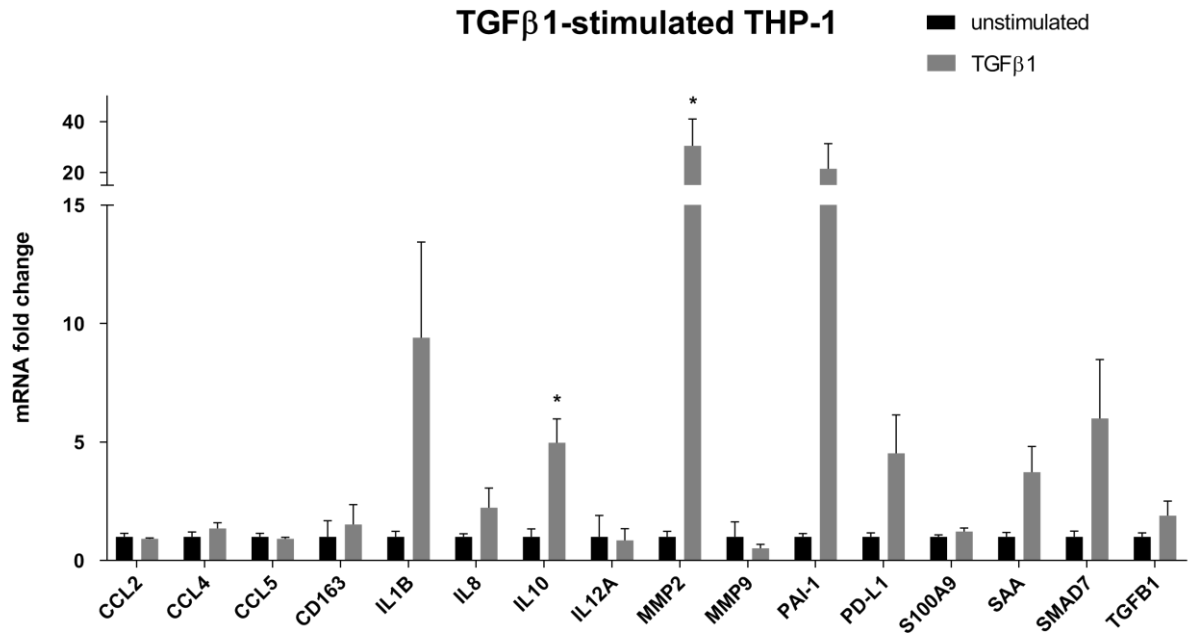

Figure 7: TGFβ1-stimulated THP-1 cells upregulate mRNA expression of different molecules. The ordinate represents the mRNA expression fold change relative to the unstimulated control. The error bars indicate standard errors of the mean and an unpaired t-test was used to compare the unstimulated vs. the TGFβ1-stimulated condition (n=3). Statistically significant differences between stimulated and unstimulated cells ($p<0.05$) are indicated by "*".

TGFβ1-stimulated THP-1 cells expressed significantly more IL10 and MMP2 at the mRNA level. Additionally, they showed a strong trend towards upregulating IL1B, PAI-1, PD-L1, SAA and Smad7 mRNA expression after TGFβ1 stimulation. As explained in detail later, these factors play a role in melanoma progression and metastasis. In contrast to the case with SAA stimulation, exposure to TGFβ1 did not upregulate CCL2, CCL5, IL8, MMP9 or S100A9 mRNA expression in THP-1 cells.

To investigate the response of HDF to TGFβ1, cells were stimulated with 2.5 ng/ml TGFβ1 for 24 h. Figure 8 represents the qPCR analysis of genes whose expression was found to be increased in a larger preliminary screen, which included genes involved in tumor progression and functional roles of CAFs (Table 4). Additionally, markers for TGFβ1-stimulated HDF mentioned in Table 15 are shown in Figure 8.

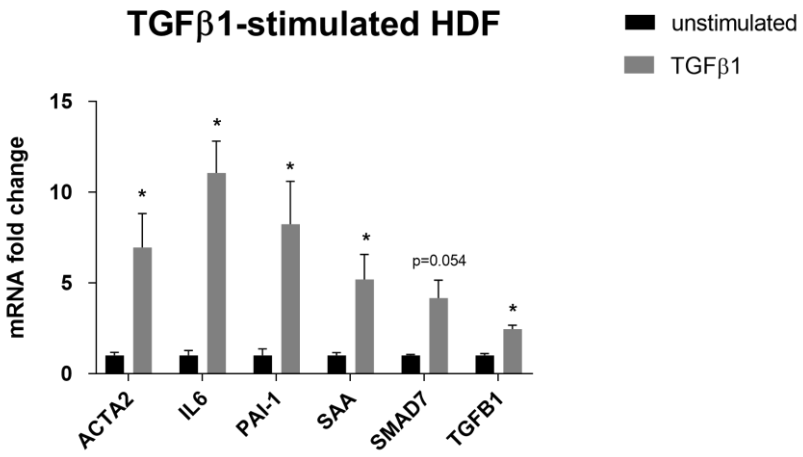

Figure 8: HDF upregulate various factors upon stimulation with TGFβ1. The ordinate displays the relative mRNA fold changes of these genes normalized to the unstimulated control. The error bars indicate standard errors of the mean. A paired t-test was used to compare the stimulated vs. unstimulated HDF (n=4). Statistically significant differences between stimulated and unstimulated cells ($p<0.05$) are indicated by "*".

TGFβ1 significantly induced its own expression in HDF, as well as the CAF marker α-SMA (ACTA2). Several other CAF markers (FAP, PGRFRα and β and S100A4) were assessed, but none of them was upregulated (data not shown), indicating that TGFβ1 alone does not upregulate them *in vitro*. TGFβ1-stimulated HDF also expressed more Smad7, which was narrowly not significant ($p=0.054$). Additionally, IL6, PAI-1 and SAA were expressed at higher levels upon TGFβ1 stimulation. As explained later, these factors are associated with the protumorigenic CAF phenotype and play a role in melanoma progression.

5.4 Local overexpression of PAI-1 correlates with poor prognosis in melanoma

Stimulation of HDF and THP-1 cells by SAA and TGFβ1 resulted in significant upregulation of a number of cancer-relevant genes. Of these, PAI-1 was strongly upregulated at the mRNA level in both TGFβ1-stimulated HDF and THP-1 cells. PAI-1, encoded by the gene SerpinE1, is a serine protease inhibitor that can be induced through TGFβ1 signaling[171]. Recently, PAI-1 has been associated with poor outcome in various tumors[172]. In breast cancer, it is already established as a marker for poor outcome, and high PAI-1 predicts the benefit from adjuvant chemotherapy in early, lymph node negative breast cancer[173]. Recent studies suggest a potential mechanistic involvement of PAI-1 in melanoma metastasis. For instance, in the B16 mouse melanoma model, stromal cell-derived PAI-1 promoted both the size of subcutaneous tumors and the extent of metastases[174], and stimulated macrophage infiltration[175]. In human melanoma, increased expression of PAI-1 has been observed in cutaneous metastases[176]. Therefore, I next asked whether PAI-1 might be associated with poor outcome of melanoma patients.

To address this question, PAI-1 protein expression in tumor samples of the TCGA Skin Cutaneous Melanoma cohort was correlated with overall survival (OS) and progression-free survival (PFS). Clinical data and protein expression z-scores were obtained from the online portal cBio Portal of cancer genomics[168, 169]. For OS analysis, there were 348 samples for which clinical data and PAI-1 expression was available, while for PFS analysis, there were 308 samples for which both were available.

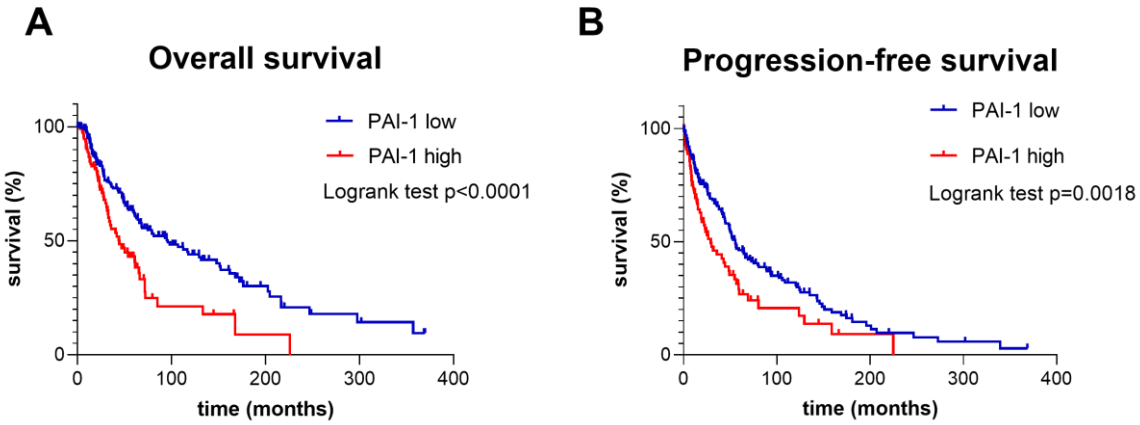

Figure 9: Higher PAI-1 protein expression levels in tumor samples of the TCGA melanoma cohort correlate with worse overall survival (A) and worse progression-free survival (B). The Kaplan Meier curves were generated with data from primary melanomas of the TCGA Skin Cutaneous Melanoma cohort, retrieved from the cBioPortal of Cancer Genomics[168, 169]. The percentage of surviving patients is represented at the ordinate. Overall survival (A, n=348) or progression-free survival (B, n=308) are represented on the x-axis. The upper tertile was set as a cutoff between low and high PAI-1 protein levels. The logrank test was used to compare the patients with low PAI-1 levels (blue) to the patients with high levels (red).

As shown in Figure 9, melanoma patients with higher PAI-1 protein levels in their primary tumor had a significantly worse overall survival than patients with lower

levels (logrank test p<0.0001). Furthermore, the progression-free survival in those patients was also significantly shorter (logrank test p=0.0018).

5.5 PAI-1 is produced by both melanoma and stromal cells

TGFβ1 stimulation of HDF and THP-1 strongly upregulated PAI-1 expression (Figure 7 and Figure 8), suggesting that CAFs and/or TAMs might be a source of PAI-1 in melanoma. Therefore, I carried out further experiments to investigate which cell types express PAI-1 in primary melanoma lesions.

5.5.1 Immunohistochemistry

To visualize the expression of PAI-1 in primary melanoma, costainings of PAI-1 together with S100B were performed as described previously. Additionally, costainings with CD163 and PAI-1 were carried out.

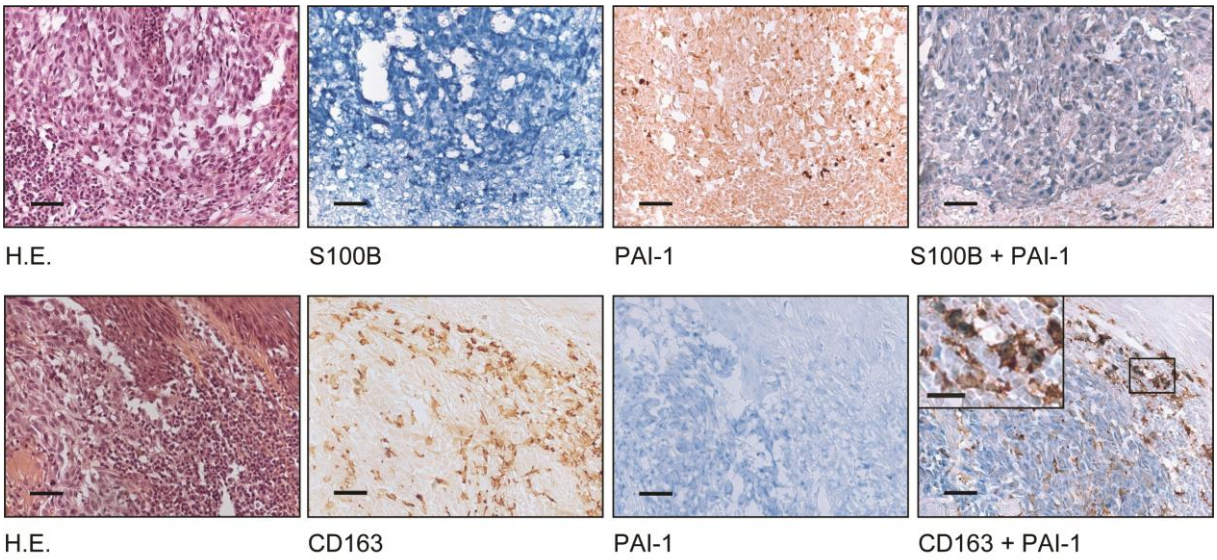

Figure 10: Expression of PAI-1 in human melanoma. Immunohistochemical stainings against S100B (blue) and PAI-1 (brown) as well as CD163 (brown) and PAI-1 (blue) in human primary melanoma. H.E.=hematoxylin-eosin staining. The scaling bar represents 50 µm or 20 µm (inset).

In these stainings, PAI-1 was present in S100B-positive melanoma cells, but also in the S100B-negative stroma. Certain stromal and tumor cells showed a stronger staining than others. Additionally, some CD163-positive stromal cells also expressed PAI-1. This suggests that melanoma cells and stromal cells including CD163-positive TAMs contribute to PAI-1 production in primary melanoma lesions.

5.5.2 Some melanoma cell lines and unstimulated HDF produce PAI-1

Next, I determined the levels of PAI-1 transcription and protein secretion in melanoma cells, macrophages and fibroblasts. Transcription of PAI-1 in human melanoma cell lines, THP-1 cells and HDF was therefore assessed by qPCR as previously described. Expression was normalized to the melanoma cell line G361. To determine the levels of secreted PAI-1, conditioned medium from the eight melanoma cell lines as well as from unstimulated HDF and THP-1 cells was analyzed by ELISA.

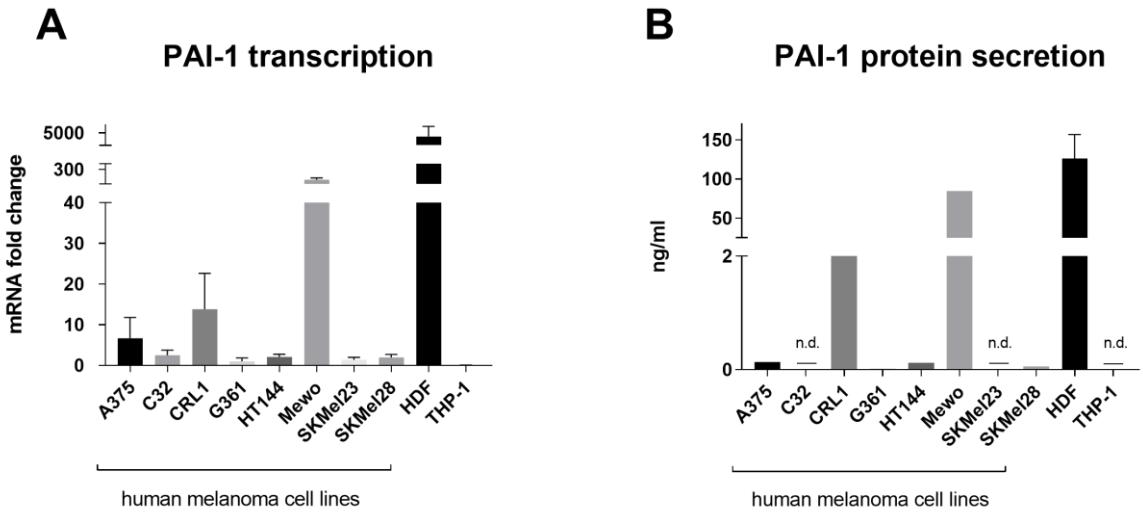

Figure 11: PAI-1 transcription (A) and protein secretion (B) in melanoma cell lines, unstimulated HDF and THP-1. (A) PAI-1 mRNA is expressed by eight human melanoma cell lines (n=3) and also by unstimulated HDF (n=4) and THP-1 cells (n=3). The ordinate represents the relative mRNA expression normalized to the melanoma cell line G361. (B) PAI-1 protein is secreted by some melanoma cell lines (n=1) and unstimulated HDF (n=4), but not by unstimulated THP-1 cells (n=1). The ordinate represents the amounts of PAI-1 measured by ELISA in ng/ml. Error bars refer to standard errors of the mean. PAI-1 protein levels of unstimulated HDF and THP-1 in this figure were from the same experiments as in **Figure 12**. N.d.=not detectable.

HDF and some melanoma cell lines (Mewo, CRL1) produced high amounts of PAI-1, whereas the other melanoma cell lines showed low mRNA expression and protein secretion levels of PAI-1. The melanoma cell lines C32 and SKMel23 as well as unstimulated THP-1 cells produced undetectable amounts of PAI-1. The melanoma cell lines with lower levels of TGFβ1 (Figure 4, e.g. C32, G361, HT144, SKMel28), also expressed lower levels of PAI-1.

In conclusion, these results show that certain melanoma cell lines as well as unstimulated HDF express and secrete PAI-1 constitutively.

5.5.3 TGFβ1 stimulation induces PAI-1 secretion in THP-1 cells and HDF

PAI-1 mRNA expression was upregulated in TGFβ1-stimulated HDF and THP-1 (Figure 7 and Figure 8). To determine whether TGFβ1 also increases PAI-1 secretion in these cells, conditioned medium from THP-1 and HDF cells treated with and without TGFβ1 stimulation for 48 h was analyzed by ELISA. As shown in the following Figure 12, upon stimulation with TGFβ1, both HDF and THP-1 cells secreted significantly more PAI-1.

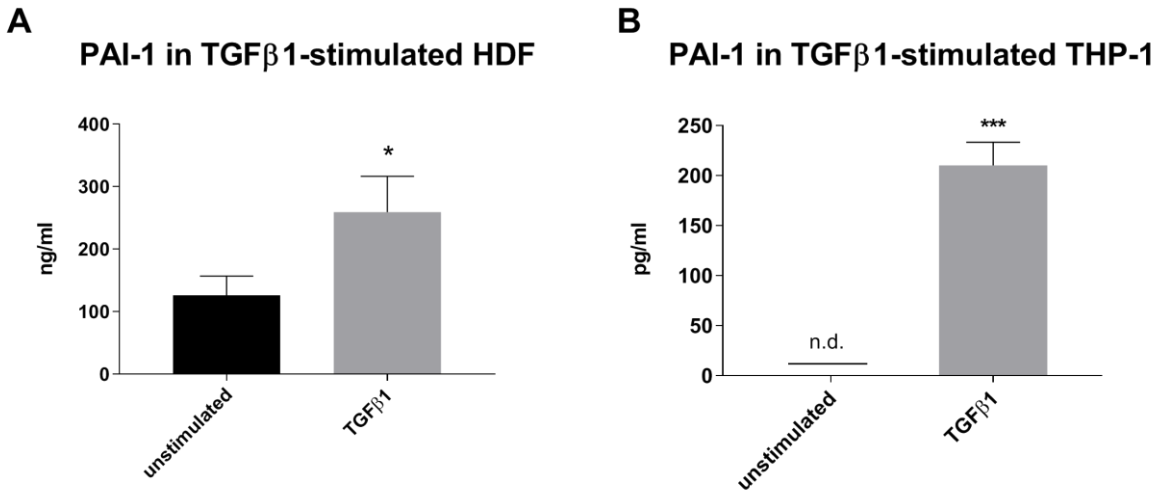

Figure 12: PAI-1 protein levels secreted by THP-1 cells and HDF upon TGFβ1 stimulation. The ordinate represents the concentration of PAI-1 measured by ELISA in ng/ml (A) or pg/ml (B) respectively. The error bars indicate standard errors of the mean. A paired t-test was used to compare unstimulated and TGFβ1-stimulated HDF (n=4); an unpaired t-test was used in the case of THP-1 cells (n=2). Statistically significant differences between stimulated and unstimulated cells are indicated by "*" ($0.01<p<0.05$) and "***" ($p<0.001$) respectively. PAI-1 protein levels of unstimulated HDF and THP-1 in this Figure as well as in **Figure 11** were from the same experiment. N.d.=not detectable.

Taken together, these results suggest that CAFs are likely to be an important source of PAI-1 in melanoma. Non-stimulated fibroblasts already produced relatively high amounts of PAI-1 protein, which was enhanced through TGFβ1 stimulation (Figure 11 and Figure 12). The concentrations of PAI-1 protein produced by unstimulated HDF (mean around 150 ng/ml) as well as TGFβ1-stimulated HDF (mean around 250 ng/ml) exceed those detected in melanoma cell lines (highest production by the cell line Mewo: mean around 100 ng/ml). Consistently, some stromal cells in Figure 10 showed a strong staining for PAI-1, supporting a participation of stromal cells in PAI-1 production.

Although THP-1 cells increased PAI-1 production after TGFβ1 stimulation, the absolute protein levels were relatively low compared to melanoma cell lines or HDF. The positive staining for PAI-1 in CD163-positive cells in Figure 10 supports the notion that TAMs *in situ* produce PAI-1.

5.5.4 Systemic levels of TGFβ1 and PAI-1 in melanoma patients correlate with each other

The above findings indicate that increased transcription of PAI-1 in melanoma samples is associated with poor prognosis, and that TGFβ1 plays an important role in upregulating PAI-1. This might lead to the hypothesis that there could be a correlation between TGFβ1 and PAI-1 protein levels in the blood of melanoma patients. To determine if this is the case, I analyzed the levels of TGFβ1 and PAI-1 by ELISA in the plasma of 22 patients. As shown in Figure 13, the plasma levels of PAI-1 strongly correlated with those of TGFβ1 in melanoma patients.

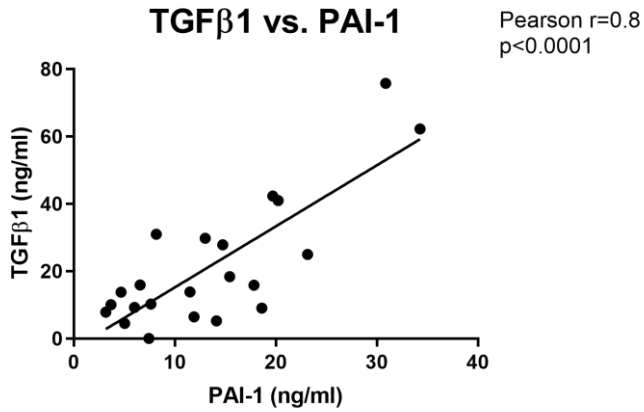

Figure 13: Plasma levels of TGFβ1 and PAI-1 in melanoma patients positively correlate with each other. The Pearson correlation coefficient r for the plasma levels of TGFβ1 and PAI-1 in melanoma was calculated (r=0.8, 95 % confidence interval: 0.5725 to 0.9139, p<0.0001).

6 DISCUSSION

Increased TGFβ1 and SAA expression is associated with melanoma progression and poor prognosis. The data presented in this thesis show that melanoma cells represent an important source of TGFβ1 and SAA. Furthermore, my results suggest that TGFβ1 and SAA are involved in the crosstalk between melanoma cells on one hand, and tumor-associated fibroblasts and macrophages on the other, because both proteins induced the transcription of a number of genes related to tumor progression in HDF and THP-1 cells. Of particular interest, TGFβ1 significantly enhanced PAI-1 production in both HDF and THP-1 cells, a protein associated with tumor progression in other contexts. Consistently, the systemic levels of TGFβ1 and PAI-1 positively correlated with each other in melanoma patients. Fibroblasts expressed relatively high PAI-1 levels before stimulation, which was strongly increased after treatment with TGFβ1, suggesting an important role for these cells as a source for locally increased PAI-1 levels. Importantly, increased local PAI-1 levels were associated with poor prognosis in melanoma patients. These results will further be contextualized in the following sections.

6.1 Expression of TGFβ1 and SAA in the primary melanoma niche

My results show that TGFβ isoforms are expressed in different melanoma cell lines (Figure 4), which is in line with previous studies[112]. TGFβ isoforms were found not only in melanoma cells, but also in stromal cells (Figure 3 and Figure 4). This is in line with the finding that TGFβ can be produced by stromal cells in response to several stimuli[100].

In my experiments, relatively high mRNA levels of TGFβ2 and 3 were observed in naïve HDF (Figure 4). Others have reported that skin fibroblasts express “baseline levels” of TGFβ2[177], which is not in accordance with these results. However, TGFβ2 and 3 expression levels in naïve HDF have not yet been directly compared to levels in melanoma cells.

Interestingly, TGFβ1 upregulated its own transcription in HDF (Figure 8), which is in line with previous findings[178]. A feedforward loop might therefore contribute to the positive TGFβ staining that is observed in the stroma in Figure 3. This was only observed in HDF, and not in THP-1 cells in my experiments, suggesting that the feedforward loop that amplifies TGFβ1 might be more pronounced in HDF. Nevertheless, TAMs *in situ* can still express TGFβ1 due to other stimuli in the tumor niche[179].

SAA was also expressed in melanoma and stromal cells in Figure 3 and Figure 4. This is consistent with reported SAA expression in other malignant tissues such as lung[150], breast[148] and esophageal[152] tumors. SAA has also been detected in melanoma cells and adjacent TAMs[149], as well as CAFs isolated from pancreatic[146] and colorectal[145] carcinomas. SAA transcription was relatively weak in untreated

THP-1 cells (Figure 4), consistent with previous findings[147]. In THP-1 cells, SAA was also observed to upregulate its own mRNA expression, which is in line with self-amplifying loops reported for SAA in different cell types[124]. However, expression levels of SAA in melanoma and stromal cells have not yet been compared directly.

6.2 Responses of THP-1 cells and HDF to SAA and TGFβ1

6.2.1 SAA stimulates THP-1 cells to express a variety of tumor-promoting factors

In my experiments, the stimulation of THP-1 cells with SAA upregulated the expression of a number of cancer-relevant genes. This increased expression was mainly TLR4 dependent (Figure 5). These results suggest that SAA in the tumor milieu may stimulate monocytic macrophages to produce tumor-promoting factors.

The elevated production of IL1B by SAA-stimulated THP-1 has also been observed by others[180] and involved TLR2, 4 or FPR2[135]. In various tumors including melanoma, IL1B plays a role in invasion, proliferation and stimulation of adjacent cells to produce proangiogenic molecules including IL8 and VEGF[181]. Additionally, IL1B can promote the differentiation of Tregs, and can be considered as initiator of immunotolerance[154]. A recent study reported that macrophages produce IL1B upon stimulation with melanoma conditioned medium, initiating a crosstalk with fibroblasts, which contributed to BRAFi resistance in melanoma[82]. My experiments suggest that melanoma-derived SAA might play a role in this IL1B production by monocytic cells.
Interestingly, I also observed that PD-L1 expression was upregulated in SAA-stimulated THP-1 cells. PD-L1 can be detected in tumor cells as well as antigen-presenting cells including macrophages, where it inhibits the antitumor response of cytotoxic T cells and NK cells[42]. Hence, melanoma-derived SAA might contribute to PD-L1 expression by TAMs in the primary tumor niche, thereby suppressing antitumor immunity.
Consistent with my results, IL8 has previously been detected in SAA-stimulated monocytes and THP-1 cells, where it mediated chemotaxis together with SAA and CCL3[136]. IL8 can promote angiogenesis, tumor growth and metastasis in melanoma and other cancers[182]. Additionally, a recent study reported that high systemic levels of IL8 in melanoma patients could predict poor response to PD1/PD-L1 blockade[183]. Hence, monocytes exposed to melanoma-derived SAA might contribute to elevated levels of IL8.
SAA treatment of THP-1 cells resulted in enhanced transcription of MMP9, which is consistent with previous findings in the context of lung adenocarcinoma[150]. Others have reported that SAA-induced MMP9 upregulation in THP-1 cells *in vitro* depends on FPR2-mediated NF-κB activation[137]. In my experiments, blockade of TLR4 dampened, but did not completely inhibit SAA-induced MMP9 expression, suggesting that FPR2 and TLR4 might both be relevant. The protease MMP9 degrades ECM components[184], including basal membrane collagen type IV[185], which facilitates tumor cell invasion. Increased MMP9 expression has been associated with invasion,

metastasis, angiogenesis and poor prognosis in many different tumors[186]. In addition, MMP9 can activate many cytokines and growth factors by proteolysis, including IL1B, IL8 and TGFβ1[184]. Further research is required to understand the contribution of SAA-stimulated monocytic cells to MMP9-mediated protumorigenic effects in melanoma.
Smad7, a TGFβ1-inducible protein that suppresses the TGFβ1 pathway[101], was upregulated in SAA-stimulated THP-1 cells. Similar to SAA, Smad7 can promote NF-κB activation and cytokine secretion in macrophages[187]. Therefore, it might be interesting to test whether a crosstalk exists between intracellular pathways that are stimulated through TGFβ1 and SAA in monocytic cells, and how this impacts melanoma progression.
In my experiments, SAA upregulated S100A9 and its own expression in THP-1 cells in a TLR4-dependent manner. S100A9 is part of the family of S100 calcium-binding proteins, it can induce SAA proteins in various cell types[188] and has been associated with metastatic behavior[189]. Previously, others have shown in mouse models that melanoma-derived S100A8/A9 can induce SAA3 in distant pre-metastatic lungs. In this study, SAA initiated a positive feed-forward loop of chemoattractant secretion. This recruited monocytic cells and tumor cells to pre-metastatic lesions and promoted lung metastasis through TLR4- and NF-κB-dependent signaling[190]. The observation that SAA can induce its own expression in monocytic cells has not yet been reported to my knowledge. Melanoma patients in early tumor stages were found to have elevated systemic levels of SAA, which correlated with poor prognosis[96]. Therefore, induction of S100A9 and its own expression by SAA in monocytic cells might be part of a metastasis-initiating feedforward loop that is reflected by elevated systemic levels of SAA.

It is unclear where SAA-stimulated THP-1 cells fit into the M1/M2 paradigm. Classical M1 markers such as IL12A and M2 markers such as IL10[191] were not induced through SAA in naïve THP-1 cells. Nevertheless, some M1-related factors such as IL1B and IL8[192] were upregulated. The upregulated chemokines CCL2 and 5, known to recruit monocytic cells[136], have been linked to proinflammatory M1 macrophages[76] but also to TAMs[77]. Additionally, the TAM marker CD163 was upregulated through SAA, and mRNA levels of TAM-associated MMP9[78] were also elevated. Previous studies have reported that SAA induced the expression of a variety of M2 macrophage markers in THP-1[142], which I did not observe in my experiments. Others found that THP-1 cells pretreated with M-CSF show a mixed M1/M2 gene expression signature upon SAA stimulation, which was characterized by the expression of IL1B, IL6, IL10 and CD163[193]. Unpolarized M0 and proinflammatory M1-subpopulations of TAMs were described to be present in *in situ*-melanomas to a similar extent as M2-TAMs[194], suggesting that both phenotypes may play a role in melanoma progression.

A possible reason for the mixed M1/M2 marker profile after SAA treatment is that THP-1 cells represent a mixed population of cells in different conditions between monocyte and macrophage[195]. To induce macrophage differentiation and to maintain the number of differentiated cells in experimental conditions and between replicates,

THP-1 can be pre-treated with phorbol-12-myristate-13-acetate (PMA) or M-CSF[196]. However, PMA activates several intracellular pathways including the protein kinase C pathway[197, 198], leads to accumulation of intracellular NF-κB[199] and induces the expression of several proto-oncogenes[200]. Additionally, PMA promotes M1-differentiation of THP-1 cells[197]. Considering these limitations and since the aim was to investigate the response to a single molecule (SAA/TGFβ1), non-pretreated THP-1 cells were used in this thesis.

In conclusion, SAA alone did not polarize naïve THP-1 cells, but induced expression of TAM-associated factors that are implicated in melanoma invasion, matrix remodeling, angiogenesis, immune evasion and therapy resistance.

6.2.2 HDF were not susceptible to SAA stimulation

HDF did not significantly change the expression of the panel of tested genes in response to SAA treatment (Figure 6). Variance in gene expression was observed between different patient-derived samples. In previous studies, synovial fibroblasts stimulated with SAA were found to upregulate various inflammatory molecules, including IL6, CCL2 and CXCL1, 2, 3 and 8, in a TLR4-dependent manner[143]. Amnion fibroblasts upregulated IL1B, IL6 and cyclooxygenase 2 upon SAA treatment[134], and dermal fibroblasts were found to produce more IL6 through TLR2[165]. The different experimental context or origins of these fibroblasts might explain their enhanced responsiveness to SAA compared to the HDFs used in my experiments.

6.2.3 THP-1 cells produce protumorigenic factors upon TGFβ1 treatment, including PAI-1

TGFβ1 significantly upregulated IL10 and MMP2 transcription in THP-1 cells (Figure 7). IL10 is associated with the M2 phenotype[74] and plays an important role in the immune evasion of melanoma together with TGFβ1. For instance, melanoma-derived IL10 dampened the ability of dendritic cells to activate cytotoxic T cells and inhibited IFN production of NK cells, both at the origin of impaired antitumor immune responses[201]. Hence, monocytic macrophages conditioned by TGFβ1 might also be a source of IL10. MMPs are known to be TGFβ1-downstream targets in tumor and stromal cells[202]. Others have suggested that MMP production[73] (including MMP2[45]), but also IL10-secretion[77] are functional features of TAMs. In contrast to this, the TAM marker CD163, and also other TAM-associated molecules such as CCL2 or 5[77] and MMP9[78], were not upregulated in TGFβ1 stimulated THP-1 cells.

Although there was a strong tendency towards enhanced expression of Smad7, IL1B, SAA, PD-L1 and PAI-1 in TGFβ1-stimulated THP-1 cells, this effect was not significant. This might be explained through the context-sensitivity of the effects of TGFβ1[98], or by the experimental approach using THP-1 cells without pre-differentiation. In this regard, it is notable that M-CSF as well as PMA, which are frequently used for pre-differentiation, can induce PAI-1 in monocytic cells[203, 204]. This

might lead to an overestimation of PAI-1 production by TGFβ1-stimulated, pre-treated THP-1 cells, especially when comparing secreted levels with those of naïve HDF or melanoma cells. Nevertheless, although transcription of PAI-1 was not significantly increased, protein secretion of PAI-1 measured by ELISA was significantly enhanced in THP-1 cells in response to TGFβ1 stimulation (Figure 12).

Taken together, my results suggest that TGFβ1 does not completely polarize naïve THP-1 cells towards TAMs, but upregulates molecules linked to immune evasion and matrix remodeling.

6.2.4 TGFβ1 activates HDF to produce PAI-1

HDF upregulate α-SMA expression in response to TGFβ1 treatment (Figure 8). This observation is consistent with expression signatures that have been reported for different models of CAFs, including TGFβ1-stimulated fibroblasts[205] and melanoma CAFs *in vivo*[66]. Additionally, TGFβ1-stimulated HDF increased mRNA expression of IL6 in my experiments. In previous studies, CAF-derived IL6 was found to promote tumor cell migration and invasion in the context of melanoma[59]. Thus, tumor-derived TGFβ1 might contribute to the production of migratory signals by HDF.
Upon TGFβ1 treatment, I found that HDF expressed significantly more SAA and PAI-1. Consistently, others have characterized the expression and secretion of various factors in CAFs isolated from murine colorectal carcinoma, and found that SAA and PAI-1 were upregulated compared to normal fibroblasts[145]. Since colorectal tumors are known to produce TGFβ1[61], elevated expression of SAA and PAI-1 in this context could conceivably be induced by tumor-derived TGFβ1. Taken together, my results indicate that TGFβ1 activates naïve fibroblasts to produce molecules associated with melanoma progression, including PAI-1.

Since both HDF as well as THP-1 cells upregulated PAI-1 in response to TGFβ1, the following paragraphs will consider the role of PAI-1 in my experiments and its functions in different contexts.

6.3 Plasminogen activator inhibitor 1

Plasminogen activator inhibitor 1 (PAI-1, encoded by the gene SerpinE1) is a serine protease inhibitor that represses tissue- and urokinase plasminogen activators (tPA and uPA)[206]. Additionally, SerpinE1 is considered to be a target gene of TGFβ1 signaling[171]. Plasmin degrades the ECM either directly or through the activation of effector enzymes. Therefore, PAI-1 is a potent inhibitor of proteolysis in many processes, including wound healing, thrombosis, tissue growth and many others[172]. Additionally, PAI-1 has tumor-promoting roles beyond proteolysis inhibition, which will be explained in detail later on.

6.3.1 PAI-1 is present in melanoma

In human melanoma sections, I observed expression of PAI-1 in both melanoma cells and stromal cells of the cancer microenvironment. Consistently, positive staining for PAI-1 has previously been found in human primary melanoma and cutaneous metastases[176], fibroblasts and the ECM of murine melanomas[207]. Similar findings have also been made in other cancer types, with evidence that both CAFs and TAMs contribute to the PAI-1 content of the primary tumor milieu. In lung adenocarcinoma, PAI-1 staining correlated with that of α-SMA[208], suggesting an important role for CAFs in PAI-1 production. The histological presence of PAI-1 in breast CAFs was linked to enhanced invasiveness[209]. In addition, an association between PAI-1 staining and TAM infiltration in non-small cell lung carcinoma (NSCLC)[210], and renal cell carcinoma[211] has been reported, consistent with the co-localization of CD163 and PAI-1 in my experiments (Figure 10). Additional work will be required to determine whether these findings also hold true in melanoma.

6.3.2 PAI-1 production by melanoma cells

Figure 11 shows that some melanoma cell lines produced high levels of PAI-1 compared to others. Interestingly, human melanoma cell lines that express high PAI-1 levels were more likely to form lung metastases in mice compared to low-PAI-1 cell lines[212]. It is therefore notable that the cell lines with the highest PAI-1 levels in my experiments derive from metastatic sites (Mewo: lymph node metastasis[161] and CRL1: cutaneous metastasis[159]). All other cell lines are from primary tumors (Table 12) and had lower PAI-1 levels.

6.3.3 PAI-1 production by HDF

Unstimulated HDF produced high amounts of PAI-1 in my experiments (Figure 11). Others have also observed levels of secreted PAI-1 in unstimulated fibroblasts and melanoma cells that were approximately the same as those in my experiments[213]. Although PAI-1 was produced in relatively high amounts by naïve HDF, secretion was significantly increased by TGFβ1 stimulation. The absolute levels measured by ELISA were highest in TGFβ1-stimulated HDF (Figure 11 and Figure 12), suggesting an important role for these cells in PAI-1 production and amplification.

Enhanced PAI-1 levels secreted by TGFβ1-stimulated fibroblasts have not yet been reported in the context of melanoma. Others showed that *in situ*-CAFs isolated from colorectal carcinoma[61], OSCC[214] and melanoma[215] were sources of PAI-1, which is in line with my findings. In these studies, stimuli responsible for PAI-1 induction in fibroblasts were not investigated[61, 214, 215]. My findings suggest that TGFβ1 produced by these tumors[61, 214, 216] might play a role.

Taken together, these results suggest that fibroblasts represent an important but yet under-investigated source of PAI-1 in the context of the primary melanoma niche.

6.3.4 PAI-1 production by THP-1 cells

The strong, but not significant upregulation of PAI-1 in TGFβ1-stimulated THP-1 in Figure 7 is in accordance with detected upregulation of this molecule in different models of TGFβ1-stimulated monocytes[203, 217]. Nevertheless, absolute levels of PAI-1 in TGFβ1-stimulated THP-1 cells were lower (Figure 12) than in melanoma cell lines (Figure 11) or HDF (Figure 11 and Figure 12). It is known that other contextual factors such as glucocorticoids can enhance the responsiveness of macrophages to TGFβ1[218], and the production of PAI-1 has been reported to be enhanced in monocytic cells exposed to glucocorticoids and TGFβ1[203]. Due to these context-dependent effects of TGFβ1, melanoma-TAMs *in situ* may still be an important source of PAI-1. Therefore, positive PAI-1 staining in CD163-positive TAMs in Figure 10 and the low absolute levels of PAI-1 in Figure 12 might not contradict each other.

6.3.5 PAI-1 production is stimulated by TGFβ1-mediated crosstalk

My results clearly show that TGFβ1 increases PAI-1 production in THP-1 cells as well as HDF. As TGFβ1 is produced by both tumor and stromal cells, these results suggest that TGFβ1-mediated crosstalk between these cellular compartments serves to amplify PAI-1 expression in the primary melanoma niche. Consistent with this notion, I found a positive correlation between plasma levels of PAI-1 and TGFβ1 in the blood from 22 melanoma patients. Cell lines with high TGFβ1 levels in my experiments (Mewo, CRL1, Figure 4) also expressed more PAI-1 (Figure 11) than cell lines with lower TGFβ1 mRNA levels (e.g. G361, C32, SKMel 28). This suggests that autocrine TGFβ1 signaling may also serve to increase local PAI-1 levels, which deserves to be investigated further.

6.3.6 Functional roles of PAI-1 in melanoma and other tumors

PAI-1 has been reported to have pleiotropic effects on tumor growth, angiogenesis, therapy resistance, invasion and metastasis. These effects will be described in detail in this section.

PAI-1 can promote tumor cell proliferation directly. For example, PAI-1-downregulation impaired the entry into the S-phase of the cell cycle through the depletion of cyclin D3/cdk4/6 and cyclin E/cdk2 in urothelial and cervical cancer cell lines. In this study, ectopic PAI-1 expression could also promote tumor growth in a mouse xenograft model[219].

PAI-1 is also thought to play a major role in cell migration, invasion and metastasis, since it influences cellular adhesion-deadhesion-cycles. Nevertheless, the literature suggests that PAI-1 can have both positive and negative effects on cell migration and invasion. On the one hand, the active form of PAI-1 dampens the activation of plasmin, inhibiting the degradation of the ECM[206]. Therefore, it might be argued that PAI-1 rather impedes tumor cell invasion. On the other hand, active, cleaved as well as latent PAI-1 can bind to low-density lipoprotein receptor-related protein1 (LRP1),

which promotes cell migration independently of plasmin[220]. LRP1 is a receptor involved in tumor progression, that is expressed (amongst others) in fibroblasts, macrophages, smooth muscle cells, and malignant cells[221]. Binding of PAI-1 to LRP1 induces the internalization of cell surface bound integrins, resulting in cellular detachment from the ECM, which is necessary for migration[222].

Given these observations, the question arises whether the pro- or the antimigratory effects of PAI-1 predominate in the context of cancer. In OSCC, PAI-1 overexpression enhances tumor cell migration by activation of the PI3K/AKT pathway[223]. Similarly, invasion and migration of colorectal carcinoma[224] and glioma cells[225] depends on PAI-1. In contrast to this, Humbert et al. found that PAI-1, derived from TGFβ-signaling, inhibited migration and invasion in several melanoma cell lines *in vitro*[226]. This latter study is the only one to report an inhibitory role for PAI-1 in tumor progression. The effect may be unspecific, as the authors reported that cell viability was significantly decreased after 48 h of TGFβ stimulation compared to unstimulated cells, which might at least partly explain impaired migration and invasion. In this study, decreased plasmin activity was implicated in the impaired migration and invasion. However, the researchers did not consider other target structures of PAI-1 involved in migration, e.g. LRP1. Nor the role of stromal PAI-1, neither the impact of PAI-1 on melanoma metastasis *in vivo* were considered in this study[226].
Other reports contradict the conclusion that PAI-1 has an inhibitory role in melanoma invasion and metastasis. For example, melanoma cell lines with enhanced metastatic potential *in vivo* have higher PAI-1 levels compared to non-metastatic cell lines[227]. In mice bearing B16-derived tumors, PAI-1 depletion limited melanoma metastasis[174]. In the same study, the inhibition of stromal PAI-1 had more pronounced effects on metastasis and angiogenesis compared to the depletion of tumor-derived PAI-1[174]. Therefore, it is important to consider the role of PAI-1 produced by tumor-associated stromal cells. Additionally, the effects of PAI-1 on migration, invasion and metastasis might also depend on the precise experimental conditions.

In addition to effects on tumor cell migration and invasion, PAI-1 might play a role in stimulating the migration and recruitment of immune cells into the tumor microenvironment. For example, PAI-1 can cause macrophage migration into murine melanomas via binding to LRP1 and intracellular FAK-phosphorylation[175]. In another study, NSCLC-derived PAI-1 activated macrophages via TLR4, which recruited CD163-positive TAMs and induced TGFβ1 expression. TAM-derived TGFβ1 in turn stimulated tumor cells to produce more PAI-1, generating a feed-forward loop[210]. Therefore, it might be interesting to study the role of TGFβ1 and PAI-1 in a co-culture model of human melanoma cell lines and THP-1 cells. Others observed that the interaction between murine colorectal carcinoma cells and CAFs promotes the production of α-SMA, PAI-1 and TGFβ1 by CAFs. This over-activated the TGFβ1 pathway in both tumor cells and CAFs, fostering tumor progression *in vivo*[61].

PAI-1 can exert both positive and negative effects on angiogenesis. The role of PAI-1 in angiogenesis is dependent on the source (tumor or host cells) and the concentration of PAI-1. At lower levels, PAI-1 stimulates angiogenesis in murine melanoma, whereas it has antiangiogenic effects at high concentrations[228]. Proangiogenic roles of PAI-1 were also reported in various other tumors including breast cancer and NSCLC[229]. In mice bearing B16-derived melanomas, inhibition of PAI-1 reduced both angiogenesis and tumor growth[174], but the mechanisms remain subject to further research. In another study, PAI-1 averted apoptosis of vascular endothelial cells by cleaving the surface bound apoptosis ligand FasL[230]. This may also contribute to the proangiogenic effects of PAI-1.

In addition to promoting growth, PAI-1 plays a role in therapy resistance in different tumors. PAI-1 has been found to rescue OSCC from cisplatin-induced apoptosis[223] through activation of the PI3K/AKT pathway. Similar observations have been made in fibrosarcoma[231], esophageal squamous cell carcinoma[232] and NSCLC[233]. In melanoma, PAI-1 has been identified as one of the proteins upregulated in BRAFi resistant cell lines[234], but the exact mechanisms explaining the role of PAI-1 in BRAFi resistance remain to be investigated.

6.3.7 PAI-1 as a therapeutic target

Since PAI-1 exerts many tumor promoting functions, therapeutic strategies targeting PAI-1 are currently being investigated. The small molecule PAI-1 inhibitor tiplaxtinin (=PAI-039) reduces the growth of urothelial carcinoma cell lines and xenografts[219] as well as the cervical carcinoma cell line HeLa[235]. Additionally, it impairs the self-renewal of tumor initiating cells in head and neck cancer by inhibition of SOX2 expression[236]. Interestingly, SOX2 overexpression is induced in melanoma through TGFβ1, promoting invasion and metastasis[237]. Therefore, it might be interesting to test the effects of tiplaxtinin in melanoma. To date, only one study has investigated the effect of a PAI-1 inhibitor (SK-216) in melanoma. Here, SK-216 impaired tumor vascularization and growth in B16-derived melanoma xenografts[174]. Besides, there are numerous other small molecule inhibitors, small interfering RNAs and also natural compounds targeting PAI-1 that have been tested in breast cancer, colorectal carcinoma and other tumors[229]. Nevertheless, further studies are needed to identify side effects of these molecules. PAI-1 inhibition enhances plasmin-mediated fibribolysis[206] and therefore might cause severe spontaneous bleeding. By inhibiting epithelial cell migration, collagen deposition and the differentiation of myofibroblasts, functional PAI-1 blockade impairs wound healing[238]. Further, most of the small molecule inhibitors targeting PAI-1 exhibit short plasma half-lives of around 2 to 3 hours, and concentrations in the range of mmol/l were often needed in order to observe antitumor effects[239]. Nevertheless, recent approaches have been made to overcome these limitations. PAI-1 inhibitors are being modified to prolong their plasma half-life[240] or to reduce spontaneous bleeding[241].

6.3.8 PAI-1: a potential diagnostic tool in melanoma?

Importantly, I found that local PAI-1 upregulation was strongly associated with poor overall and progression-free survival in a cohort of melanoma patients (Figure 9). These are the first data to show a correlation between locally elevated levels of PAI-1 and poor prognosis in melanoma. Consistent with these findings, PAI-1 has been associated with poor outcome in many tumors[172]. In lymph node negative breast cancer, it is already established as a marker for poor prognosis and high PAI-1 levels predict the benefit from adjuvant chemotherapy[173]. These data suggest that PAI-1 may represent a useful prognostic and theranostic tool for assessment of melanoma patients.

To date, few studies have considered the prognostic value of PAI-1 in melanoma. PAI-1 is upregulated in thick versus thin melanoma, or in atypical nevi and melanoma compared to benign nevi[242]. In cutaneous metastases, local PAI-1 levels were higher compared to primary melanoma lesions[176]. Increased PAI-1 levels were also observed in metastatic compared to nonmetastatic cell lines[88]. In 2002, a small study (including 18 patients with advanced melanoma, 12 cases with locally limited melanoma and 39 healthy controls) indicated that the active fraction of plasma-PAI-1 might predict the metastatic risk of early stage melanoma[243]. Importantly, in this thesis, increased PAI-1 protein levels in melanomas from the TCGA cohort were associated with significantly worse overall and progression-free survival (Figure 9), further emphasizing a potential diagnostic role of PAI-1 in melanoma.

The therapeutic landscape of melanoma is getting more and more complex and individualized. Therefore, there is an urgent need for new markers to facilitate clinical decisions in melanoma, e.g. in order to identify patients that might profit from a more expensive or more aggressive therapy, or patients that need to be monitored in shorter intervals. Therefore, based on the results of this thesis, I strongly recommend investigating the diagnostic, prognostic and theranostic potential of PAI-1 in melanoma.

6.4 Conclusion

This thesis shows that melanoma cells condition fibroblasts and monocytic macrophages via TGFβ1 and SAA to create a tumor promoting milieu. TGFβ1 enhanced PAI-1 production in both cell types, which was associated with poor outcome in melanoma patients. The following figure proposes a summarizing scheme of these results.

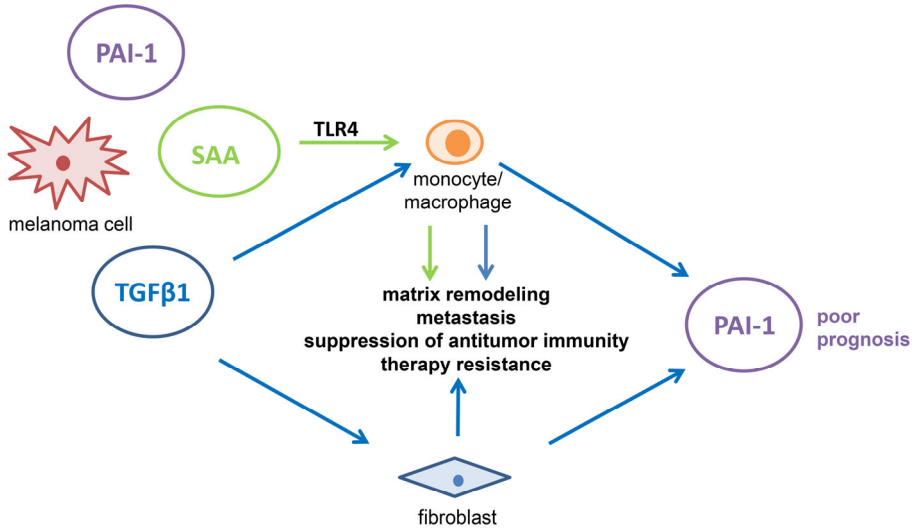

Figure 14: Melanoma cells influence fibroblasts and monocytes through SAA and TGFβ1. Melanoma cells produce TGFβ1 and SAA, which have the capacity to condition fibroblasts and monocytes to create a tumor promoting milieu. Additionally, TGFβ1 induces PAI-1 in macrophages and fibroblasts. The most important sources of locally enhanced PAI-1 levels in this context are TGFβ1-stimulated HDF and melanoma cells. This local overexpression of PAI-1 significantly correlated with worse overall and disease-free survival in melanoma patients. PAI-1=plasminogen activator inhibitor 1, SAA=serum amyloid A, TGFβ1=transforming growth factor beta 1, TLR4=toll-like receptor 4.

SAA stimulation only had a significant effect on gene expression in THP-1 cells. In the main, SAA mediated these effects by activating TLR4. The upregulated genes have been implicated in melanoma invasion, matrix remodeling, angiogenesis, immune evasion and therapy resistance. These results emphasize an important role for monocytic macrophages in melanoma progression.

TGFβ1 upregulated molecules linked to immune evasion and matrix remodeling in THP-1 cells. It also induced a CAF-like phenotype in HDF and promoted PAI-1 production in both cell types. Currently, potential sources of enhanced PAI-1 levels in melanoma have been under-investigated. My results reveal that TGFβ1-activated stromal cells, especially fibroblasts, represent an important source of PAI-1, in addition to melanoma cells.

Importantly, in melanoma patient samples of the TCGA cohort, I found that local overexpression of PAI-1 protein significantly correlated with worse overall and disease-free survival. This is the first cohort, in which a link between locally elevated PAI-1 and prognosis of melanoma patients has been investigated, underlining the potential role of PAI-1 as a new prognostic tool in melanoma.

7 TABLES

8 REFERENCES

1. Gordon, R: Skin cancer: an overview of epidemiology and risk factors. *Semin Oncol Nurs,* 29**:** 160-169, 2013.
2. Tolleson, WH: Human melanocyte biology, toxicology, and pathology. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev,* 23**:** 105-161, 2005.
3. Ali, Z, Yousaf, N, Larkin, J: Melanoma epidemiology, biology and prognosis. *EJC Suppl,* 11**:** 81-91, 2013.
4. Rosendahl, C, Cameron, A, Wilkinson, D, Belt, P, Williamson, R, Weedon, D: Nail matrix melanoma: consecutive cases in a general practice. *Dermatol Pract Concept,* 2**:** 202a213, 2012.
5. Tas, F, Keskin, S, Karadeniz, A, Dagoglu, N, Sen, F, Kilic, L, Yildiz, I: Noncutaneous melanoma have distinct features from each other and cutaneous melanoma. *Oncology,* 81**:** 353-358, 2011.
6. Holmes, D: The cancer that rises with the sun. *Nature,* 515**:** S110-111, 2014.
7. Zentrum für Krebsregisterdaten im Robert-Koch-Institut: *Datenbankabfrage der altersstandardisierten Inzidenzrate des malignen Melanoma der Haut (C43) pro 100.000 Einwohner in Deutschland von 1999 bis 2016*. Online: https://www.krebsdaten.de/Krebs/DE/Home/homepage_node.html, Stand: 27.12.2019.
8. Whiteman, DC, Green, AC, Olsen, CM: The Growing Burden of Invasive Melanoma: Projections of Incidence Rates and Numbers of New Cases in Six Susceptible Populations through 2031. *J Invest Dermatol,* 136**:** 1161-1171, 2016.
9. Svedman, FC, Pillas, D, Taylor, A, Kaur, M, Linder, R, Hansson, J: Stage-specific survival and recurrence in patients with cutaneous malignant melanoma in Europe - a systematic review of the literature. *Clin Epidemiol,* 8**:** 109-122, 2016.
10. Braeuer, RR, Watson, IR, Wu, C-J, Mobley, AK, Kamiya, T, Shoshan, E, Bar-Eli, M: Why is melanoma so metastatic? *Pigment Cell Melanoma Res,* 27**:** 19-36, 2014.
11. Villanueva, J, Herlyn, M: Melanoma and the tumor microenvironment. *Curr Oncol Rep,* 10**:** 439-446, 2008.
12. Willyard, C: Risk factors: riddle of the rays. *Nature,* 515**:** S112-113, 2014.
13. Garibyan, L, Fisher, DE: How sunlight causes melanoma. *Curr Oncol Rep,* 12**:** 319-326, 2010.
14. Emri, G, Paragh, G, Tosaki, A, Janka, E, Kollar, S, Hegedus, C, Gellen, E, Horkay, I, Koncz, G, Remenyik, E: Ultraviolet radiation-mediated development of cutaneous melanoma: An update. *J Photochem Photobiol B,* 185**:** 169-175, 2018.
15. Psaty, EL, Scope, A, Halpern, AC, Marghoob, AA: Defining the patient at high risk for melanoma. *Int J Dermatol,* 49**:** 362-376, 2010.
16. Sun, Y, Liu, WZ, Liu, T, Feng, X, Yang, N, Zhou, HF: Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J Recept Signal Transduct Res,* 35**:** 600-604, 2015.
17. Reddy, BY, Miller, DM, Tsao, H: Somatic driver mutations in melanoma. *Cancer,* 123**:** 2104-2117, 2017.

18. Domingues, B, Lopes, JM, Soares, P, Populo, H: Melanoma treatment in review. *Immunotargets Ther,* 7**:** 35-49, 2018.
19. Ersahin, T, Tuncbag, N, Cetin-Atalay, R: The PI3K/AKT/mTOR interactive pathway. *Mol Biosyst,* 11**:** 1946-1954, 2015.
20. Munoz-Couselo, E, Adelantado, EZ, Ortiz, C, Garcia, JS, Perez-Garcia, J: NRAS-mutant melanoma: current challenges and future prospect. *Onco Targets Ther,* 10**:** 3941-3947, 2017.
21. Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft, Deutsche Krebshilfe, AWMF): *Diagnostik, Therapie und Nachsorge des Melanoms, Langversion 3.2, 2019*, AWMF Registernummer: 032/024OL. Online: www.leitlinienprogramm-onkologie.de/leitlinien/melanom/, Stand: 26.12.2019.
22. Breslow, A: Thickness, cross-sectional areas and depth of invasion in the prognosis of cutaneous melanoma. *Ann Surg,* 172**:** 902-908, 1970.
23. Griewank, KG: Biomarkers in melanoma. *Scand J Clin Lab Invest Suppl,* 245**:** S104-112, 2016.
24. Passarelli, A, Mannavola, F, Stucci, LS, Tucci, M, Silvestris, F: Immune system and melanoma biology: a balance between immunosurveillance and immune escape. *Oncotarget,* 8**:** 106132-106142, 2017.
25. Ladanyi, A: Prognostic and predictive significance of immune cells infiltrating cutaneous melanoma. *Pigment Cell Melanoma Res,* 28**:** 490-500, 2015.
26. Emri, E, Egervari, K, Varvolgyi, T, Rozsa, D, Miko, E, Dezso, B, Veres, I, Mehes, G, Emri, G, Remenyik, E: Correlation among metallothionein expression, intratumoural macrophage infiltration and the risk of metastasis in human cutaneous malignant melanoma. *J Eur Acad Dermatol Venereol,* 27**:** e320-327, 2013.
27. Petrelli, F, Cabiddu, M, Coinu, A, Borgonovo, K, Ghilardi, M, Lonati, V, Barni, S: Prognostic role of lactate dehydrogenase in solid tumors: a systematic review and meta-analysis of 76 studies. *Acta Oncol,* 54**:** 961-970, 2015.
28. Karagiannis, P, Fittall, M, Karagiannis, SN: Evaluating biomarkers in melanoma. *Front Oncol,* 4**:** 383, 2014.
29. Mouawad, R, Spano, JP, Khayat, D: Old and new serological biomarkers in melanoma: where we are in 2009. *Melanoma Res,* 20**:** 67-76, 2010.
30. Gaynor, R, Irie, R, Morton, D, Herschman, HR: S100 protein is present in cultured human malignant melanomas. *Nature,* 286**:** 400-401, 1980.
31. Vereecken, P, Cornelis, F, Van Baren, N, Vandersleyen, V, Baurain, JF: A synopsis of serum biomarkers in cutaneous melanoma patients. *Dermatol Res Pract,* 2012**:** 260643, 2012.
32. Morton, DL, Thompson, JF, Cochran, AJ, Mozzillo, N, Nieweg, OE, Roses, DF, Hoekstra, HJ, Karakousis, CP, Puleo, CA, Coventry, BJ, Kashani-Sabet, M, Smithers, BM, Paul, E, Kraybill, WG, McKinnon, JG, Wang, HJ, Elashoff, R, Faries, MB: Final trial report of sentinel-node biopsy versus nodal observation in melanoma. *N Engl J Med,* 370**:** 599-609, 2014.
33. Corrie, P, Hategan, M, Fife, K, Parkinson, C: Management of melanoma. *Br Med Bull,* 111**:** 149-162, 2014.
34. Wang, M, Zhao, J, Zhang, L, Wei, F, Lian, Y, Wu, Y, Gong, Z, Zhang, S, Zhou, J, Cao, K, Li, X, Xiong, W, Li, G, Zeng, Z, Guo, C: Role of tumor microenvironment in tumorigenesis. *J Cancer,* 8**:** 761-773, 2017.
35. Ives, NJ, Suciu, S, Eggermont, AMM, Kirkwood, J, Lorigan, P, Markovic, SN, Garbe, C, Wheatley, K: Adjuvant interferon-alpha for the treatment of high-risk melanoma: An individual patient data meta-analysis. *Eur J Cancer,* 82**:** 171-183, 2017.

36. Eggermont, AM, Suciu, S, Rutkowski, P, Kruit, WH, Punt, CJ, Dummer, R, Sales, F, Keilholz, U, de Schaetzen, G, Testori, A: Long term follow up of the EORTC 18952 trial of adjuvant therapy in resected stage IIB-III cutaneous melanoma patients comparing intermediate doses of interferon-alpha-2b (IFN) with observation: Ulceration of primary is key determinant for IFN-sensitivity. *Eur J Cancer,* 55**:** 111-121, 2016.
37. Chapman, PB, Hauschild, A, Robert, C, Haanen, JB, Ascierto, P, Larkin, J, Dummer, R, Garbe, C, Testori, A, Maio, M, Hogg, D, Lorigan, P, Lebbe, C, Jouary, T, Schadendorf, D, Ribas, A, O'Day, SJ, Sosman, JA, Kirkwood, JM, Eggermont, AM, Dreno, B, Nolop, K, Li, J, Nelson, B, Hou, J, Lee, RJ, Flaherty, KT, McArthur, GA: Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med,* 364**:** 2507-2516, 2011.
38. Merlino, G, Herlyn, M, Fisher, DE, Bastian, BC, Flaherty, KT, Davies, MA, Wargo, JA, Curiel-Lewandrowski, C, Weber, MJ, Leachman, SA, Soengas, MS, McMahon, M, Harbour, JW, Swetter, SM, Aplin, AE, Atkins, MB, Bosenberg, MW, Dummer, R, Gershenwald, JE, Halpern, AC, Herlyn, D, Karakousis, GC, Kirkwood, JM, Krauthammer, M, Lo, RS, Long, GV, McArthur, G, Ribas, A, Schuchter, L, Sosman, JA, Smalley, KS, Steeg, P, Thomas, NE, Tsao, H, Tueting, T, Weeraratna, A, Xu, G, Lomax, R, Martin, A, Silverstein, S, Turnham, T, Ronai, ZA: The state of melanoma: challenges and opportunities. *Pigment Cell Melanoma Res,* 29**:** 404-416, 2016.
39. Wolchok, JD, Chiarion-Sileni, V, Gonzalez, R, Rutkowski, P, Grob, JJ, Cowey, CL, Lao, CD, Wagstaff, J, Schadendorf, D, Ferrucci, PF, Smylie, M, Dummer, R, Hill, A, Hogg, D, Haanen, J, Carlino, MS, Bechter, O, Maio, M, Marquez-Rodas, I, Guidoboni, M, McArthur, G, Lebbe, C, Ascierto, PA, Long, GV, Cebon, J, Sosman, J, Postow, MA, Callahan, MK, Walker, D, Rollin, L, Bhore, R, Hodi, FS, Larkin, J: Overall Survival with Combined Nivolumab and Ipilimumab in Advanced Melanoma. *N Engl J Med,* 377**:** 1345-1356, 2017.
40. Schadendorf, D, Hodi, FS, Robert, C, Weber, JS, Margolin, K, Hamid, O, Patt, D, Chen, TT, Berman, DM, Wolchok, JD: Pooled Analysis of Long-Term Survival Data From Phase II and Phase III Trials of Ipilimumab in Unresectable or Metastatic Melanoma. *J Clin Oncol,* 33**:** 1889-1894, 2015.
41. Pasquali, S, Hadjinicolaou, AV, Chiarion Sileni, V, Rossi, CR, Mocellin, S: Systemic treatments for metastatic cutaneous melanoma. *Cochrane Database Syst Rev,* 2**:** Cd011123, 2018.
42. Merelli, B, Massi, D, Cattaneo, L, Mandala, M: Targeting the PD1/PD-L1 axis in melanoma: biological rationale, clinical challenges and opportunities. *Crit Rev Oncol Hematol,* 89**:** 140-165, 2014.
43. Hoag, H: Drug development: a chance of survival. *Nature,* 515**:** S118-120, 2014.
44. Hui, L, Chen, Y: Tumor microenvironment: Sanctuary of the devil. *Cancer Lett,* 368**:** 7-13, 2015.
45. Yuan, Y, Jiang, YC, Sun, CK, Chen, QM: Role of the tumor microenvironment in tumor progression and the clinical applications (Review). *Oncol Rep,* 35**:** 2499-2515, 2016.
46. Zhou, L, Yang, K, Andl, T, Wickett, RR, Zhang, Y: Perspective of Targeting Cancer-Associated Fibroblasts in Melanoma. *J Cancer,* 6**:** 717-726, 2015.
47. Labonte, AC, Tosello-Trampont, AC, Hahn, YS: The role of macrophage polarization in infectious and inflammatory diseases. *Mol Cells,* 37**:** 275-285, 2014.

48. Wang, H, Yang, L, Wang, D, Zhang, Q, Zhang, L: Pro-tumor activities of macrophages in the progression of melanoma. *Hum Vaccin Immunother,* 13**:** 1556-1562, 2017.
49. Ziani, L, Chouaib, S, Thiery, J: Alteration of the Antitumor Immune Response by Cancer-Associated Fibroblasts. *Front Immunol,* 9**:** 414, 2018.
50. Kalluri, R: The biology and function of fibroblasts in cancer. *Nat Rev Cancer,* 16**:** 582-598, 2016.
51. Cocco, E, Bellone, S, El-Sahwi, K, Cargnelutti, M, Buza, N, Tavassoli, FA, Schwartz, PE, Rutherford, TJ, Pecorelli, S, Santin, AD: Serum amyloid A: a novel biomarker for endometrial cancer. *Cancer,* 116**:** 843-851, 2010.
52. LeBleu, VS, Kalluri, R: A peek into cancer-associated fibroblasts: origins, functions and translational impact. *Dis Model Mech,* 11, 2018.
53. Alkasalias, T, Moyano-Galceran, L, Arsenian-Henriksson, M, Lehti, K: Fibroblasts in the Tumor Microenvironment: Shield or Spear? *Int J Mol Sci,* 19, 2018.
54. Santi, A, Kugeratski, FG, Zanivan, S: Cancer Associated Fibroblasts: The Architects of Stroma Remodeling. *Proteomics,* 18**:** e1700167, 2018.
55. Evans, RA, Tian, YC, Steadman, R, Phillips, AO: TGF-beta1-mediated fibroblast-myofibroblast terminal differentiation-the role of Smad proteins. *Exp Cell Res,* 282**:** 90-100, 2003.
56. Nurmik, M, Ullmann, P, Rodriguez, F, Haan, S, Letellier, E: In search of definitions: Cancer-associated fibroblasts and their markers. *Int J Cancer*, 2019.
57. Kinugasa, Y, Matsui, T, Takakura, N: CD44 expressed on cancer-associated fibroblasts is a functional molecule supporting the stemness and drug resistance of malignant cancer cells in the tumor microenvironment. *Stem Cells,* 32**:** 145-156, 2014.
58. Li, G, Satyamoorthy, K, Herlyn, M: N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res,* 61**:** 3819-3825, 2001.
59. Jobe, NP, Rosel, D, Dvorankova, B, Kodet, O, Lacina, L, Mateu, R, Smetana, K, Brabek, J: Simultaneous blocking of IL-6 and IL-8 is sufficient to fully inhibit CAF-induced human melanoma cell invasiveness. *Histochem Cell Biol,* 146**:** 205-217, 2016.
60. McCarthy, JB, El-Ashry, D, Turley, EA: Hyaluronan, Cancer-Associated Fibroblasts and the Tumor Microenvironment in Malignant Progression. *Front Cell Dev Biol,* 6**:** 48, 2018.
61. Hawinkels, LJ, Paauwe, M, Verspaget, HW, Wiercinska, E, van der Zon, JM, van der Ploeg, K, Koelink, PJ, Lindeman, JH, Mesker, W, ten Dijke, P, Sier, CF: Interaction with colon cancer cells hyperactivates TGF-beta signaling in cancer-associated fibroblasts. *Oncogene,* 33**:** 97-107, 2014.
62. Leivonen, SK, Ala-Aho, R, Koli, K, Grenman, R, Peltonen, J, Kahari, VM: Activation of Smad signaling enhances collagenase-3 (MMP-13) expression and invasion of head and neck squamous carcinoma cells. *Oncogene,* 25**:** 2588-2600, 2006.
63. Prakash, M, Kale, S, Ghosh, I, Kundu, GC, Datta, K: Hyaluronan-binding protein 1 (HABP1/p32/gC1qR) induces melanoma cell migration and tumor growth by NF-kappa B dependent MMP-2 activation through integrin alpha(v)beta(3) interaction. *Cell Signal,* 23**:** 1563-1577, 2011.
64. Berking, C, Takemoto, R, Schaider, H, Showe, L, Satyamoorthy, K, Robbins, P, Herlyn, M: Transforming growth factor-beta1 increases survival of human melanoma through stroma remodeling. *Cancer Res,* 61**:** 8306-8316, 2001.

65. Meierjohann, S, Hufnagel, A, Wende, E, Kleinschmidt, MA, Wolf, K, Friedl, P, Gaubatz, S, Schartl, M: MMP13 mediates cell cycle progression in melanocytes and melanoma cells: in vitro studies of migration and proliferation. *Mol Cancer,* 9**:** 201, 2010.
66. Ziani, L, Safta-Saadoun, TB, Gourbeix, J, Cavalcanti, A, Robert, C, Favre, G, Chouaib, S, Thiery, J: Melanoma-associated fibroblasts decrease tumor cell susceptibility to NK cell-mediated killing through matrix-metalloproteinases secretion. *Oncotarget,* 8**:** 19780-19794, 2017.
67. Hirata, E, Girotti, MR, Viros, A, Hooper, S, Spencer-Dene, B, Matsuda, M, Larkin, J, Marais, R, Sahai, E: Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin beta1/FAK signaling. *Cancer Cell,* 27**:** 574-588, 2015.
68. Zhao, F, Evans, K, Xiao, C, DeVito, N, Theivanthiran, B, Holtzhausen, A, Siska, PJ, Blobe, GC, Hanks, BA: Stromal Fibroblasts Mediate Anti-PD-1 Resistance via MMP-9 and Dictate TGFbeta Inhibitor Sequencing in Melanoma. *Cancer Immunol Res,* 6**:** 1459-1471, 2018.
69. Fujii, N, Shomori, K, Shiomi, T, Nakabayashi, M, Takeda, C, Ryoke, K, Ito, H: Cancer-associated fibroblasts and CD163-positive macrophages in oral squamous cell carcinoma: their clinicopathological and prognostic significance. *J Oral Pathol Med,* 41**:** 444-451, 2012.
70. Tommelein, J, Verset, L, Boterberg, T, Demetter, P, Bracke, M, De Wever, O: Cancer-associated fibroblasts connect metastasis-promoting communication in colorectal cancer. *Front Oncol,* 5**:** 63, 2015.
71. Orimo, A, Gupta, PB, Sgroi, DC, Arenzana-Seisdedos, F, Delaunay, T, Naeem, R, Carey, VJ, Richardson, AL, Weinberg, RA: Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell,* 121**:** 335-348, 2005.
72. Hutchenreuther, J, Vincent, K, Norley, C, Racanelli, M, Gruber, SB, Johnson, TM, Fullen, DR, Raskin, L, Perbal, B, Holdsworth, DW, Postovit, LM, Leask, A: Activation of cancer-associated fibroblasts is required for tumor neovascularization in a murine model of melanoma. *Matrix Biol*, 2018.
73. Shapouri-Moghaddam, A, Mohammadian, S, Vazini, H, Taghadosi, M, Esmaeili, SA, Mardani, F, Seifi, B, Mohammadi, A, Afshari, JT, Sahebkar, A: Macrophage plasticity, polarization, and function in health and disease. *J Cell Physiol,* 233**:** 6425-6440, 2018.
74. Martinez, FO, Gordon, S: The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep,* 6**:** 13, 2014.
75. Chanmee, T, Ontong, P, Konno, K, Itano, N: Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers (Basel),* 6**:** 1670-1690, 2014.
76. Hao, NB, Lu, MH, Fan, YH, Cao, YL, Zhang, ZR, Yang, SM: Macrophages in tumor microenvironments and the progression of tumors. *Clin Dev Immunol,* 2012**:** 948098, 2012.
77. Chavez-Galan, L, Olleros, ML, Vesin, D, Garcia, I: Much More than M1 and M2 Macrophages, There are also CD169(+) and TCR(+) Macrophages. *Front Immunol,* 6**:** 263, 2015.
78. Lewis, CE, Harney, AS, Pollard, JW: The Multifaceted Role of Perivascular Macrophages in Tumors. *Cancer Cell,* 30**:** 18-25, 2016.
79. Pieniazek, M, Matkowski, R, Donizy, P: Macrophages in skin melanoma-the key element in melanomagenesis. *Oncol Lett,* 15**:** 5399-5404, 2018.

80. Belgiovine, C, D'Incalci, M, Allavena, P, Frapolli, R: Tumor-associated macrophages and anti-tumor therapies: complex links. *Cell Mol Life Sci,* 73**:** 2411-2424, 2016.
81. Yang, L, Zhang, Y: Tumor-associated macrophages: from basic research to clinical application. *J Hematol Oncol,* 10**:** 58, 2017.
82. Young, HL, Rowling, EJ, Bugatti, M, Giurisato, E, Luheshi, N, Arozarena, I, Acosta, JC, Kamarashev, J, Frederick, DT, Cooper, ZA, Reuben, A, Gil, J, Flaherty, KT, Wargo, JA, Vermi, W, Smith, MP, Wellbrock, C, Hurlstone, A: An adaptive signaling network in melanoma inflammatory niches confers tolerance to MAPK signaling inhibition. *J Exp Med,* 214**:** 1691-1710, 2017.
83. Erdag, G, Schaefer, JT, Smolkin, ME, Deacon, DH, Shea, SM, Dengel, LT, Patterson, JW, Slingluff, CL, Jr.: Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res,* 72**:** 1070-1080, 2012.
84. Roszer, T: Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators Inflamm,* 2015**:** 816460, 2015.
85. Zhang, J, Yan, Y, Yang, Y, Wang, L, Li, M, Wang, J, Liu, X, Duan, X, Wang, J: High Infiltration of Tumor-Associated Macrophages Influences Poor Prognosis in Human Gastric Cancer Patients, Associates With the Phenomenon of EMT. *Medicine (Baltimore),* 95**:** e2636, 2016.
86. He, KF, Zhang, L, Huang, CF, Ma, SR, Wang, YF, Wang, WM, Zhao, ZL, Liu, B, Zhao, YF, Zhang, WF, Sun, ZJ: CD163+ tumor-associated macrophages correlated with poor prognosis and cancer stem cells in oral squamous cell carcinoma. *Biomed Res Int,* 2014**:** 838632, 2014.
87. Zhang, BC, Gao, J, Wang, J, Rao, ZG, Wang, BC, Gao, JF: Tumor-associated macrophages infiltration is associated with peritumoral lymphangiogenesis and poor prognosis in lung adenocarcinoma. *Med Oncol,* 28**:** 1447-1452, 2011.
88. Sousa, S, Brion, R, Lintunen, M, Kronqvist, P, Sandholm, J, Monkkonen, J, Kellokumpu-Lehtinen, PL, Lauttia, S, Tynninen, O, Joensuu, H, Heymann, D, Maatta, JA: Human breast cancer cells educate macrophages toward the M2 activation status. *Breast Cancer Res,* 17**:** 101, 2015.
89. Falleni, M, Savi, F, Tosi, D, Agape, E, Cerri, A, Moneghini, L, Bulfamante, GP: M1 and M2 macrophages' clinicopathological significance in cutaneous melanoma. *Melanoma Res,* 27**:** 200-210, 2017.
90. Zaidi, MR, Davis, S, Noonan, FP, Graff-Cherry, C, Hawley, TS, Walker, RL, Feigenbaum, L, Fuchs, E, Lyakh, L, Young, HA, Hornyak, TJ, Arnheiter, H, Trinchieri, G, Meltzer, PS, De Fabo, EC, Merlino, G: Interferon-gamma links ultraviolet radiation to melanomagenesis in mice. *Nature,* 469**:** 548-553, 2011.
91. Wang, T, Xiao, M, Ge, Y, Krepler, C, Belser, E, Lopez-Coral, A, Xu, X, Zhang, G, Azuma, R, Liu, Q, Liu, R, Li, L, Amaravadi, RK, Xu, W, Karakousis, G, Gangadhar, TC, Schuchter, LM, Lieu, M, Khare, S, Halloran, MB, Herlyn, M, Kaufman, RE: BRAF Inhibition Stimulates Melanoma-Associated Macrophages to Drive Tumor Growth. *Clin Cancer Res,* 21**:** 1652-1664, 2015.
92. Tham, M, Tan, KW, Keeble, J, Wang, X, Hubert, S, Barron, L, Tan, NS, Kato, M, Prevost-Blondel, A, Angeli, V, Abastado, JP: Melanoma-initiating cells exploit M2 macrophage TGFbeta and arginase pathway for survival and proliferation. *Oncotarget,* 5**:** 12027-12042, 2014.
93. Fujimura, T, Kambayashi, Y, Fujisawa, Y, Hidaka, T, Aiba, S: Tumor-Associated Macrophages: Therapeutic Targets for Skin Cancer. *Front Oncol,* 8**:** 3, 2018.

94. Sleeman, JP: The metastatic niche and stromal progression. *Cancer Metastasis Rev,* 31**:** 429-440, 2012.
95. Krasagakis, K, Tholke, D, Farthmann, B, Eberle, J, Mansmann, U, Orfanos, CE: Elevated plasma levels of transforming growth factor (TGF)-beta1 and TGF-beta2 in patients with disseminated malignant melanoma. *Br J Cancer,* 77**:** 1492-1494, 1998.
96. Findeisen, P, Zapatka, M, Peccerella, T, Matzk, H, Neumaier, M, Schadendorf, D, Ugurel, S: Serum amyloid A as a prognostic marker in melanoma identified by proteomic profiling. *J Clin Oncol,* 27**:** 2199-2208, 2009.
97. Kajdaniuk, D, Marek, B, Borgiel-Marek, H, Kos-Kudla, B: Transforming growth factor beta1 (TGFbeta1) in physiology and pathology. *Endokrynol Pol,* 64**:** 384-396, 2013.
98. Massague, J: TGFbeta signalling in context. *Nat Rev Mol Cell Biol,* 13**:** 616-630, 2012.
99. Jullien, P, Berg, TM, Lawrence, DA: Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF. *Int J Cancer,* 43**:** 886-891, 1989.
100. Miyazono, K, Katsuno, Y, Koinuma, D, Ehata, S, Morikawa, M: Intracellular and extracellular TGF-beta signaling in cancer: some recent topics. *Front Med*, 2018.
101. Nakao, A, Afrakhte, M, Moren, A, Nakayama, T, Christian, JL, Heuchel, R, Itoh, S, Kawabata, M, Heldin, NE, Heldin, CH, ten Dijke, P: Identification of Smad7, a TGFbeta-inducible antagonist of TGF-beta signalling. *Nature,* 389**:** 631-635, 1997.
102. Furler, RL, Nixon, DF, Brantner, CA, Popratiloff, A, Uittenbogaart, CH: TGF-beta Sustains Tumor Progression through Biochemical and Mechanical Signal Transduction. *Cancers (Basel),* 10, 2018.
103. Hata, A, Chen, YG: TGF-beta Signaling from Receptors to Smads. *Cold Spring Harb Perspect Biol,* 8, 2016.
104. Caja, L, Dituri, F, Mancarella, S, Caballero-Diaz, D, Moustakas, A, Giannelli, G, Fabregat, I: TGF-beta and the Tissue Microenvironment: Relevance in Fibrosis and Cancer. *Int J Mol Sci,* 19, 2018.
105. Moustakas, A, Heldin, CH: Mechanisms of TGFbeta-Induced Epithelial-Mesenchymal Transition. *J Clin Med,* 5, 2016.
106. Bellomo, C, Caja, L, Moustakas, A: Transforming growth factor beta as regulator of cancer stemness and metastasis. *Br J Cancer*, 2016.
107. Huang, JJ, Blobe, GC: Dichotomous roles of TGF-beta in human cancer. *Biochem Soc Trans,* 44**:** 1441-1454, 2016.
108. Bruna, A, Darken, RS, Rojo, F, Ocana, A, Penuelas, S, Arias, A, Paris, R, Tortosa, A, Mora, J, Baselga, J, Seoane, J: High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. *Cancer Cell,* 11**:** 147-160, 2007.
109. Lampropoulos, P, Zizi-Sermpetzoglou, A, Rizos, S, Kostakis, A, Nikiteas, N, Papavassiliou, AG: Prognostic significance of transforming growth factor beta (TGF-beta) signaling axis molecules and E-cadherin in colorectal cancer. *Tumour Biol,* 33**:** 1005-1014, 2012.
110. Zhao, J, Liang, Y, Yin, Q, Liu, S, Wang, Q, Tang, Y, Cao, C: Clinical and prognostic significance of serum transforming growth factor-beta1 levels in patients with pancreatic ductal adenocarcinoma. *Braz J Med Biol Res,* 49, 2016.

111. Hou, YL, Chen, H, Dong, ZH, Xue, CJ, Wu, YF, Luo, HX, Wu, YC, Li, CH: Clinical significance of serum transforming growth factor-beta1 in lung cancer. *Cancer Epidemiol,* 37**:** 750-753, 2013.
112. Tang, MR, Wang, YX, Guo, S, Han, SY, Li, HH, Jin, SF: Prognostic significance of in situ and plasma levels of transforming growth factor beta1, -2 and -3 in cutaneous melanoma. *Mol Med Rep,* 11**:** 4508-4512, 2015.
113. Datto, MB, Li, Y, Panus, JF, Howe, DJ, Xiong, Y, Wang, XF: Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. *Proc Natl Acad Sci U S A,* 92**:** 5545-5549, 1995.
114. Elliott, RL, Blobe, GC: Role of transforming growth factor Beta in human cancer. *J Clin Oncol,* 23**:** 2078-2093, 2005.
115. Siegel, PM, Massague, J: Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. *Nat Rev Cancer,* 3**:** 807-821, 2003.
116. Lasfar, A, Cohen-Solal, KA: Resistance to transforming growth factor beta-mediated tumor suppression in melanoma: are multiple mechanisms in place? *Carcinogenesis,* 31**:** 1710-1717, 2010.
117. Krasagakis, K, Kruger-Krasagakes, S, Fimmel, S, Eberle, J, Tholke, D, von der Ohe, M, Mansmann, U, Orfanos, CE: Desensitization of melanoma cells to autocrine TGF-beta isoforms. *J Cell Physiol,* 178**:** 179-187, 1999.
118. Dewing, D, Emmett, M, Pritchard Jones, R: The Roles of Angiogenesis in Malignant Melanoma: Trends in Basic Science Research over the Last 100 Years. *ISRN Oncol,* 2012**:** 546927, 2012.
119. Cantelli, G, Orgaz, JL, Rodriguez-Hernandez, I, Karagiannis, P, Maiques, O, Matias-Guiu, X, Nestle, FO, Marti, RM, Karagiannis, SN, Sanz-Moreno, V: TGF-beta-Induced Transcription Sustains Amoeboid Melanoma Migration and Dissemination. *Curr Biol,* 25**:** 2899-2914, 2015.
120. Wilson, EB, El-Jawhari, JJ, Neilson, AL, Hall, GD, Melcher, AA, Meade, JL, Cook, GP: Human tumour immune evasion via TGF-beta blocks NK cell activation but not survival allowing therapeutic restoration of anti-tumour activity. *PLoS One,* 6**:** e22842, 2011.
121. Hadaschik, EN, Enk, AH: TGF-beta1-induced regulatory T cells. *Hum Immunol,* 76**:** 561-564, 2015.
122. McCarter, MD, Baumgartner, J, Escobar, GA, Richter, D, Lewis, K, Robinson, W, Wilson, C, Palmer, BE, Gonzalez, R: Immunosuppressive dendritic and regulatory T cells are upregulated in melanoma patients. *Ann Surg Oncol,* 14**:** 2854-2860, 2007.
123. Gong, D, Shi, W, Yi, SJ, Chen, H, Groffen, J, Heisterkamp, N: TGFbeta signaling plays a critical role in promoting alternative macrophage activation. *BMC Immunol,* 13**:** 31, 2012.
124. De Buck, M, Gouwy, M, Wang, JM, Van Snick, J, Opdenakker, G, Struyf, S, Van Damme, J: Structure and Expression of Different Serum Amyloid A (SAA) Variants and their Concentration-Dependent Functions During Host Insults. *Curr Med Chem,* 23**:** 1725-1755, 2016.
125. Kluve-Beckerman, B, Drumm, ML, Benson, MD: Nonexpression of the human serum amyloid A three (SAA3) gene. *DNA Cell Biol,* 10**:** 651-661, 1991.
126. Tannock, LR, De Beer, MC, Ji, A, Shridas, P, Noffsinger, VP, den Hartigh, L, Chait, A, De Beer, FC, Webb, NR: Serum amyloid A3 is a high density lipoprotein-associated acute-phase protein. *J Lipid Res,* 59**:** 339-347, 2018.
127. Whitehead, AS, de Beer, MC, Steel, DM, Rits, M, Lelias, JM, Lane, WS, de Beer, FC: Identification of novel members of the serum amyloid A protein

superfamily as constitutive apolipoproteins of high density lipoprotein. *J Biol Chem,* 267**:** 3862-3867, 1992.
128. Dong, Z, Wu, T, Qin, W, An, C, Wang, Z, Zhang, M, Zhang, Y, Zhang, C, An, F: Serum amyloid A directly accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Mol Med,* 17**:** 1357-1364, 2011.
129. Sun, L, Ye, RD: Serum amyloid A1: Structure, function and gene polymorphism. *Gene,* 583**:** 48-57, 2016.
130. Benditt, EP, Eriksen, N: Amyloid protein SAA is associated with high density lipoprotein from human serum. *Proc Natl Acad Sci U S A,* 74**:** 4025-4028, 1977.
131. Ye, RD, Sun, L: Emerging functions of serum amyloid A in inflammation. *J Leukoc Biol,* 98**:** 923-929, 2015.
132. Malle, E, Sodin-Semrl, S, Kovacevic, A: Serum amyloid A: an acute-phase protein involved in tumour pathogenesis. *Cell Mol Life Sci,* 66**:** 9-26, 2009.
133. Butcher, MJ, Galkina, EV: wRAPping up early monocyte and neutrophil recruitment in atherogenesis via Annexin A1/FPR2 signaling. *Circ Res,* 116**:** 774-777, 2015.
134. Li, W, Wang, W, Zuo, R, Liu, C, Shu, Q, Ying, H, Sun, K: Induction of pro-inflammatory genes by serum amyloid A1 in human amnion fibroblasts. *Sci Rep,* 7**:** 693, 2017.
135. De Buck, M, Gouwy, M, Wang, JM, Van Snick, J, Proost, P, Struyf, S, Van Damme, J: The cytokine-serum amyloid A-chemokine network. *Cytokine Growth Factor Rev,* 30**:** 55-69, 2016.
136. Gouwy, M, De Buck, M, Portner, N, Opdenakker, G, Proost, P, Struyf, S, Van Damme, J: Serum amyloid A chemoattracts immature dendritic cells and indirectly provokes monocyte chemotaxis by induction of cooperating CC and CXC chemokines. *Eur J Immunol,* 45**:** 101-112, 2015.
137. Lee, HY, Kim, MK, Park, KS, Bae, YH, Yun, J, Park, JI, Kwak, JY, Bae, YS: Serum amyloid A stimulates matrix-metalloproteinase-9 upregulation via formyl peptide receptor like-1-mediated signaling in human monocytic cells. *Biochem Biophys Res Commun,* 330**:** 989-998, 2005.
138. Vaure, C, Liu, Y: A comparative review of toll-like receptor 4 expression and functionality in different animal species. *Front Immunol,* 5**:** 316, 2014.
139. Flo, TH, Halaas, O, Torp, S, Ryan, L, Lien, E, Dybdahl, B, Sundan, A, Espevik, T: Differential expression of Toll-like receptor 2 in human cells. *J Leukoc Biol,* 69**:** 474-481, 2001.
140. Bell, E: TLR4 signalling. *Nat,* 8**:** 241, 2008.
141. Kawai, T, Akira, S: The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol,* 11**:** 373-384, 2010.
142. Sun, L, Zhou, H, Zhu, Z, Yan, Q, Wang, L, Liang, Q, Ye, RD: Ex vivo and in vitro effect of serum amyloid a in the induction of macrophage M2 markers and efferocytosis of apoptotic neutrophils. *J Immunol,* 194**:** 4891-4900, 2015.
143. de Seny, D, Cobraiville, G, Charlier, E, Neuville, S, Esser, N, Malaise, D, Malaise, O, Calvo, FQ, Relic, B, Malaise, MG: Acute-phase serum amyloid a in osteoarthritis: regulatory mechanism and proinflammatory properties. *PLoS One,* 8**:** e66769, 2013.
144. Moshkovskii, SA: Why do cancer cells produce serum amyloid A acute-phase protein? *Biochemistry (Mosc),* 77**:** 339-341, 2012.
145. Torres, S, Bartolome, RA, Mendes, M, Barderas, R, Fernandez-Acenero, MJ, Pelaez-Garcia, A, Pena, C, Lopez-Lucendo, M, Villar-Vazquez, R, de Herreros, AG, Bonilla, F, Casal, JI: Proteome profiling of cancer-associated

fibroblasts identifies novel proinflammatory signatures and prognostic markers for colorectal cancer. *Clin Cancer Res,* 19**:** 6006-6019, 2013.
146. Djurec, M, Grana, O, Lee, A, Troule, K, Espinet, E, Cabras, L, Navas, C, Blasco, MT, Martin-Diaz, L, Burdiel, M, Li, J, Liu, Z, Vallespinos, M, Sanchez-Bueno, F, Sprick, MR, Trumpp, A, Sainz, B, Jr., Al-Shahrour, F, Rabadan, R, Guerra, C, Barbacid, M: Saa3 is a key mediator of the protumorigenic properties of cancer-associated fibroblasts in pancreatic tumors. *Proc Natl Acad Sci U S A,* 115**:** E1147-e1156, 2018.
147. Urieli-Shoval, S, Meek, RL, Hanson, RH, Eriksen, N, Benditt, EP: Human serum amyloid A genes are expressed in monocyte/macrophage cell lines. *Am J Pathol,* 145**:** 650-660, 1994.
148. Yang, M, Liu, F, Higuchi, K, Sawashita, J, Fu, X, Zhang, L, Zhang, L, Fu, L, Tong, Z, Higuchi, K: Serum amyloid A expression in the breast cancer tissue is associated with poor prognosis. *Oncotarget,* 7**:** 35843-35852, 2016.
149. De Santo, C, Arscott, R, Booth, S, Karydis, I, Jones, M, Asher, R, Salio, M, Middleton, M, Cerundolo, V: Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol,* 11**:** 1039-1046, 2010.
150. Sung, HJ, Ahn, JM, Yoon, YH, Rhim, TY, Park, CS, Park, JY, Lee, SY, Kim, JW, Cho, JY: Identification and validation of SAA as a potential lung cancer biomarker and its involvement in metastatic pathogenesis of lung cancer. *J Proteome Res,* 10**:** 1383-1395, 2011.
151. Urieli-Shoval, S, Finci-Yeheskel, Z, Dishon, S, Galinsky, D, Linke, RP, Ariel, I, Levin, M, Ben-Shachar, I, Prus, D: Expression of serum amyloid a in human ovarian epithelial tumors: implication for a role in ovarian tumorigenesis. *J Histochem Cytochem,* 58**:** 1015-1023, 2010.
152. Wang, JY, Zheng, YZ, Yang, J, Lin, YH, Dai, SQ, Zhang, G, Liu, WL: Elevated levels of serum amyloid A indicate poor prognosis in patients with esophageal squamous cell carcinoma. *BMC Cancer,* 12**:** 365, 2012.
153. Lee, JM, Kim, EK, Seo, H, Jeon, I, Chae, MJ, Park, YJ, Song, B, Kim, YS, Kim, YJ, Ko, HJ, Kang, CY: Serum amyloid A3 exacerbates cancer by enhancing the suppressive capacity of myeloid-derived suppressor cells via TLR2-dependent STAT3 activation. *Eur J Immunol,* 44**:** 1672-1684, 2014.
154. Nguyen, KD, Macaubas, C, Truong, P, Wang, N, Hou, T, Yoon, T, Mellins, ED: Serum amyloid A induces mitogenic signals in regulatory T cells via monocyte activation. *Mol Immunol,* 59**:** 172-179, 2014.
155. Hansen, MT, Forst, B, Cremers, N, Quagliata, L, Ambartsumian, N, Grum-Schwensen, B, Klingelhofer, J, Abdul-Al, A, Herrmann, P, Osterland, M, Stein, U, Nielsen, GH, Scherer, PE, Lukanidin, E, Sleeman, JP, Grigorian, M: A link between inflammation and metastasis: serum amyloid A1 and A3 induce metastasis, and are targets of metastasis-inducing S100A4. *Oncogene,* 34**:** 424-435, 2015.
156. Herskind, C, Bentzen, SM, Overgaard, J, Overgaard, M, Bamberg, M, Rodemann, HP: Differentiation state of skin fibroblast cultures versus risk of subcutaneous fibrosis after radiotherapy. *Radiother Oncol,* 47**:** 263-269, 1998.
157. Giard, DJ, Aaronson, SA, Todaro, GJ, Arnstein, P, Kersey, JH, Dosik, H, Parks, WP: In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst,* 51**:** 1417-1423, 1973.
158. Chen, TR, Shaw, MW: Stable chromosome changes in human malignant melanoma. *Cancer Res,* 33**:** 2042-2047, 1973.

159. Herlyn, M, Balaban, G, Bennicelli, J, Guerry, Dt, Halaban, R, Herlyn, D, Elder, DE, Maul, GG, Steplewski, Z, Nowell, PC, et al.: Primary melanoma cells of the vertical growth phase: similarities to metastatic cells. *J Natl Cancer Inst,* 74**:** 283-289, 1985.
160. Deschodt-Lanckman, M, Vanneste, Y, Loir, B, Michel, A, Libert, A, Ghanem, G, Lejeune, F: Degradation of alpha-melanocyte stimulating hormone (alpha-MSH) by CALLA/endopeptidase 24.11 expressed by human melanoma cells in culture. *Int J Cancer,* 46**:** 1124-1130, 1990.
161. Fogh, J, Fogh, JM, Orfeo, T: One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. *J Natl Cancer Inst,* 59**:** 221-226, 1977.
162. Tsuchiya, S, Yamabe, M, Yamaguchi, Y, Kobayashi, Y, Konno, T, Tada, K: Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer,* 26**:** 171-176, 1980.
163. Baranova, IN, Bocharov, AV, Vishnyakova, TG, Kurlander, R, Chen, Z, Fu, D, Arias, IM, Csako, G, Patterson, AP, Eggerman, TL: CD36 is a novel serum amyloid A (SAA) receptor mediating SAA binding and SAA-induced signaling in human and rodent cells. *J Biol Chem,* 285**:** 8492-8506, 2010.
164. Lee, HY, Kim, SD, Shim, JW, Lee, SY, Lee, H, Cho, KH, Yun, J, Bae, YS: Serum amyloid A induces CCL2 production via formyl peptide receptor-like 1-mediated signaling in human monocytes. *J Immunol,* 181**:** 4332-4339, 2008.
165. O'Reilly, S, Cant, R, Ciechomska, M, Finnigan, J, Oakley, F, Hambleton, S, van Laar, JM: Serum amyloid A induces interleukin-6 in dermal fibroblasts via Toll-like receptor 2, interleukin-1 receptor-associated kinase 4 and nuclear factor-kappaB. *Immunology,* 143**:** 331-340, 2014.
166. Morizane, S, Kajita, A, Mizuno, K, Takiguchi, T, Iwatsuki, K: Toll-like receptor signalling induces the expression of serum amyloid A in epidermal keratinocytes and dermal fibroblasts. *Clin Exp Dermatol,* 44**:** 40-46, 2019.
167. Barros, MH, Hauck, F, Dreyer, JH, Kempkes, B, Niedobitek, G: Macrophage polarisation: an immunohistochemical approach for identifying M1 and M2 macrophages. *PLoS One,* 8**:** e80908, 2013.
168. Gao, J, Aksoy, BA, Dogrusoz, U, Dresdner, G, Gross, B, Sumer, SO, Sun, Y, Jacobsen, A, Sinha, R, Larsson, E, Cerami, E, Sander, C, Schultz, N: Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal,* 6**:** pl1, 2013.
169. Cerami, E, Gao, J, Dogrusoz, U, Gross, BE, Sumer, SO, Aksoy, BA, Jacobsen, A, Byrne, CJ, Heuer, ML, Larsson, E, Antipin, Y, Reva, B, Goldberg, AP, Sander, C, Schultz, N: The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov,* 2**:** 401-404, 2012.
170. Hildenbrand, R, Schaaf, A: The urokinase-system in tumor tissue stroma of the breast and breast cancer cell invasion. *Int J Oncol,* 34**:** 15-23, 2009.
171. Datta, PK, Blake, MC, Moses, HL: Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-beta -induced physical and functional interactions between smads and Sp1. *J Biol Chem,* 275**:** 40014-40019, 2000.
172. Zheng, D, Chen, H, Davids, J, Bryant, M, Lucas, A: Serpins for diagnosis and therapy in cancer. *Cardiovasc Hematol Disord Drug Targets,* 13**:** 123-132, 2013.

173. Duffy, MJ, McGowan, PM, Harbeck, N, Thomssen, C, Schmitt, M: uPA and PAI-1 as biomarkers in breast cancer: validated for clinical use in level-of-evidence-1 studies. *Breast Cancer Res,* 16**:** 428, 2014.
174. Masuda, T, Hattori, N, Senoo, T, Akita, S, Ishikawa, N, Fujitaka, K, Haruta, Y, Murai, H, Kohno, N: SK-216, an inhibitor of plasminogen activator inhibitor-1, limits tumor progression and angiogenesis. *Mol Cancer Ther,* 12**:** 2378-2388, 2013.
175. Thapa, B, Koo, BH, Kim, YH, Kwon, HJ, Kim, DS: Plasminogen activator inhibitor-1 regulates infiltration of macrophages into melanoma via phosphorylation of FAK-Tyr(9)(2)(5). *Biochem Biophys Res Commun,* 450**:** 1696-1701, 2014.
176. Klein, RM, Bernstein, D, Higgins, SP, Higgins, CE, Higgins, PJ: SERPINE1 expression discriminates site-specific metastasis in human melanoma. *Exp Dermatol,* 21**:** 551-554, 2012.
177. Nakagawa, H, Liyanarachchi, S, Davuluri, RV, Auer, H, Martin, EW, Jr., de la Chapelle, A, Frankel, WL: Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene,* 23**:** 7366-7377, 2004.
178. Lin, RY, Sullivan, KM, Argenta, PA, Meuli, M, Lorenz, HP, Adzick, NS: Exogenous transforming growth factor-beta amplifies its own expression and induces scar formation in a model of human fetal skin repair. *Ann Surg,* 222**:** 146-154, 1995.
179. Liu, Z, Kuang, W, Zhou, Q, Zhang, Y: TGF-beta1 secreted by M2 phenotype macrophages enhances the stemness and migration of glioma cells via the SMAD2/3 signalling pathway. *Int J Mol Med,* 42**:** 3395-3403, 2018.
180. Song, C, Hsu, K, Yamen, E, Yan, W, Fock, J, Witting, PK, Geczy, CL, Freedman, SB: Serum amyloid A induction of cytokines in monocytes/macrophages and lymphocytes. *Atherosclerosis,* 207**:** 374-383, 2009.
181. Lewis, AM, Varghese, S, Xu, H, Alexander, HR: Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. *J Transl Med,* 4**:** 48, 2006.
182. Waugh, DJ, Wilson, C: The interleukin-8 pathway in cancer. *Clin Cancer Res,* 14**:** 6735-6741, 2008.
183. Sanmamed, MF, Perez-Gracia, JL, Schalper, KA, Fusco, JP, Gonzalez, A, Rodriguez-Ruiz, ME, Onate, C, Perez, G, Alfaro, C, Martin-Algarra, S, Andueza, MP, Gurpide, A, Morgado, M, Wang, J, Bacchiocchi, A, Halaban, R, Kluger, H, Chen, L, Sznol, M, Melero, I: Changes in serum interleukin-8 (IL-8) levels reflect and predict response to anti-PD-1 treatment in melanoma and non-small-cell lung cancer patients. *Ann Oncol,* 28**:** 1988-1995, 2017.
184. Farina, AR, Mackay, AR: Gelatinase B/MMP-9 in Tumour Pathogenesis and Progression. *Cancers (Basel),* 6**:** 240-296, 2014.
185. Ballin, M, Gomez, DE, Sinha, CC, Thorgeirsson, UP: Ras oncogene mediated induction of a 92 kDa metalloproteinase; strong correlation with the malignant phenotype. *Biochem Biophys Res Commun,* 154**:** 832-838, 1988.
186. Huang, H: Matrix Metalloproteinase-9 (MMP-9) as a Cancer Biomarker and MMP-9 Biosensors: Recent Advances. *Sensors (Basel),* 18, 2018.
187. MohanKumar, K, Namachivayam, K, Chapalamadugu, KC, Garzon, SA, Premkumar, MH, Tipparaju, SM, Maheshwari, A: Smad7 interrupts TGF-beta signaling in intestinal macrophages and promotes inflammatory activation of these cells during necrotizing enterocolitis. *Pediatr Res,* 79**:** 951-961, 2016.

188. Bettum, IJ, Vasiliauskaite, K, Nygaard, V, Clancy, T, Pettersen, SJ, Tenstad, E, Maelandsmo, GM, Prasmickaite, L: Metastasis-associated protein S100A4 induces a network of inflammatory cytokines that activate stromal cells to acquire pro-tumorigenic properties. *Cancer Lett,* 344**:** 28-39, 2014.
189. Lukanidin, E, Sleeman, JP: Building the niche: the role of the S100 proteins in metastatic growth. *Semin Cancer Biol,* 22**:** 216-225, 2012.
190. Hiratsuka, S, Watanabe, A, Sakurai, Y, Akashi-Takamura, S, Ishibashi, S, Miyake, K, Shibuya, M, Akira, S, Aburatani, H, Maru, Y: The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat Cell Biol,* 10**:** 1349-1355, 2008.
191. Baay, M, Brouwer, A, Pauwels, P, Peeters, M, Lardon, F: Tumor cells and tumor-associated macrophages: secreted proteins as potential targets for therapy. *Clin Dev Immunol,* 2011**:** 565187, 2011.
192. Mantovani, A, Biswas, SK, Galdiero, MR, Sica, A, Locati, M: Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol,* 229**:** 176-185, 2013.
193. Anthony, D, McQualter, JL, Bishara, M, Lim, EX, Yatmaz, S, Seow, HJ, Hansen, M, Thompson, M, Hamilton, JA, Irving, LB, Levy, BD, Vlahos, R, Anderson, GP, Bozinovski, S: SAA drives proinflammatory heterotypic macrophage differentiation in the lung via CSF-1R-dependent signaling. *FASEB J,* 28**:** 3867-3877, 2014.
194. Scali, E, Mignogna, C, Di Vito, A, Presta, I, Camastra, C, Donato, G, Bottoni, U: Inflammation and macrophage polarization in cutaneous melanoma: Histopathological and immunohistochemical study. *Int J Immunopathol Pharmacol,* 29**:** 715-719, 2016.
195. Schwende, H, Fitzke, E, Ambs, P, Dieter, P: Differences in the state of differentiation of THP-1 cells induced by phorbol ester and 1,25-dihydroxyvitamin D3. *J Leukoc Biol,* 59**:** 555-561, 1996.
196. Chanput, W, Mes, JJ, Wichers, HJ: THP-1 cell line: an in vitro cell model for immune modulation approach. *Int Immunopharmacol,* 23**:** 37-45, 2014.
197. Maess, MB, Wittig, B, Cignarella, A, Lorkowski, S: Reduced PMA enhances the responsiveness of transfected THP-1 macrophages to polarizing stimuli. *J Immunol Methods,* 402**:** 76-81, 2014.
198. Liu, WS, Heckman, CA: The sevenfold way of PKC regulation. *Cell Signal,* 10**:** 529-542, 1998.
199. Takashiba, S, Van Dyke, TE, Amar, S, Murayama, Y, Soskolne, AW, Shapira, L: Differentiation of monocytes to macrophages primes cells for lipopolysaccharide stimulation via accumulation of cytoplasmic nuclear factor kappaB. *Infect Immun,* 67**:** 5573-5578, 1999.
200. Auwerx, J: The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia,* 47**:** 22-31, 1991.
201. Diaz-Valdes, N, Basagoiti, M, Dotor, J, Aranda, F, Monreal, I, Riezu-Boj, JI, Borras-Cuesta, F, Sarobe, P, Feijoo, E: Induction of monocyte chemoattractant protein-1 and interleukin-10 by TGFbeta1 in melanoma enhances tumor infiltration and immunosuppression. *Cancer Res,* 71**:** 812-821, 2011.
202. Krstic, J, Santibanez, JF: Transforming growth factor-beta and matrix metalloproteinases: functional interactions in tumor stroma-infiltrating myeloid cells. *ScientificWorldJournal,* 2014**:** 521754, 2014.

203. Hamilton, JA, Whitty, GA, Wojta, J, Gallichio, M, McGrath, K, Ianches, G: Regulation of plasminogen activator inhibitor-1 levels in human monocytes. *Cell Immunol,* 152**:** 7-17, 1993.
204. Lopez, S, Peiretti, F, Morange, P, Laouar, A, Fossat, C, Bonardo, B, Huberman, E, Juhan-Vague, I, Nalbone, G: Activation of plasminogen activator inhibitor-1 synthesis by phorbol esters in human promyelocyte HL-60--roles of PCKbeta and MAPK p42. *Thromb Haemost,* 81**:** 415-422, 1999.
205. Casey, TM, Eneman, J, Crocker, A, White, J, Tessitore, J, Stanley, M, Harlow, S, Bunn, JY, Weaver, D, Muss, H, Plaut, K: Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-β1) increase invasion rate of tumor cells: a population study. *Breast Cancer Res Tr,* 110**:** 39-49, 2008.
206. Binder, BR, Christ, G, Gruber, F, Grubic, N, Hufnagl, P, Krebs, M, Mihaly, J, Prager, GW: Plasminogen activator inhibitor 1: physiological and pathophysiological roles. *News Physiol Sci,* 17**:** 56-61, 2002.
207. de Vries, TJ, Kitson, JL, Silvers, WK, Mintz, B: Expression of plasminogen activators and plasminogen activator inhibitors in cutaneous melanomas of transgenic melanoma-susceptible mice. *Cancer Res,* 55**:** 4681-4687, 1995.
208. Masuda, T, Nakashima, T, Namba, M, Yamaguchi, K, Sakamoto, S, Horimasu, Y, Miyamoto, S, Iwamoto, H, Fujitaka, K, Miyata, Y, Hamada, H, Okada, M, Hattori, N: Inhibition of PAI-1 limits chemotherapy resistance in lung cancer through suppressing myofibroblast characteristics of cancer-associated fibroblasts. *J Cell Mol Med,* 23**:** 2984-2994, 2019.
209. Dublin, E, Hanby, A, Patel, NK, Liebman, R, Barnes, D: Immunohistochemical expression of uPA, uPAR, and PAI-1 in breast carcinoma. Fibroblastic expression has strong associations with tumor pathology. *Am J Pathol,* 157**:** 1219-1227, 2000.
210. Zhu, C, Shen, H, Zhu, L, Zhao, F, Shu, Y: Plasminogen Activator Inhibitor 1 Promotes Immunosuppression in Human Non-Small Cell Lung Cancers by Enhancing TGF-Beta1 Expression in Macrophage. *Cell Physiol Biochem,* 44**:** 2201-2211, 2017.
211. Ohba, K, Miyata, Y, Kanda, S, Koga, S, Hayashi, T, Kanetake, H: Expression of urokinase-type plasminogen activator, urokinase-type plasminogen activator receptor and plasminogen activator inhibitors in patients with renal cell carcinoma: correlation with tumor associated macrophage and prognosis. *J Urol,* 174**:** 461-465, 2005.
212. Quax, PH, van Muijen, GN, Weening-Verhoeff, EJ, Lund, LR, Dano, K, Ruiter, DJ, Verheijen, JH: Metastatic behavior of human melanoma cell lines in nude mice correlates with urokinase-type plasminogen activator, its type-1 inhibitor, and urokinase-mediated matrix degradation. *J Cell Biol,* 115**:** 191-199, 1991.
213. Buo, L, Bjornland, K, Karlsrud, TS, Kvale, D, Kjonniksen, I, Fodstad, O, Brandtzaeg, P, Johansen, HT, Aasen, AO: Expression and release of plasminogen activators, their inhibitors and receptor by human tumor cell lines. *Anticancer Res,* 14**:** 2445-2451, 1994.
214. Bagordakis, E, Sawazaki-Calone, I, Macedo, CC, Carnielli, CM, de Oliveira, CE, Rodrigues, PC, Rangel, AL, Dos Santos, JN, Risteli, J, Graner, E, Salo, T, Paes Leme, AF, Coletta, RD: Secretome profiling of oral squamous cell carcinoma-associated fibroblasts reveals organization and disassembly of extracellular matrix and collagen metabolic process signatures. *Tumour Biol,* 37**:** 9045-9057, 2016.

215. Slany, A, Meshcheryakova, A, Beer, A, Ankersmit, HJ, Paulitschke, V, Gerner, C: Plasticity of fibroblasts demonstrated by tissue-specific and function-related proteome profiling. *Clin Proteomics,* 11**:** 41, 2014.
216. Perrot, CY, Javelaud, D, Mauviel, A: Insights into the Transforming Growth Factor-beta Signaling Pathway in Cutaneous Melanoma. *Ann Dermatol,* 25**:** 135-144, 2013.
217. Lundgren, CH, Sawa, H, Sobel, BE, Fujii, S: Modulation of expression of monocyte/macrophage plasminogen activator activity and its implications for attenuation of vasculopathy. *Circulation,* 90**:** 1927-1934, 1994.
218. Gratchev, A, Kzhyshkowska, J, Kannookadan, S, Ochsenreiter, M, Popova, A, Yu, X, Mamidi, S, Stonehouse-Usselmann, E, Muller-Molinet, I, Gooi, L, Goerdt, S: Activation of a TGF-beta-specific multistep gene expression program in mature macrophages requires glucocorticoid-mediated surface expression of TGF-beta receptor II. *J Immunol,* 180**:** 6553-6565, 2008.
219. Giacoia, EG, Miyake, M, Lawton, A, Goodison, S, Rosser, CJ: PAI-1 leads to G1-phase cell-cycle progression through cyclin D3/cdk4/6 upregulation. *Mol Cancer Res,* 12**:** 322-334, 2014.
220. Czekay, RP, Wilkins-Port, CE, Higgins, SP, Freytag, J, Overstreet, JM, Klein, RM, Higgins, CE, Samarakoon, R, Higgins, PJ: PAI-1: An Integrator of Cell Signaling and Migration. *Int J Cell Biol,* 2011**:** 562481, 2011.
221. Xing, P, Liao, Z, Ren, Z, Zhao, J, Song, F, Wang, G, Chen, K, Yang, J: Roles of low-density lipoprotein receptor-related protein 1 in tumors. *Chin J Cancer,* 35**:** 6, 2016.
222. Degryse, B, Neels, JG, Czekay, RP, Aertgeerts, K, Kamikubo, Y, Loskutoff, DJ: The low density lipoprotein receptor-related protein is a motogenic receptor for plasminogen activator inhibitor-1. *J Biol Chem,* 279**:** 22595-22604, 2004.
223. Pavon, MA, Arroyo-Solera, I, Tellez-Gabriel, M, Leon, X, Viros, D, Lopez, M, Gallardo, A, Cespedes, MV, Casanova, I, Lopez-Pousa, A, Mangues, MA, Quer, M, Barnadas, A, Mangues, R: Enhanced cell migration and apoptosis resistance may underlie the association between high SERPINE1 expression and poor outcome in head and neck carcinoma patients. *Oncotarget,* 6**:** 29016-29033, 2015.
224. Chen, H, Peng, H, Liu, W, Sun, Y, Su, N, Tang, W, Zhang, X, Wang, J, Cui, L, Hu, P, Liu, S: Silencing of plasminogen activator inhibitor-1 suppresses colorectal cancer progression and liver metastasis. *Surgery,* 158**:** 1704-1713, 2015.
225. Wu, DM, Wang, S, Wen, X, Han, XR, Wang, YJ, Fan, SH, Zhang, ZF, Shan, Q, Lu, J, Zheng, YL: MircoRNA-1275 promotes proliferation, invasion and migration of glioma cells via SERPINE1. *J Cell Mol Med*, 2018.
226. Humbert, L, Lebrun, JJ: TGF-beta inhibits human cutaneous melanoma cell migration and invasion through regulation of the plasminogen activator system. *Cell Signal,* 25**:** 490-500, 2013.
227. de Souza, CF, Xander, P, Monteiro, AC, Silva, AG, da Silva, DC, Mai, S, Bernardo, V, Lopes, JD, Jasiulionis, MG: Mining gene expression signature for the detection of pre-malignant melanocytes and early melanomas with risk for metastasis. *PLoS One,* 7**:** e44800, 2012.
228. McMahon, GA, Petitclerc, E, Stefansson, S, Smith, E, Wong, MK, Westrick, RJ, Ginsburg, D, Brooks, PC, Lawrence, DA: Plasminogen activator inhibitor-1 regulates tumor growth and angiogenesis. *J Biol Chem,* 276**:** 33964-33968, 2001.

229. Li, S, Wei, X, He, J, Tian, X, Yuan, S, Sun, L: Plasminogen activator inhibitor-1 in cancer research. *Biomed Pharmacother,* 105**:** 83-94, 2018.
230. Bajou, K, Peng, H, Laug, WE, Maillard, C, Noel, A, Foidart, JM, Martial, JA, DeClerck, YA: Plasminogen activator inhibitor-1 protects endothelial cells from FasL-mediated apoptosis. *Cancer Cell,* 14**:** 324-334, 2008.
231. Romer, MU, Larsen, L, Offenberg, H, Brunner, N, Lademann, UA: Plasminogen activator inhibitor 1 protects fibrosarcoma cells from etoposide-induced apoptosis through activation of the PI3K/Akt cell survival pathway. *Neoplasia,* 10**:** 1083-1091, 2008.
232. Che, Y, Wang, J, Li, Y, Lu, Z, Huang, J, Sun, S, Mao, S, Lei, Y, Zang, R, Sun, N, He, J: Cisplatin-activated PAI-1 secretion in the cancer-associated fibroblasts with paracrine effects promoting esophageal squamous cell carcinoma progression and causing chemoresistance. *Cell Death Dis,* 9**:** 759, 2018.
233. Kang, J, Kim, W, Kwon, T, Youn, H, Kim, JS, Youn, B: Plasminogen activator inhibitor-1 enhances radioresistance and aggressiveness of non-small cell lung cancer cells. *Oncotarget,* 7**:** 23961-23974, 2016.
234. Szasz, I, Koroknai, V, Kiss, T, Vizkeleti, L, Adany, R, Balazs, M: Molecular alterations associated with acquired resistance to BRAFV600E targeted therapy in melanoma cells. *Melanoma Res,* 29**:** 390-400, 2019.
235. Gomes-Giacoia, E, Miyake, M, Goodison, S, Rosser, CJ: Targeting plasminogen activator inhibitor-1 inhibits angiogenesis and tumor growth in a human cancer xenograft model. *Mol Cancer Ther,* 12**:** 2697-2708, 2013.
236. Lee, YC, Yu, CC, Lan, C, Lee, CH, Lee, HT, Kuo, YL, Wang, PH, Chang, WW: Plasminogen activator inhibitor-1 as regulator of tumor-initiating cell properties in head and neck cancers. *Head Neck,* 38 Suppl 1**:** E895-904, 2016.
237. Weina, K, Wu, H, Knappe, N, Orouji, E, Novak, D, Bernhardt, M, Huser, L, Larribere, L, Umansky, V, Gebhardt, C, Utikal, J: TGF-beta induces SOX2 expression in a time-dependent manner in human melanoma cells. *Pigment Cell Melanoma Res,* 29**:** 453-458, 2016.
238. Simone, TM, Longmate, WM, Law, BK, Higgins, PJ: Targeted Inhibition of PAI-1 Activity Impairs Epithelial Migration and Wound Closure Following Cutaneous Injury. *Adv Wound Care (New Rochelle),* 4**:** 321-328, 2015.
239. Placencio, VR, DeClerck, YA: Plasminogen Activator Inhibitor-1 in Cancer: Rationale and Insight for Future Therapeutic Testing. *Cancer Res,* 75**:** 2969-2974, 2015.
240. Peng, S, Xue, G, Gong, L, Fang, C, Chen, J, Yuan, C, Chen, Z, Yao, L, Furie, B, Huang, M: A long-acting PAI-1 inhibitor reduces thrombus formation. *Thromb Haemost,* 117**:** 1338-1347, 2017.
241. Izuhara, Y, Yamaoka, N, Kodama, H, Dan, T, Takizawa, S, Hirayama, N, Meguro, K, van Ypersele de Strihou, C, Miyata, T: A novel inhibitor of plasminogen activator inhibitor-1 provides antithrombotic benefits devoid of bleeding effect in nonhuman primates. *J Cereb Blood Flow Metab,* 30**:** 904-912, 2010.
242. Delbaldo, C, Masouye, I, Saurat, JH, Vassalli, JD, Sappino, AP: Plasminogen activation in melanocytic neoplasia. *Cancer Res,* 54**:** 4547-4552, 1994.
243. Hanekom, GS, Stubbings, HM, Kidson, SH: The active fraction of plasmatic plasminogen activator inhibitor type 1 as a possible indicator of increased risk for metastatic melanoma. *Cancer Detect Prev,* 26**:** 50-59, 2002.

9 ACKNOWLEDGEMENTS

First of all, I would like to thank Prof. Dr. Jonathan P. Sleeman for supervising my doctoral thesis. I am very grateful for his support and guidance throughout the whole process of research, analysis, re-search and writing. I also thank him for establishing collaborations without which this thesis would not have been possible.

I especially thank Dr. Georg Sedlmeier for supervising my “Forschungsarbeit” as well as the experimental part of my thesis and teaching me various experimental methods. It was admirable how he could always be patient with me and never lost his sense of humor, even though he was working for his own thesis and supervising my experiments simultaneously.

Next, I thank all former and current members of the Sleeman lab with whom I have had the pleasure to work. Thanks to Annette Gruber for teaching me immunohistochemistry and for being patient and indulgent with a clumsy med student at her first days in the lab. I also want to thank Sven Rosswag for helping me optimize RNA isolation and western blot protocols and for all the post-it blocks. And I thank Dr. Wilko Thiele for long and fruitful discussions about my thesis, but also about a bunch of other inspiring subjects. I want to thank Dr. Boyan Garvalov for his support concerning statistical analysis, for productive discussions and for critical proofreading, as well as for his admirable patience during these processes.

I also want to thank the Medical Faculty Mannheim of the University of Heidelberg. It was possible in an uncomplicated way to pause studies for two semesters in order to learn the required experimental methods and to carry out most of the experiments. Additionally, during this year, the scholarship of the faculty allowed me to invest a maximum of time in my thesis. Therefore, I express my thanks to everyone who made this scholarship possible. Importantly, I want to thank Prof. Dr. Hammes, who offered an incredible support through the program of the Junior Scientific Masterclass. I am very thankful how much I was allowed to learn about experimental research, scientific writing and presentation and also about myself through this program.

Next, I would like to express my thanks to all the collaborators for their contribution to this project. I thank Prof. Dr. Utikal for providing me with melanoma cell lines, patient-derived primary melanoma samples and for his advice on immunohistochemistry. Additionally, Prof. Dr. Kzyshkovska and the kind help of her lab members enabled me to carry out experiments with the cell line THP-1. Furthermore, I want to thank PD Dr. Herskind for providing me with primary HDF and for his advice on how to make fibroblasts happy. Thanks to Dr. Quagliata and the University Hospital of Zurich, blood samples of melanoma patients could be part of this project, for which I am very grateful.

Finally, my deepest gratitude belongs to my family and friends for their constant support. I thank my parents for encouraging me to go on, even though it could all have been simpler, easier and faster. I thank Tabi for proofreading my thesis and for fruitful discussions about punctuation, spaces and everything else. And I thank Patrick, without whom I cannot imagine my life.

10 CURRICULUM VITAE

PERSONAL DATA:

Name and surname:	Kathrin Metzger
Date of birth:	16.12.1994
Place of birth:	St. Ingbert, Germany
Marital status:	Single
Father:	Udo Metzger
Mother:	Anne-Michaela Moritz

SCHOOL EDUCATION:

2005 – 2013	Deutsch-Französisches Gymnasium Saarbrücken
07/ 2010	„Diplôme National du Brevet“ (french intermediate school leaving certificate)
06/ 2013	“Abitur” and “Baccalauréat” (german and french high school diploma)

UNIVERSITY CAREER:

Since 10/2013	Study of Human Medicine Medizinische Fakultät Mannheim der Universität Heidelberg
10/2014 – 12/2019	Founding member of the College Journal Club (CJC) Mannheim
09/2015	“Erster Abschnitt der Ärztlichen Prüfung“ (first medical degree)
02/2016 – 07/2017	Junior Scientific Masterclass
12/2016	„Forschungsarbeit“ (scientific research work) Topic: Literature research and data analysis concerning the coexpression of TGFβ1 and SAA in melanoma
11/2016 – 10/2017	Scholarship of the faculty for MD students
10/2019	“Zweiter Abschnitt der Ärztlichen Prüfung“ (second medical degree)